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JOURNAL OF CHROMATOGRAPHY

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CHROMBIO. 2572

APPLICATION OF RADIOISOTOPE TRACER TECHNIQUES TO ANALYTICAL GAS CHROMATOGRAPHY: DETERMINATION OF GAS CHROMATOGRAPHIC PEAK YIELD

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(First received October 31st, 1984; revised manuscript received January 28th, 1985)

SUMMARY

The determination of gas chromatographic peak yields using a radio-gas chromatography system, in which ^{14}C-labelled substances eluted from a gas chromatography column are burnt to $^{14}CO_2$ through a combustion tube, is described. As the first step of the study, the adequacy of the combustion tube was investigated by a radioisotope tracer technique. Consequently, it was found that almost complete combustion could be achieved by the combustion tube for the substances investigated.

INTRODUCTION

Gas chromatography (GC) is a useful technique for the identification and quantification of substances and has been widely employed because of its high sensitivity and resolution, rapidity and ease of handling. Flame-ionization detection (FID) and electron-capture detection (ECD) are usually used in GC analyses, but they suffer from the following disadvantages. The detector response differs considerably with the kind of substance involved, so calibration graphs for every component of a sample must be prepared in order to be able to quantify simultaneously many components. Further, it is impossible to know what percentage of the amount of an injected substance reaches the detector system. We define this percentage as "GC peak yield" [1].

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We previously developed a radio-gas chromatography (RGC) system equipped with a new radiodetector system, synchronized accumulating radiodetection (SARD) [2], which makes it possible to improve the detection efficiency without sacrificing the resolution, and to obtain the radioactive intensity on the chromatogram in digital form. We have undertaken a series of studies of the fundamental problems in GC such as GC peak yields and the adsorption of substances on column packings, utilizing this RGC—SARD system. As the first step, an attempt to determine GC peak yields by RGC—SARD is reported in this paper.

EXPERIMENTAL

Radioactive samples and reagents

n-[1-^{14}C]Hexadecane (53.6 mCi/mmol), [4-^{14}C]testosterone (58.0 mCi/mmol) and *L*-[ring-2-^{14}C]histidine (59.0 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). The radioactive substances showed a single peak on thin-layer chromatography. Solutions of [^{14}C]hexadecane, [^{14}C]testosterone and [^{14}C]histidine were prepared in cyclohexane, ethanol and water, respectively. All reagents were purchased from Wako (Tokyo, Japan) and were of analytical-reagent grade.

Liquid scintillation counting

Unless stated otherwise, the radioactivity of a sample was measured in a mixture of 10 ml of toluene-base scintillation cocktail and 0.5 ml of methanol with a liquid scintillation counter (Aloka LSC 502 or 903).

Combustion furnace

The combustion tube placed in the electric furnace was a 180 × 5 mm I.D. quartz tube as described previously [2], and was packed with only copper oxide wire of dimensions 5 × 1 mm (unless otherwise stated, about 10 g). The temperature of the electric furnace during operation was about 800°C.

Injection technique

Aliquots (4 μl) from each solution were injected by the usual method with a 10-μl Hamilton microsyringe in all experiments.

Radio-gas chromatography

A glass column (1 m × 3 mm I.D.) was packed with 1.5% OV-17 on Shimalite W (80—100 mesh). The carrier gas (nitrogen) and the counting gas (methane) flow-rates were 50 and 250 ml/min, respectively.

Assembly for measurement of combustion efficiency (combustion assembly)

The injection port was connected directly to the combustion tube by a stainless-steel capillary tube (30 cm × 1 mm I.D.) placed in an oven. The sample was introduced into the combustion tube through the capilllary tube with the carrier gas (nitrogen). Unless stated otherwise, the gas exhausted from the combustion tube was introduced into the gas-flow proportional counters used in the RGC—SARD after mixing with the counting gas (methane), the carrier

gas and counting gas flow-rates were set at 50 and 250 ml/min, respectively, and the injection port and the oven were heated to 250°C and 160°C, respectively.

Derivatization of [^{14}C]histidine with pentafluoropropionic anhydride (PFPA) [3]

An aqueous solution of [^{14}C]histidine (containing 5—10 μg of histidine) was lyophilized, 50 μl of PFPA were added to the residue and the solution was heated at 70°C for 1.5 h. The excess of PFPA was evaporated under a gentle stream of nitrogen, 50 μl of ethyl acetate were added to the residue and a portion of the solution was injected without any purification.

Radio-gas chromatograms of [^{14}C]hexadecane, [^{14}C]testosterone and [^{14}C]histidine derivatives

[^{14}C]Hexadecane (1.85 nCi, 0.69 mCi/mmol), [^{14}C]testosterone, (1.83 nCi, 0.67 mCi/mmol) and [^{14}C]histidine (1.79 nCi, 0.60 mCi/mmol, after derivatization with PFPA) were injected into the RGC—SARD system. The column oven and injection port temperatures for the three samples were as follows: [^{14}C]hexadecane, 160 and 250; [^{14}C]testosterone, 275 and 295; and [^{14}C]-histidine, PFPA derivative, 160 and 210°C, respectively.

Measurement of combustion efficiency

Method A. [^{14}C]Hexadecane (53.6 mCi/mmol), [^{14}C]testosterone (58.0 mCi/mmol) and [^{14}C]histidine (3.4 mCi/mmol, after derivatization with PFPA) were injected into the combustion assembly and the gas exhausted from the combustion tube was introduced into a series of two absorption traps for $^{14}CO_2$ [4] containing 6 ml of monoethanolamine—methanol (1:1) for 5 min. One twentieth of the trapping solution was taken and the radioactivity was measured in 10 ml of toluene-base scintillation cocktail with a liquid scintillation counter. The injected radioactivity was 9—20 nCi for any substance.

Method B. About 2 nCi of [^{14}C]hexadecane (5.73 mCi/mmol) and [^{14}C]-testosterone (5.87 mCi/mmol) were injected into the combustion assembly and the radioactive peak intensities were measured with the gas-flow proportional counters.

Capability of copper oxide wire for oxidation

[^{14}C]Hexadecane (about 1.8 nCi, 53.6 mCi/mmol) dissolved in cyclohexane (4 μl) and pure cyclohexane (4 μl) were injected into the RGC—SARD system alternately and the radioactive peak intensities were meausred. The radioactive peak intensities obtained by the first four injections of the radioactive sample were averaged and the mean value was taken as 100%. The injection port and the column oven were heated to 250°C and 160°C, respectively.

Relationship between carrier gas flow-rate and radioactive peak intensity

The injection port and the oven of the combustion assembly were heated to 200°C. About 2 nCi of [^{14}C]hexadecane (5.73 mCi/mmol) and [^{14}C]-testosterone (5.87 mCi/mmol) were injected into the assembly at carrier gas flow-rates of 20, 50 and 80 ml/min and the radioactive peak intensities were measured with the gas-flow proportional counters.

Relationship between temperature of injection port oven and radioactive peak intensity

About 2 nCi of [^{14}C]hexadecane (5.73 mCi/mmol), [^{14}C]testosterone (5.87 mCi/mmol) and [^{14}C]histidine (0.60 mCi/mmol, after derivatization with PFPA) were injected into the combustion assembly with temperatures of the injection port oven of 150, 200, 250, 300 and 350°C and the radioactive peak intensities were measured with the gas-flow proportional counters.

RESULTS AND DISCUSSION

In GC analyses, the kinds of substances that can be detected have increased rapidly and microanalyses below the nanogram level have been possible owing to the development of highly sensitive detectors and low-loaded column packings and to progress with derivatization techniques. However, the increase in the range of applications has been accompanied by some problems. For example, adequate accuracy cannot be obtained when analysing a very small amount of a substance because there is poor linearity between the amount of substance injected and the peak intensity. It is considered that this effect arises from incompleteness of derivatization, thermal decomposition of sample substances and adsorption of substances on the column packing and/or the injection port, etc. The response factor in GC may be represented by the following expression:

Response factor = GC peak yield (%) x detector response

It is essentially impossible to determine GC peak yields because the peak intensity shows only indirectly the amount of substance that reaches the detector and the detector response varies with the kind of substance involved.

In the RGC—SARD system, effluents from a GC column are introduced into the combustion furnace, where ^{14}C-labelled substances are burnt to produce $^{14}CO_2$, and the gas, after mixing with the counting gas (methane), is introduced into five gas-flow proportional counters arranged longitudinally in series. The response factor of the RGC—SARD system for ^{14}C-labelled substances may be represented by the following expression:

Response factor = GC peak yield (%) x combustion efficiency (%) x counting efficiency (%)

The counting efficiency is considered to be constant, irrespective of the kind of substance involved, and can be determined by using an authentic sample. Then, if the combustion efficiency can be determined, it may be possible to determine the GC peak yields of various substances from the ratio of the radioactive intensity on the chromatogram to the injected radioactivity.

Fig. 1 shows typical examples of radio-gas chromatograms that were obtained by the injection of almost identical radioactivities of [^{14}C]hexadecane, [^{14}C]-testosterone and [^{14}C]histidine (after derivatization) into the RGC—SARD system. The radioactive peak intensities varied widely with the kind of substance injected.

The counting efficiency, which was calculated from the data in Fig. 1 on the assumption that the adsorption of [^{14}C]hexadecane was negligible because of its low polarity, was 96.5%. This value is reasonable for this type of gas-flow

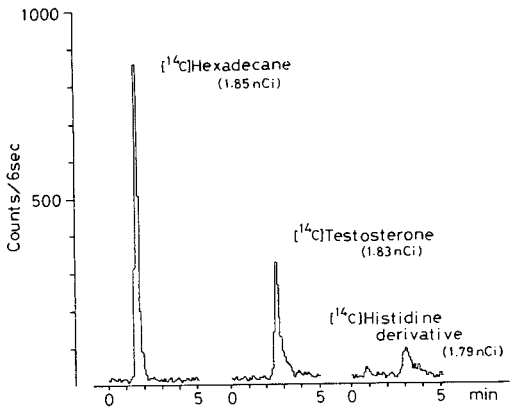

Fig. 1. Radiochromatograms of ^{14}C-labelled substances. Injected radioactivity in parentheses.

proportional counter and will be used as the counting efficiency of this counting system [5, 6]. Therefore, it was concluded that all of the injected radioactivity of [^{14}C]hexadecane reaches the detection system, that is, the GC peak yield of [^{14}C]hexadecane is 100%. Now we can evaluate GC peak yields of various substances on the basis of [^{14}C]hexadecane. Consequently, if the combustion efficiency is 100%, the GC peak yield of [^{14}C]testosterone is calculated to be 74.7 ± 1.64%. When a substance is injected into a GC column after derivatization (without any purification), it was considered that the GC peak yield includes the influence of derivatization. As shown in Fig. 1, when [^{14}C]histidine was injected after derivatization with PFPA, [^{14}C]histidine-di-PFP appeared on the radio-gas chromatogram as the main derivative together with an unknown minor component. If the combustion efficiency is 100%, the GC peak yields are calculated to be 25.9 ± 1.18% and 7.2 ± 0.86%, respectively, and the total GC peak yield is 33.1 ± 1.89%, on the basis of the injected radioactivity.

In RGC, substances eluted from a GC column must be burnt quantitatively or at least with a constant efficiency, independent of the chemical structure. It has been reported [7, 8] that ^{14}C-labelled substances in the effluents from a GC column can be quantitatively burnt to $^{14}CO_2$ through a narrow-bore quartz tube packed with copper oxide that is maintained at 700—800°C. The investigations cited, however, dealt exclusively with readily combustible substances and the combustion efficiencies of [^{14}C]testosterone and [^{14}C]histidine derivatives, which may be more or less non-combustible, were compared with that of readily combustible [^{14}C]hexadecane.

The known radioactivities of these substances were injected into the combustion assembly and the radioactivities in the gas exhausted from the combustion tube were measured by methods A and B (see Experimental). In method A, the proportions of the radioactivity recovered as $^{14}CO_2$ to that injected (recovery yield) were calculated as shown in Table I. Although the recovery yield of [^{14}C]hexadecane was almost quantitative, those of [^{14}C]-testosterone and [^{14}C]histidine derivatives were low. In method B, the ratios of the radioactive peak intensity to the injected radioactivity were calculated as shown in Table II. The time course of elution of the radioactivity from the

TABLE I

RECOVERY OF ^{14}C RADIOACTIVITY FROM COMBUSTION TUBE

Compound	Recovery yield (%)*
[^{14}C]Hexadecane	97.7 ± 1.89
[^{14}C]Testosterone	62.7 ± 3.46
[^{14}C]Histidine derivative	42.7 ± 3.15

*Mean ± S.D. (n = 7).

TABLE II

RATIO OF RADIOACTIVE PEAK INTENSITY TO RADIOACTIVITY INJECTED INTO COMBUSTION ASSEMBLY

Compound	Ratio (%)*
[^{14}C]Hexadecane	100.0 ± 1.81
[^{14}C]Testosterone	65.1 ± 3.98

*Mean ± S.D. (n = 5).

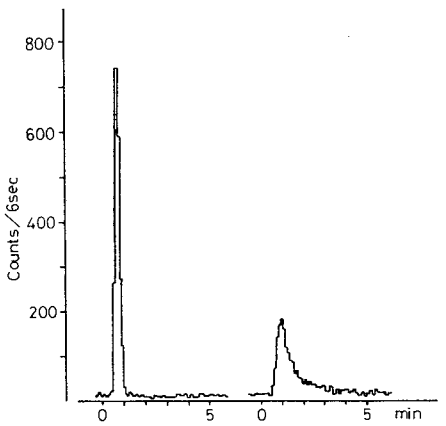

Fig. 2. Radioactive peaks obtained by injection of [^{14}C]hexadecane (left) and [^{14}C]-testosterone (right) into the combustion assembly.

combustion tube is shown in Fig. 2. The ratio for [^{14}C]testosterone was lower than that for [^{14}C]hexadecane. It is considered that this low ratio for [^{14}C]-testosterone and the low recovery yields of [^{14}C]testosterone and [^{14}C]-histidine derivatives can be ascribed to the incompleteness of the combustion, and/or adsorption on the injection port and the stainless-steel capillary tube. The recovery yields in Table I were essentially identical with the ratios in Table II, and it was concluded that the recovery yield could be substituted for the ratio in order to compare combustion efficiencies.

The combustion efficiency may vary with the time during which a substance is in contact with the copper oxide wire. Consequently, the factors that influence the combustion efficiency are thought to be the amount of copper oxide wire packed in the combustion tube and the carrier gas flow-rate. Moreover, the degree of consumption of copper oxide wire, which is gradually

consumed and changed into copper with its usage, is also a factor that influences the combustion efficiency.

First, the relationship between the degree of consumption of copper oxide wire and the combustion efficiency was investigated. [^{14}C]Hexadecane in cyclohexane and the pure cyclohexane were injected into the RGC—SARD system as described under Experimental. As shown in Fig. 3, the ratio of the radioactive peak intensity to the injected radioactivity was kept almost constant during the experiment. About four-fifths of the copper oxide wire from the inlet side of the combustion tube turned red on its surface after the injection of 200 μl of cyclohexane. The experiment revealed that the combustion tube used in this study retained a sufficient capability for combustion until the volume of the injected solvent amounted to at least 200 μl.

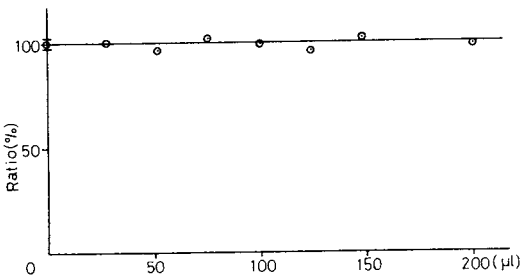

Fig. 3. Capability of combustion tube. Radioactive sample: [^{14}C]hexadecane.

Second, the relationship between the amount of copper oxide wire and the combustion efficiency was investigated. [^{14}C]Testosterone was injected into the combustion assembly, the combustion tube of which was packed with 2.5, 5.0 and 7.5 g of copper oxide wire. Consequently, the proportion of the radioactivity recovered as $^{14}CO_2$ to that injected was more than 90% at any amount of copper oxide wire, which suggested that [^{14}C]testosterone could be burnt almost completely in the combustion tube used. This result also means that a combustion tube packed with about 10 g of copper oxide wire can fulfil its function efficiently even if three quarters of the wire are consumed. This result agrees approximately with that in the experiment on the consumption of copper oxide wire.

Third, the relationship between the carrier gas flow-rate and the combustion efficiency was investigated. [^{14}C]Hexadecane and [^{14}C]testosterone were injected into the combustion assembly at carrier gas flow-rates of 20, 50 and 80 ml/min. The ratio of the radioactive peak intensity to the injected radioactivity of [^{14}C]hexadecane was essentially constant at any flow-rate. The ratio of [^{14}C]testosterone at each flow-rate is shown in Table III and the peak shape in Fig. 4. The times during which the substance is in contact with copper oxide wire at flow-rates of 80, 50 and 20 ml/min are calculated to be 1.6, 2.6 and 6.4 sec, respectively. The ratios at flow-rates of 80 and 50 ml/min were essentially the same. In contrast, the ratio at a flow-rate of 20 ml/min was nearly 10% lower. It seems that this phenomenon results from the increase in adsorption of the substance due to the extremely low flow-rate. In this experiment, the counting gas flow-rate was set so that the total gas flow-rate through

TABLE III

RELATIONSHIP BETWEEN CARRIER GAS FLOW-RATE AND RATIO OF RADIOACTIVE PEAK INTENSITY TO RADIOACTIVITY INJECTED INTO COMBUSTION ASSEMBLY

	Flow-rate (ml/min)		Ratio (%)*
	Carrier gas	Counting gas	
A	80	220	74.1 ± 0.20
B	50	250	75.4 ± 0.54
C	20	280	64.9 ± 2.29

*Mean ± S.D. (n = 3).

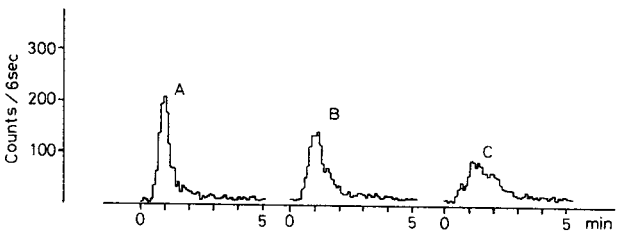

Fig. 4. Effect of carrier gas flow-rate on peak shape. A, B and C correspond to A, B and C, respectively, in Table III.

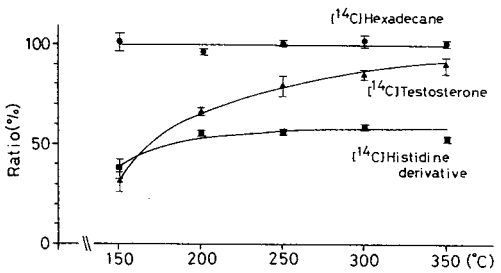

Fig. 5. Relationship between the temperature of injection port oven and the ratio of the radioactive peak intensity to the radioactivity injected into the combustion assembly.

the counters amounted to 300 ml/min at any carrier gas flow-rate, so the concentration of the counting gas passing the counters varied (73, 83 and 93%). However, it was considered that the counting efficiency did not vary because the gas in the counters, except for the counting gas, was almost all nitrogen, which is not a quencher. This experiment suggested that [^{14}C]testosterone, similarly to [^{14}C]hexadecane, could be burnt almost completely in the combustion tube used.

The relationship between the temperatures of the injection port and the oven and the ratio of the radioactive peak intensity to the injected radioactivity was investigated. [^{14}C]Hexadecane, [^{14}C]testosterone and [^{14}C]-histidine (after derivatization) were injected into the combustion assembly. The result is shown in Fig. 5. With [^{14}C]hexadecane the ratio was essentially constant at any temperature. With [^{14}C]testosterone the ratio inceased with increase in temperature and reached to 90% at 350°C. Consequently, it was

concluded that the low ratio for [^{14}C]testosterone shown in Table II resulted from adsorption on the injection port and the stainless-steel capillary tube. With [^{14}C]histidine derivatives the ratio was approximately constant (about 55%) at temperatures higher than 200°C. It is reasonable to conclude that this results from incompleteness of derivatization rather than the low combustion efficiency.

It was found that the combustion tube could burn more or less non-combustible substances, [^{14}C]testosterone and [^{14}C]histidine derivatives, similarly to [^{14}C]hexadecane, almost quantitatively and possessed sufficient capability for combustion. Consequently, it was concluded that GC peak yields can be determined by the radioisotope tracer technique using the RGC—SARD system and that [^{14}C]hexadecane is a useful substance for the standardization of GC peak yields. The derivatization yield, thermal decomposition and adsorption of substances on the column packings and/or the injection port, etc., will be investigated in subsequent studies.

REFERENCES

1 S. Baba, M. Utoh and M. Horie, J. Chromatogr., 307 (1984) 1.
2 S. Baba and Y. Kasuya, J. Chromatogr., 196 (1980) 144.
3 N. Mahy and E. Gelpi, Chromatographia, 11 (1978) 573.
4 S. Baba, T. Konishi and H. Ido, Yakugaku Zasshi, 93 (1973) 532.
5 B. Kolb and E. Wiedeking, Z. Anal. Chem., 243 (1968) 129.
6 T.H. Simpson, J. Chromatogr., 38 (1968) 24.
7 M. Matucha and E. Smolkova, J. Chromatogr., 127 (1976) 163.
8 J. Winkelman and A. Karmen, Anal. Chem., 34 (1962) 1067.

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CHROMBIO. 2562

QUANTITATIVE DETERMINATION OF BETAMETHASONE AND ITS MAJOR METABOLITE IN EQUINE URINE BY MICRO-LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received August 22nd, 1984; revised manuscript received January 22nd, 1985)

SUMMARY

Micro-liquid chromatography—mass spectrometry (micro-LC—MS) was utilized to quantitatively determine betamethasone and its major unconjugated metabolite, 6β-hydroxybetamethasone, in equine plasma and urine. The advantage of micro-LC—MS over conventional gas chromatography—mass spectrometry in corticosteroid determination is illustrated and the reliable, steadfast nature of micro-LC—MS is demonstrated through example.

INTRODUCTION

Micro-liquid chromatography—mass spectrometry (micro-LC—MS) has continued to find new and novel applications in both qualitative [1—3] and comparative [4] determinations. The power of this analytical tool affords difficult drug determinations and although proven sensitive and specific, micro-LC—MS utilization in precision studies such as quantitative determination has found very limited application [5, 6].

Quantitative determination can profile drug concentrations over time and in turn be used to estimate pharmacokinetic parameters such as drug half-life, volume of distribution, body clearance, elimination rate and drug compartmentalization. To do this, determinations must discern between administered drugs and endogenous material as well as between parent drugs and their metabolites. Many techniques utilized for quantitative determination are unable to make both distinctions. Radioimmunoassay [7, 8], competitive protein binding [8, 9] and enzyme immunoassay [10] fail to consistently distinguish parent drugs from metabolites, while thin-layer chromatography (TLC), gas chroma-

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tography (GC) or liquid chromatography (LC) alone can not confirm analyte identities.

MS, on the other hand, when coupled to these chromatographic techniques can afford sensitve, unequivocal identification of parent drugs and metabolites. Still, the polar, non-volatile, heat-labile nature of the corticosteroids [11] makes analysis by TLC—direct inlet probe-MS or GC—MS difficult while micro-LC—MS remains a viable, non-destructive alternative.

In this study we illustrate how the sensitive, specific capabilities of micro-LC—MS can be applied to the quantitative determination of corticosteroids in biological fluids. In doing so, we demonstrate the stable, reliable nature of direct liquid introduction (DLI) micro-LC—MS in precision studies over extended periods and illustrate the advantage of micro-LC—MS over conventional GC—MS in corticosteroid determination.

MATERIALS AND METHODS

Drug administration and chemicals

Betamethasone sodium phosphate (Shering, Kenilworth, NJ, U.S.A.) was administered intravenously (60 mg) to healthy Standardbred horses (ca. 500 kg each). Reference betamethasone was purchased from Sigma (St. Louis, MO, U.S.A.) and 6α-methylprednisolone from Steraloids (Wilton, NH, U.S.A.). Dr. R. Draper of Shering (Bloomfield, NJ, U.S.A.) generously donated 6β-hydroxybetamethasone (U.S. Patent No. 4,201,778).

Micro-LC methanol was distilled-in-glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) while the water was HPLC grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Eluents were suction-filtered through a 0.45-μm pore-size filter (Millipore, Bedford, MA, U.S.A.) and then continuously purged with helium during micro-LC operation to eliminate dissolved gases. All sample solutions injected into the micro-LC system were filtered through a 0.45-μm pore-size Millipore filter. Derivatizing agents, methoxyamine hydrochloride and trimethylsilylimidazole, were purchased from Pierce (Rockford, IL, U.S.A.).

Drug extraction and TLC clean-up

Plasma and urine were extracted by the method of Skrabalak and Maylin [12] with the following modifications. The extraction solvent was diethyl ether—methylene chloride—isopropanol (2:1:1) and only the initial TLC system was used. Further separation of TLC-prepared samples was afforded by micro-LC—MS.

Micro-LC

A modified Waters M660 solvent programmer [13] and M6000A pump were equipped with a Rheodyne Model 7410 micro-loop (0.5 μl) injector (Rheodyne, Cotati, CA, U.S.A.) and a 25 cm × 1 mm I.D. Whatman Partisil 10 ODS-3 microbore column (Whatman, Clifton, NJ, U.S.A.). Micro-LC separations were accomplished with methanol—water (70:30) at a flow-rate of 40 μl/min.

Micro-LC—MS and GC—MS

An unmodified Hewlett-Packard Model 5985B GC—MS instrument equipped with option 01 (Hewlett-Packard, Palo Alto, CA, U.S.A.) was utilized for micro-LC—MS operation. The liquid nitrogen cooled cryopump was used in all chemical ionization (CI) experiments as recommended by the manufacturer.

The normal CI operating parameters of the mass spectrometer for all micro-LC—MS experiments were as follows: electron energy, 230 eV; emission current, 300 μA; ion source pressure; 0.5—0.6 Torr (66.5—80 Pa) as measured at the GC—CI-MS interface thermocouple; repeller, 5 V; electron multiplier, 2400 V. The micro-LC and MS systems were interfaced via an on-line DLI micro-LC—MS probe as previously described [1].

A Hewlett-Packard Model 5992 GC—MS system (Hewlett-Packard) equipped with a silicone membrane separator was utilized for electron-impact ionization (EI) GC—MS acquisitions. EI spectra were obtained with 220 μA emission current and 70 eV ionization energy. GC was carried out on a 1 m × 2 mm I.D. packed column of 3% OV-101, temperature programmed from 220°C (isothermal for 2 min) to 260°C at 10°C/min and purged with helium carrier gas at a flow-rate of 30 ml/min. Urine extracts for GC—EI-MS were derivatized as previously described [14].

Quantitation

All plasma and urine samples were spiked with known amounts (100 ng/ml) of 6α-methylprednisolone before being extracted and analyzed as described. Immediately before and after sample analysis, a mixture containing equal amounts of 6β-hydroxybetamethasone, betamethasone and 6α-methylprednisolone were determined by micro-LC—MS. Individual drug levels within these mixtures ranged from 75 to 150 ng each; their determinations afforded

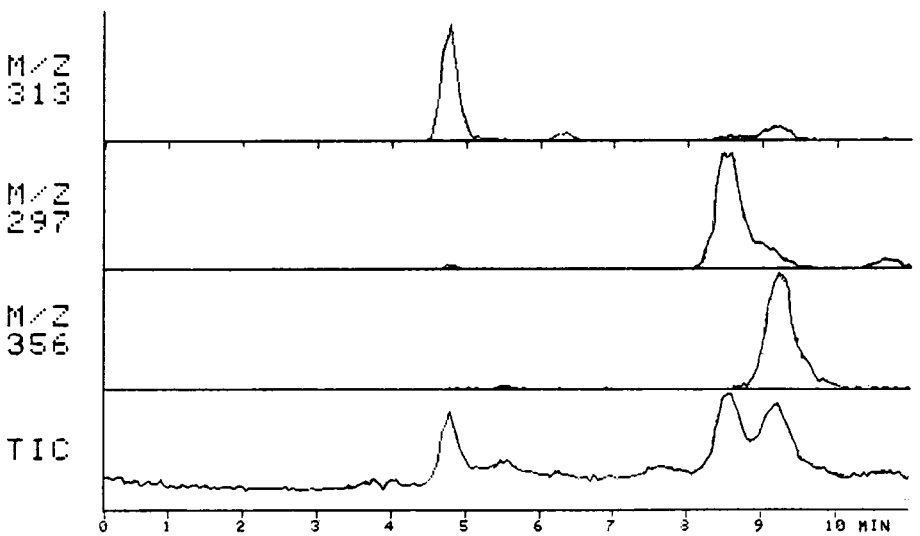

Fig. 1. Selected-ion profiles of 6β-hydroxybetamethasone (m/z 313), betamethasone (m/z 297) and internal standard, 6α-methylprednisolone (m/z 356) and NICI—TIC micro-LC—MS chromatograms of a representative betamethasone administration 2-h plasma extract.

comparison of the system's responses to these drugs under similar conditions over a wide range. Selected-ion abundances of 6β-hydroxybetamethasone (*m/z* 313) and betamethasone (*m/z* 297) were compared to those of 6α-methylprednisolone (*m/z* 356) and the respective standard ratios calculated (n = 4; 6β-hydroxybetamethasone/6α-methylprednisolone = 10.78 ± 2.49, x ± S.E.; betamethasone/6α-methylprednisolone = 4.89 ± 0.89, $\bar{x}$ ± S.E.).

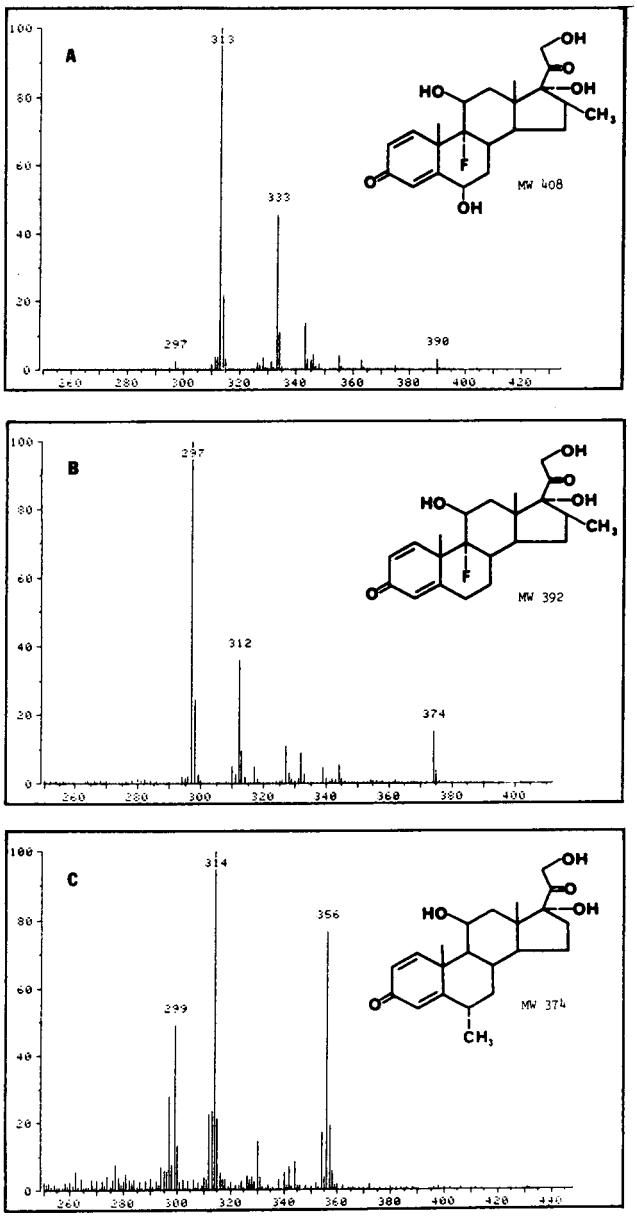

Fig. 2. Structures and mass spectra of (A) 6β-hydroxybetamethasone, (B) betamethasone and (C) 6α-methylprednisolone.

Corresponding sample ratios were later compared to respective standard ratios and multiplied by the spike concentration to estimate drug concentrations in plasma and urine. Recoveries of the three drugs from both plasma and urine were shown by re-isolation procedures and high-performance liquid chromatography (HPLC) to be in the ratio 1:1:1; extraction efficiency was 40%.

RESULTS AND DISCUSSION

The selected-ion profiles of 6β-hydroxybetamethasone (*m/z* 313); betamethasone (*m/z* 297) and 6α-methylprednisolone (*m/z* 356) as well as the negative-ion chemical ionization—total-ion current (NICI—TIC) chromatogram of a representative betamethasone administration plasma extract are shown in Fig. 1. The structures and mass spectra of these corticosteroids are given in Fig. 2. Under the conditions used, each sample was analyzed in less than 12 min. This relatively short run time affords efficient use of the micro-LC—MS system and allows numerous samples to be run within a given work period. The ability of the mass spectrometer and its associated computer system to profile selected-ions enables one to distinguish peaks of interest from both endogenous interferences as well as other analyte peaks. This ability is most evident near the end of the chromatogram where betamethasone and 6α-methylprednisolone peaks are not baseline resolved. Selected-ion profiles of betamethasone (*m/z* 297) and 6α-methylprednisolone (*m/z* 356) produce baseline resolution, thereby affording quantitation of their respective peaks.

Contrary to the results afforded by micro-LC—MS, corticosteroid determination by GC—MS usually requires derivatization of the drugs. Inevitably the derivatizing procedures command considerable preparation and time while producing multiple products that can hinder quantitative

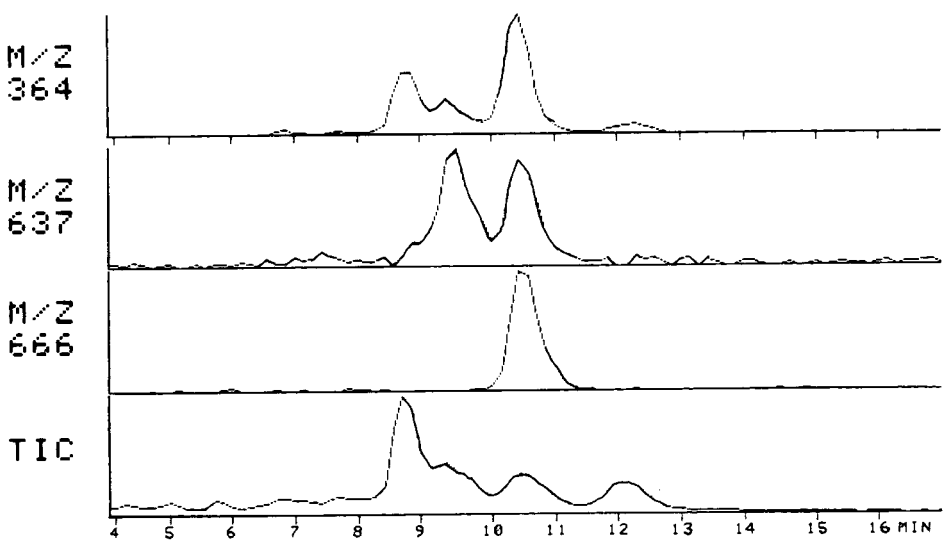

Fig. 3. GC—MS selected-ion profiles and total-ion current of spiked equine urine extract derivatized with methoxyamine hydrochloride and trimethylsilylimidazole. Urine was spiked with 6β-hydroxybetamethasone, betamethasone and 6α-methylprednisolone at 10, 10 and 0.1 μg/ml, respectively, before extraction.

measurement [14, 15]. Fig. 3 shows GC—MS selected-ion profiles and TIC of a methoxime-trimethylsilyl (MO-TMS) derivatized equine urine extract. The urine was spiked with 6β-hydroxybetamethasone, betamethasone and 6α-methylprednisolone at 10, 10 and 0.1 μg/ml, respectively, before extraction to approximate or exceed peak urine concentrations of betamethasone products quantitatively analyzed in this work by micro-LC—MS. The 6α-methylprednisolone concentrations used for standardization remained constant in all GC—MS and micro-LC—MS samples.

Betamethasone was chosen as a representative component to examine derivatized corticosteroid determination by conventional GC—MS while the scanned mass range encompassed all expected derivatization products (*m/z* 35—*m/z* 770). The TIC in Fig. 3 exhibits multiple peaks which are inconsistent with a single derivatization product for each spiked component. In fact, by inspecting the selected-ion profiles indicative of MO-TMS derivatized betamethasone, *m/z* 364 [15], we see major chromatographic peaks corresponding to both the mono-MO-tri-TMS (*m/z* 637) and bi-MO-tri-TMS (*m/z* 666) derivative. This clearly demonstrates multiple products of betamethasone derivatization and thus the incompatibility of GC—MS with quantitative corticosteroid determination.

One aspect of DLI micro-LC—MS addressed in this work is reliability over extended periods. The continually acquired NICI—TIC chromatogram of multiple injected samples is shown in Fig. 4. During these 185 min of acquisition, methanol—water (70:30) eluent continuously jetted into the mass spectrometer at 40 μl/min. Nevertheless, source ionization pressure remained at 0.6 Torr without adjustment due to the excellent performance of the liquid nitrogen cooled cryopump and the DLI micro-LC—MS probe which delivered a fine, uninterrupted stream of eluent. The entire system responded well to a

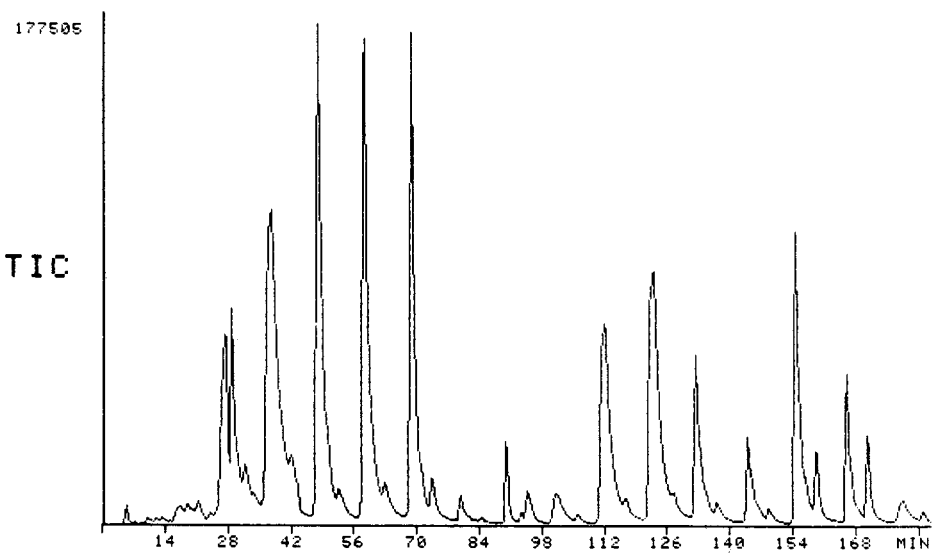

Fig. 4. Continually acquired NICI—TIC micro-LC—MS chromatogram of multiple injected samples obtained with a Whatman Partisil 10 ODS-3 microbore column (25 cm × 1 mm I.D.) and methanol—water (70:30, v/v) at a flow-rate of 40 μl/min.

TABLE I

PLASMA CONCENTRATIONS OF BETAMETHASONE AND 6β-HYDROXYBETAMETHASONE IN THE HORSE ($n = 2$), DETERMINED BY MICRO-LC—MS, FOLLOWING A SINGLE INTRAVENOUS DOSE OF BETAMETHASONE SODIUM PHOSPHATE (0.12 mg/kg)

Time (h)	Concentration ± S.E. (ng/ml)	
	Betamethasone	6β-Hydroxybetamethasone
2	61.05 ± 2.97	14.19 ± 4.74
4	40.08 ± 10.05	13.69 ± 0.41
6	18.31 ± 6.05	6.17 ± 0.79
8	11.86 ± 4.10	5.52 ± 0.32

TABLE II

URINARY EXCRETION OF BETAMETHASONE AND 6β-HYDROXYBETAMETHASONE IN THE HORSE ($n = 2$), DETERMINED BY MICRO-LC—MS, FOLLOWING A SINGLE INTRAVENOUS DOSE OF BETAMETHASONE SODIUM PHOSPHATE (0.12 mg/kg)

Time (h)	Total amount ± S.E. (μg)	
	Betamethasone	6β-Hydroxybetamethasone
1	1335.16 ± 441.02	692.46 ± 66.27
3	656.46 ± 254.27	980.77 ± 279.48
5	295.35 ± 179.06	641.35 ± 333.45
7	105.87 ± 35.28	305.89 ± 40.87
25.5	8.09 ± 1.89	54.72 ± 15.67
29	3.95 ± 0.65	27.34 ± 1.38
47.5	3.91 ± 1.77	2.38 ± 0.40

wide range of sample quantity while retaining a steady reference baseline. Such performance, essential to quantiative analysis, was achieved on multiple sample series of variable durations and thus substantiates DLI micro-LC—MS as a reliable, steadfast system.

Table I gives the plasma concentration data of bethamethasone and 6β-hydroxybetamethasone. The data illustrate relative amounts of parent drug versus metabolite over time following a single intravenous injection of bethamethasone sodium phosphate (0.12 mg/kg).

Table II gives the urinary excretion data of betamethasone and 6β-hydroxybetamethasone. These data along with those for plasma not only demonstrate the expedient excretion of drug but also demonstrate the sensitivity of micro-LC—MS in multiple samples.

Fig. 5 shows the selected-ion profiles and NICI—TIC chromatograms of a 48-h post-administration urine sample extract. The chromatograms illustrate the sensitive, selective nature of micro-LC—MS. In this extract both 6β-hydroxybetamethasone (m/z 313) and its parent, betamethasone (m/z 297) were present at very low concentrations within a complex matrix of interference. Nevertheless, micro-LC—MS was able to quantitatively determine the drugs under circumstances not amenable to conventional LC detectors.

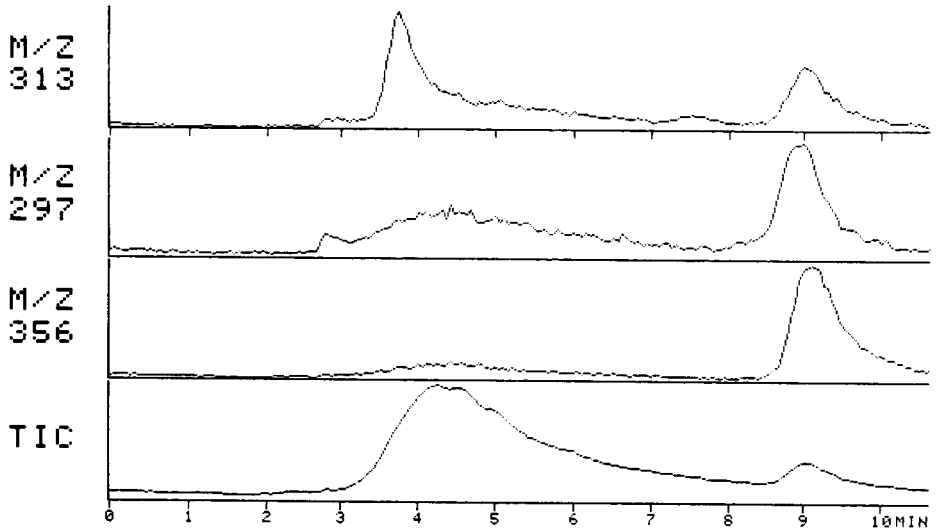

Fig. 5. Selected-ion profiles of 6β-hydroxybetamethasone (*m/z* 313), betamethasone (*m/z* 297) and internal standard, 6α-methylprednisolone (*m/z* 356) and NICI—TIC micro-LC—MS chromatograms of a 48-h post-betamethasone administration urine extract.

CONCLUSION

As shown, DLI micro-LC—MS is a reliable, stable technique which can be utilized over extended periods of time. The additional sensitivity and specificity of DLI micro-LC—MS affords its application to such precision work as quantitative determination of underivatized corticosteroids in a manner superior to that of conventional GC—MS.

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REFERENCES

1 C. Eckers, D.S. Skrabalak and J. Henion, Clin. Chem., 28 (1982) 1882.
2 C. Eckers, J.D. Henion, G.A. Maylin, D.S. Skrabalak, J. Vessman, A.M. Tivert and J.C. Greenfield, Intern. J. Mass Spectrom. Ion Phys., 46 (1983) 205.
3 J. Henion, D. Skrabalak, E. Dewey and G. Maylin, Drug Met. Rev., 14 (1983) 955.
4 D.S. Skrabalak, J.D. Henion, T.R. Covey and K.K. Cuddy, J. Chromatogr., submitted for publication.
5 F.R. Sugnaux, D.S. Skrabalak and J.D. Henion, J. Chromatogr., 264 (1983) 357.
6 R.G. Christensen, E. White, V.S. Meiselman and H.S. Hertz, J. Chromatogr., 271 (1983) 61.
7 D.I. Chapman, M.S. Moss and J. Whiteside, Vet. Rec., 100 (1977) 447.

8 A.V. Tembo, J.W. Ayres, E. Sakmar, M.R. Hallmark and J.G. Wagner, Steroids, 29 (1977) 679.
9 M. Ficher, G.C. Curtis, V.K. Ganjam, L. Joshein and S. Perry, Clin. Chem., 19 (1973) 511.
10 G. Konimani, A. Yamauchi, S. Ishihara and M. Kono, Steroids, 37 (1981) 303.
11 W.J.A. VandenHeuvel and E.C. Horning, Biochem. Biophys. Res. Commun., 3 (1960) 356.
12 D.S. Skrabalak and G.A. Maylin, J. Pharm. Methods, 8 (1982) 291.
13 P. Kucera, J. Chromatogr., 198 (1980) 93.
14 E. Houghton, P. Teale, M.C. Dumasia and J.K. Wellby, Biomed. Mass Spectrom., 9 (1982) 459.
15 J.P. Thenot and E.C. Horning, Anal. Lett., 5 (1972) 905.

Journal of Chromatography, 341 (1985) 271—278
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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DETERMINATION OF OESTRIOL IN PREGNANCY URINE BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the determination of oestriol in pregnancy urine by normal-phase high-performance liquid chromatography with electrochemical detection is described. A large-volume wall-jet cell with an $Ag—Ag^+$ reference electrode was used as the detector system. The limit of detection obtained is comparable to that of electrochemical detection following reversed-phase liquid chromatography. One of the advantages of electrochemical detection with normal-phase systems is that adsorption problems are minimized.

INTRODUCTION

During the later months of pregnancy, especially after the 12th week of gestation, large amounts of oestrogens are produced in the body and excreted [1]. The oestrogens, which are formed in the foeto-placental unit, consist mainly of oestrone, oetradiol and oestriol. Of the three, oestriol is the major oestrogen secreted. The monitoring of the levels of oestriol in urine excreted by pregnant women is a widely accepted test for determining the health of the foetus during the later stages of pregnancy [2, 3]. A deficiency in the excretion levels of oestriol is indicative of a possible malfunction of the placenta.

At present, the main method employed by hospitals for the determination of oestrone in urine is based on a highly specific Kober colour reaction [4], which indicates the total amount of oestrogen present. This can lead to an erroneous conclusion about the condition of the patient as it is really the level of oestriol that is the crucial indicator [5]. Therefore, the Kober colour reaction is usually followed by a spectrofluorimetric determination of the oestriol concentration [6].

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An alternative method to determine selectively the various oestrogen steroids would be to employ high-performance liquid chromatography (HPLC) [7]. The separated oestrogen mixture may be monitored by UV [5, 7], fluorescence or, more recently, electrochemical detection [8—13]. Reports describing the application of HPLC with electrochemical detection to oestrogen steroids have mostly involved reversed-phase systems. Claims have been made that the electrochemical detection of oestrogen steroids is about twenty times more sensitive than UV detection [11]. In contrast to numerous papers published on the electrochemical detection of oestrogen steroids following reversed-phase HPLC [5, 8, 12], little work has been reported on their detection following normal-phase HPLC. One of the few studies dealing with the latter is that of Hiroshima et al. [14]. However, their approach is completely different from that presented in this paper.

The difficulty of employing electrochemical detection in normal-phase HPLC arises from the low dielectric constant of the eluents. The choice of the supporting electrolyte and a suitable reference electrode thereby present difficulties. Indeed, several workers have precluded the use of electrochemical detection with normal-phase HPLC on these grounds [12]. Hiroshima et al. [14] solved the problem by using post-column addition of an electrolyte in a solvent with a high dielectric constant. The ratio of the volume of this conducting solution to that of the low-dielectric-constant eluent was about 3:1. Hence the solvent mixture entering the detector actually has a high dielectric constant. One of the drawbacks of using this approach is that the post-column addition of solution results in dilution in addition to an increase in the volume flow-rate. This results in a decrease in the current response because of the lower solute concentration and its decreased residence time at the working electrode. There is, therefore, a reduction in the efficiency of electrolysis and sensitivity. In the work of Hiroshima et al. [14] the reference was an Ag—AgCl system. This reference is generally not recommended for non-aqueous work because of the junction potentials that arise.

In recent work, however, it was shown that electrochemical detection in normal-phase HPLC can, in fact, be employed, with the addition of tetraalkylammonium salts as the supporting electrolyte to the eluent itself, and with the use of an $Ag—Ag^+$ electrode as the reference system [15]. High limits of detection for the oestrogen steroids (100 pg levels), comparable to those obtained with reversed-phase systems, can be achieved. This contrasts with the results of Hiroshima et al. [14], where detection limits were ten times lower. In this paper, we describe a method for the electrochemical detection of oestriol in pregnancy urine using a large-volume wall-jet cell (WJC). The benefits of using a large-volume WJC in amperometric detection have been discussed in previous papers [15, 16]. We also compare the oestriol levels determined by this electrochemical detection technique with those obtained using a spectrofluorimetric method.

EXPERIMENTAL

Electrochemical system

The large-volume WJC is, in principle, similar to the glass—PTFE cell used in

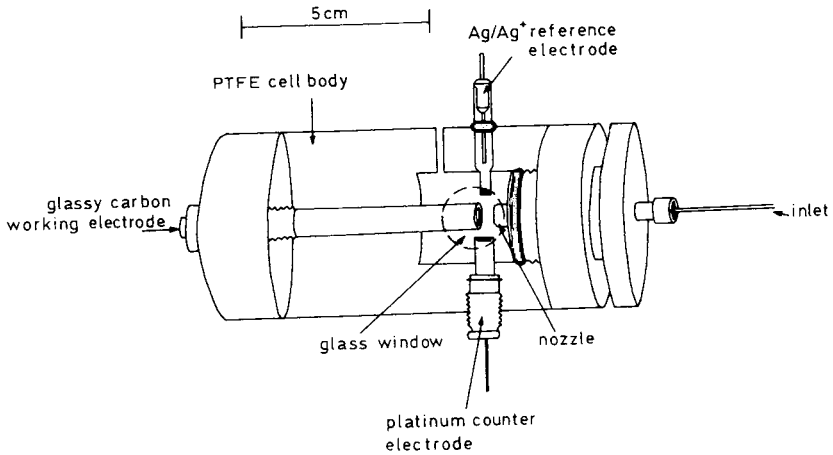

Fig. 1. Cross-section of the wall-jet cell.

previous studies [15, 16]. The main differences as as follows: (1) the cell used in this study is constructed alsmost entirely of PTFE, with the exception of a glass window press-fitted on to the cylindrical cell body; (2) the WJC in this instance operates independently of the PAR Model 303 electrode system because it has its own working, reference and counter electrodes; and (3) the cell volume is about 20 ml, compared with 35 ml for the glass-PTFE cell. Fig. 1 shows a cross-section of the PTFE WJC.

The working electrode was a 5 mm diameter glassy carbon disc (Tokai, Tokyo, Japan), which was press-fitted on to a PTFE casing. A copper lead afforded electrical contact between the glassy carbon disc and the polarographic analyser. Similarly, a 3 mm diameter platinum disc was fitted on to a PTFE casing to serve as the counter electrode. The reference electrode used was an $Ag—Ag^+$ electrode [15]. The silver wire of the reference electrode was dipped into a saturated solution of silver nitrate and tetrabutylammonium fluoroborate $[(C_4H_9)_4BF_4]$ in ethanol. The reference solution was contained in a glass tube with a ceramic frit at the end, which was in contact with the external cell solution. All potentials quoted are with respect to this reference electrode. The nozzle of the WJC was positioned 4 mm away from the working electrode to ensure the back wall of the cell did not interfere with the flow of the hydrodynamic boundary layer [16, 17]. The WJC was controlled by a PAR Model 174A polarographic analyser (Princeton Applied Research, Princeton, NJ, U.S.A.). The current output of the Model 174A was recorded on a Perkin-Elmer Model R-100 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.).

HPLC system

All chromatographic separations were performed on a Perkin-Elmer Series 4 microprocessor-controlled solvent delivery system. The eluents were deaerated with helium and kept under pressure in the solvent chamber throughout the HPLC analysis. The chromatographic column used was a Lichrosorb Si 60 (10 μm) 250 × 4.6 mm I.D. normal-phase column (Merck, Darmstadt, F.R.G.) and the pressures applied were typically 6.6—6.8 MPa. The flow-rate of the eluents was set at 1 ml/min. The samples were injected through

a Rheodyne Model 7105S 175-μl loop injector valve (Rheodyne, Berkeley, CA, U.S.A.) using a 10-μl syringe. The connection between the chromatographic column and the WJC was 10 cm × 0.16 cm I.D. PTFE tubing.

Chemicals

All reagents and chemicals were of analytical-reagent grade and used without further purification. The HPLC eluents used were *n*-hexane and ethanol (Merck). The $(C_4H_9)_4BF_4$ supporting electrolyte was prepared at a concentration of 0.05 *M* in the ethanol eluent, which also contained 0.5% ammonia solution (Ajax Chemicals, Sydney, Australia). The oestrogen standards (oestrone, oestradiol and oestriol), obtained from Sigma (St. Louis, MO, U.S.A.), were prepared in methanol.

Sample collection

The pregnancy (38—40 weeks) urine samples were supplied by Kandang Kerbau Hospital, Singapore.

Sample preparation

The hot acid hydrolysis of pregnancy urine (20 ml) was carried out according to the procedure described by Gelbke et al. [18]. After the hydrolysis and extraction steps, the extracts were evaporated to dryness under vacuum and the residue was dissolved in 1 ml of methanol.

RESULTS AND DISCUSSION

Calibration for oestrone, oestradiol and oestriol

Based on a previous study carried out using normal-phase HPLC, the three oestrogen standards were satisfactorily resolved with *n*-hexane—ethanol (80:20) as the eluent. The optimum potential applied to the working electrode was determined to be +0.4 V versus $Ag—Ag^+$. Under these operating conditions, a detection limit of the order of picomoles can be achieved, which is comparable to those quoted for reversed-phase electrochemical detection.

As the oestriol excretion level in urine samples is expected to be of the order of tens of micromoles per day, the calibration plots for the three oestrogen steroids were obtained at nmol/μl levels. Fig. 2a shows a typical electrochemical chromatogram of the three-oestrogen mixture obtained by normal-phase HPLC, and Fig. 2b shows the calibration graphs for the three oestrogen standards at the nanomole level. The reproducibility for successive injections of the oestrogen mixture was determined to be within a relative standard deviation of 3%.

Recovery studies

In order to characterize the efficiency of the sample preparation technique used, a recovery test was carried out by adding known amounts of oestrogen standards to 20-ml volumes of water and male urine samples. The oestrogen standards were then recovered by the hot acid hydrolysis and extraction steps described earlier. The recoveries of the three oestrogens were then measured using the large-volume WJC following normal-phase HPLC. Fig. 3a and b shows

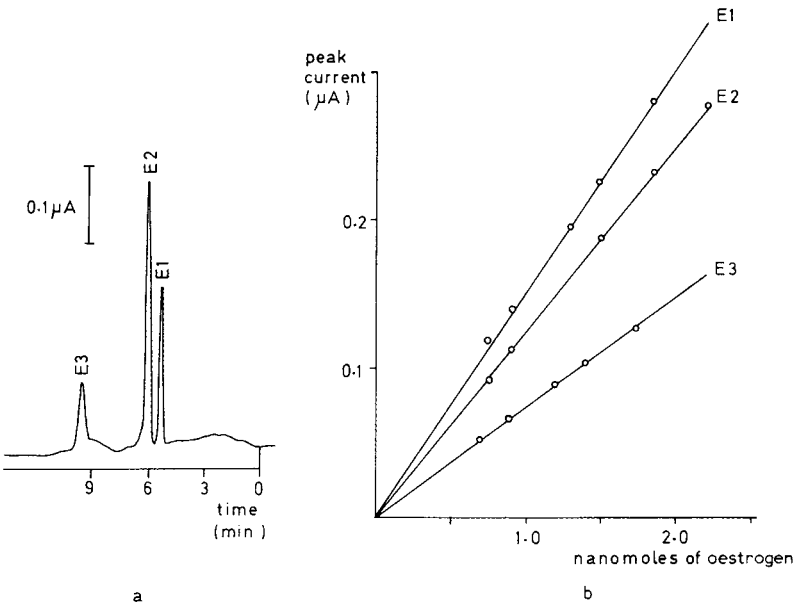

Fig. 2. (a) Chromatogram of the standard oestrogen mixture with electrochemical detection. Oestrone (E1) = 1.85 nmol; oestradiol (E2) = 3.7 nmol; oestriol (E3) = 1.74 nmol. Eluent, *n*-hexane—ethanol (80:20); flow-rate, 1.0 ml/min; working potential, +0.4 V. (b) Calibration graph for oestrogen standards.

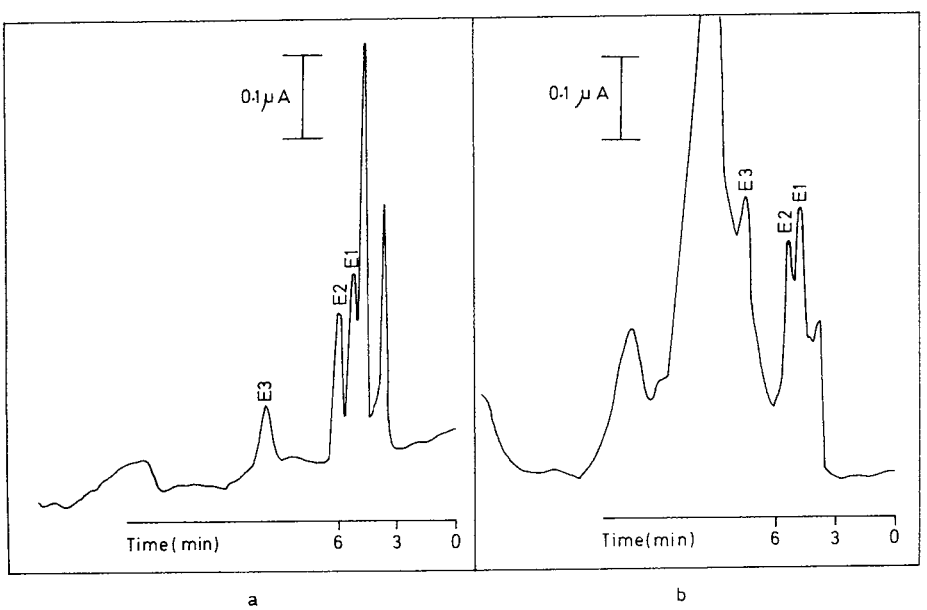

Fig. 3. Chromatogram of the recovered oestrogens in (a) a water sample and (b) a male urine sample with electrochemical detection. Conditions as in Fig. 2. E1 = oestrone; E2 = oestradiol; E3 = oestriol.

the electrochemical chromatograms of the recovered oestrogens in water and male urine samples, respectively. The oestrogen peaks in the water sample were much better resolved than those in the male urine sample, because of the greater number of interfering peaks present in the latter. The recoveries of the oestrogens in both samples are given in Table I. The slightly higher recovery for the water sample is in agreement with the work of Gelbke et al. [18] on catechol oestrogens. The recovery of oestriol in both water and urine samples was the greatest of the three oestrogen steroids studied. Although the recoveries of oestrone and oestradiol were lower, the sample preparation technique used may be judged to be reasonably efficient for the extraction of oestrogen steroids from urine samples.

TABLE I

RECOVERY OF OESTRONE, OESTRADIOL AND OESTRIOL ADDED TO WATER AND MALE URINE SAMPLES (n = 3)

Sample	Recovery (%)		
	Oestrone	Oestradiol	Oestriol
Water	68	76	91
Male urine	64	70	86

Analysis of pregnancy urine

A typical electrochemical chromatogram of a pregnancy urine extract is shown in Fig. 4. The peaks obtained for oestrone and oestradiol are much smaller than the oestriol peak. Also, the oestrone and oestradiol peaks appear to be very poorly resolved on the shoulder of an earlier eluting peak. The poor resolution of both peaks makes quantification difficult. The oestriol levels in pregnancy urine samples are given in Table II, where the results obtained by

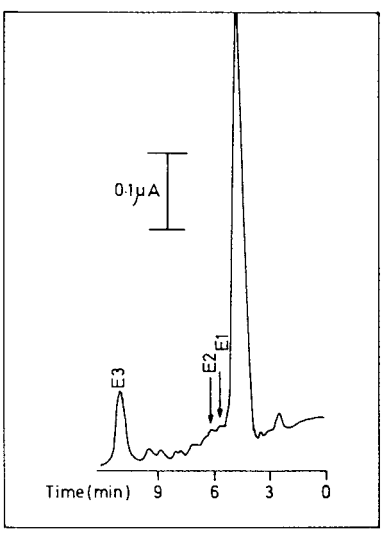

Fig. 4. Chromatogram of a pregnancy urine sample with electrochemical detection. Conditions as in Fig. 2. E1 = oestrone; E2 = oestradiol; E3 = oestriol.

TABLE II

LEVELS OF OESTRIOL IN NORMAL PREGNANCY

Subject	Weeks of gestation	Oestriol determined (μmol/day)	
		Electrochemical detection	Fluorescence detection
A	38	112.7	109.7
B	39	65.1	65.8
C	39	93.7	96.6
D	40	141.6	140.3
E	40	70.5	68.7
F	40	125.7	123.8
G	40	105.8	100.6

normal-phase electrochemical detection are compared with those obtained by spectrofluorimetry (excitation wavelength 520 nm, emission wavelength 550 nm) [7].

Potential selection

As the working potential is lowered, the peak heights of the three oestrogens decrease and the resolution is also reduced. This presumably occurs because the interfering peaks are due to compounds that have a less positive oxidation potential than the three oestrogens. Therefore, in this application, potential selection is not very useful. Although increasing the working potential generally enhances the peak height, it also results in a less stable background. In this work, we found that +0.4 V was an optimum potential that gave stable backgrounds and high sensitivity.

CONCLUSION

This work has shown that normal-phase HPLC with electrochemical detection can be used successfully to determine the oestriol excretion levels in pregnancy urine. The detection limits compare favourably with those reported for electrochemical detection following normal-phase separation and the adsorption problems are significantly less. The difficulty in quantifying oestrone and oestradiol levels in pregnancy urine is chromatographic in nature rather than a detector problem.

ACKNOWLEDGEMENTS

The award of a University Research Scholarship to B.T.T. and a Research Grant to K.P.A. and H.G. from the University are gratefully acknowledged. The authors are indebted to Prof. S.S. Ratnam, Dr. S. Arulkumaran and Mr. Stephen Koh of the Kandang Kerbau Hospital, Singapore, for the supply of urine samples. Thanks are due to Ms. Irene Teo for valuable technical assistance.

REFERENCES

1 F. Spielman, M.A. Golberger and R.T. Frank, J. Amer. Med. Ass., 101 (1933) 266.
2 A. Klopper, Obstet. Gynecol. Surv., 23 (1968) 813.
3 M.C. McNaughton, Amer. J. Obstet. Gynecol., 97 (1967) 998.
4 S. Kober, Biochem. Z., 239 (1931) 209.
5 R.J. Dolphin, J. Chromatogr., 83 (1973) 421.
6 S. Koh, Department of Obstetrics and Gynaecology, Kandang Kerbau Hospital, Singapore, 1983, personal communication.
7 J.F.K. Huber, J.A.R. Hulsman and C.A.M. Meijers, J. Chromatogr., 62 (1971) 79.
8 R.J. Dolphin and P.J. Pergande, J. Chromatogr., 143 (1977) 267.
9 W.R. Prescott, Jr., B.K. Boyd and J.F. Seaton, J. Chromatogr., 234 (1982) 513.
10 K. Shimada, F. Xie and T. Nambara, J. Chromatogr., 232 (1982) 13.
11 Z.K. Shihabi, J. Scaro and B.F. Thomas, J. Chromatogr., 224 (1981) 99.
12 K. Shimada, T. Tanaka and T. Nambara, J. Chromatogr., 178 (1979) 350.
13 K. Shimada, T. Tanaka and T. Nambara, J. Chromatogr., 223 (1981) 33.
14 O. Hiroshima, S. Ikenoya, M. Ohmae and K. Kawabe, Chem. Pharm. Bull., 29 (1981) 451.
15 H. Gunasingham, B.T. Tay and K.P. Ang, Anal. Chem., 56 (1984) 2422.
16 H. Gunasingham, B.T. Tay, K.P. Ang and L.L. Koh, J. Chromatogr., 285 (1984) 103.
17 H. Gunasingham and B. Fleet, Anal. Chem., 55 (1983) 1409.
18 H.P. Gelbke, M. Kreth and R. Knuppen, Steroids, 28 (1973) 665.

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CHROMBIO. 2569

DETERMINATION OF THE SERUM CONCENTRATION OF SPIRONOLACTONE AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and rapid high-performance liquid chromatographic assay is described for the simultaneous determination in serum of the aldosterone antagonist spironolactone and its metabolites 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone. Ultraviolet absorption at 240 nm was used to detect the different compounds after elution on a normal-phase column. Endogenous serum substances did not interfere with the assay. This method provides a convenient tool in pharmacokinetic studies of spironolactone, in contrast to previously reported aspecific fluorimetric assays or time-consuming thin-layer chromatographic analyses of radioactive biological material.

INTRODUCTION

Spironolactone, a synthetic steroid, has been used for more than twenty years

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in the therapy of oedematous conditions, as an adjunct to thiazide diuretics in the treatment of essential hypertension and in all pathological conditions associated with hyperaldosteronism [1, 2]. It is extensively metabolized in the body into a large number of metabolites (Fig. 1) [3—6].

For a long time, canrenone was thought by many authors to be the major metabolite [7, 8], and its serum levels were most commonly measured by a fluorimetric assay developed by Gochman and Gantt [9], which was modified by other investigators [10].

With the introduction of high-performance liquid chromatographic (HPLC) methods to measure canrenone concentrations, it became clear that the fluorimetric method was not specific for canrenone but measured other fluorigenic metabolites as well [11—13]. True canrenone concentrations (i.e. measured by HPLC) were only ca. one third of the concentrations measured with the fluorimetric method [14, 15]. This finding gave support to the thought that canrenone was not the major metabolite [16]. Other metabolites,

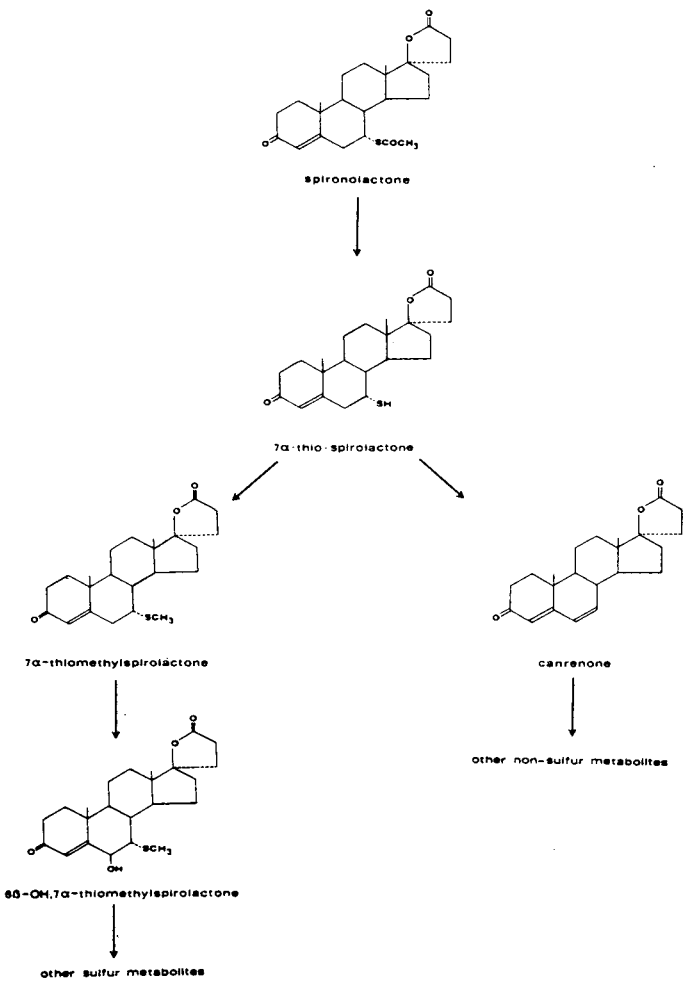

Fig. 1. Routes of metabolism of spironolactone.

or perhaps spironolactone itself, might also be responsible for the activity of spironolactone [17, 18].

The aim of this study was to develop a rapid and sensitive HPLC assay for the simultaneous determination in serum of spironolactone and its metabolites 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone. These are the compounds we found in serum of human volunteers who had taken 200 mg of spironolactone orally.

EXPERIMENTAL

Reagents and standards

Spironolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone were kindly supplied by Searle (Chicago, IL, U.S.A.). Serum standards were prepared by spiking blank serum samples with these compounds.

As the internal standard we used megestrolacetate (Novo, Copenhagen, Denmark). Diisopropyl ether, methanol, ethanol and tetrachloromethane (Merck, Darmstadt, F.R.G.) were of analytical-reagent grade. Methanol and diisopropyl ether were filtered through a Sartorius filter (0.2 μm pore size, Type 11687; Göttingen, F.R.G.) before serving as the mobile phase.

HPLC instrumentation and conditions

The HPLC system consisted of a solvent-delivery pump (Waters Assoc., Milford, MA, U.S.A., Model 510) and a variable-wavelength UV detector (Waters, Model 481), set at 240 nm. A Partisil column (particle size 5 μm, 150 × 4.6 mm I.D., Chrompack, Middelburg, The Netherlands) was used for the separation. To protect the column a silica precolumn (Guard-Pak Precolumn Module, Waters) was installed. The mobile phase consisted of diisopropyl ether—methanol (98.25:1.75) at a flow-rate of 2.2 ml/min. Chromatography was carried out at ambient temperature.

Sample preparation

To 1 ml of serum in a 10-ml centrifuge tube were added 100 μl of internal standard solution (megestrolacetate 2 μg/ml) and 100 μl of demineralised water. For the preparation of the serum standard samples, instead of 100 μl of demineralised water, 100 μl of a standard solution were added. This solution contained spironolactone, 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone in demineralised water, each in a concentration of 2 μg/ml.

Each tube was vortexed to mix the serum with these solutions. Then 5 ml of tetrachloromethane were added and the tubes were shaken mechanically for 10 min. After centrifugation for 5 min at 700 *g*, the aqueous layer was discarded and the organic layer evaporated to dryness under a mild nitrogen stream at 40°C. The residue was reconstituted in 200 μl of the mobile phase and 100 μl were injected onto the column.

Serum samples were spiked with different amounts of spironolactone and its metabolites. Final concentrations of spiked serum samples were 0, 50, 100, 200 and 400 ng/ml.

Reproducibility and recovery studies

The reproducibility of each compound was determined in eight-fold at the concentrations 50 and 200 ng/ml. Blank human serum was spiked with the compounds to obtain these concentrations. Recovery studies were performed for the internal standard and for each of the compounds. Six replicate analyses for each compound and the internal standard were carried out at a concentration of 400 ng/ml. Peak height ratios of the compounds concerned and the internal standard were plotted against the concentrations of the compounds.

RESULTS

Under the conditions described, 7α-thiomethylspirolactone, spironolactone, canrenone and 6β-hydroxy-7α-thiomethylspirolactone are eluted after ca. 7.1, 8.4, 9.3 and 10.1 min, respectively. The internal standard has a retention time of 3.3 min. Fig. 2A shows a representative chromatogram of blank human serum spiked with the above-mentioned compounds. Chromatograms of non-spiked serum samples showed no interference from endogenous serum substances (Fig. 2B).

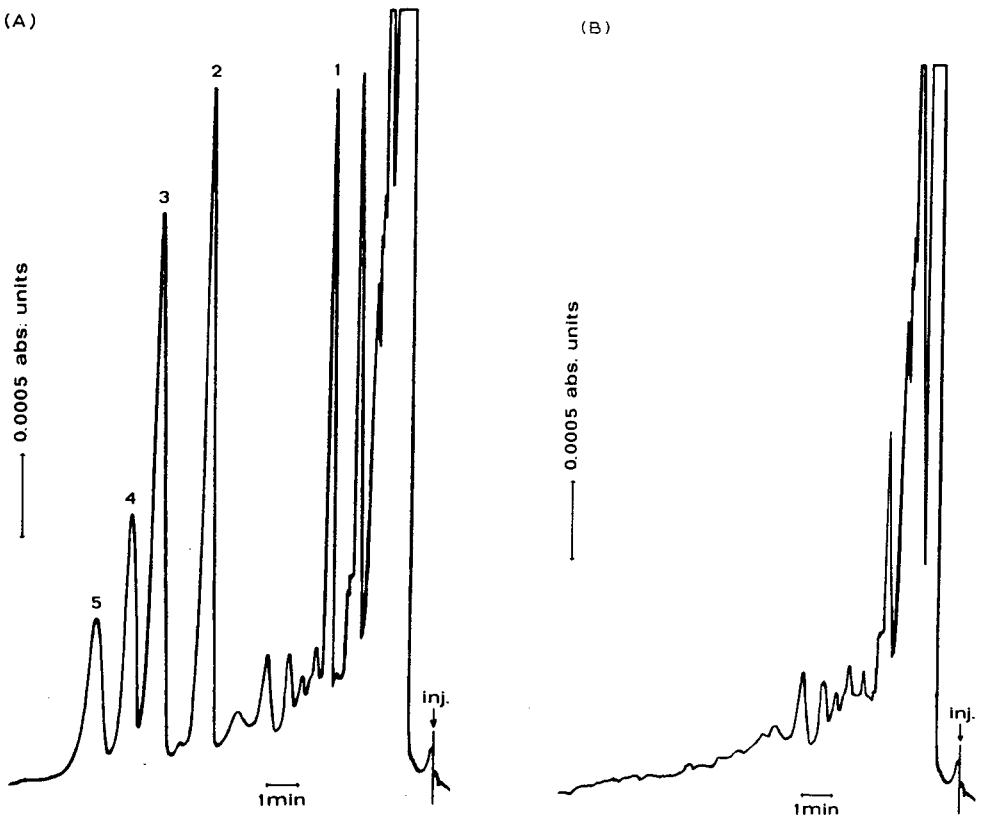

Fig. 2. (A) Chromatogram obtained after extraction from 1 ml of blank human serum spiked with 200 ng of the internal standard megestrolacetate (1), 7α-thiomethylspirolactone (2), spironolactone (3), canrenone (4) and 6β-hydroxy-7α-thiomethylspirolactone (5). (B) Chromatogram obtained after extraction from 1 ml of non-spiked blank human serum.

Methanol in a concentration of 1.75% in diisopropyl ether, at a flow-rate of 2.2 ml/min, was found to give the best resolution and reasonable elution times. In preliminary studies, small differences in the methanol concentration of the mobile phase greatly altered the retention times and the resolution of all compounds.

The detection wavelength was set at 240 nm, at which spironolactone and the thiomethyl- and the hydroxythiomethyl compounds exhibit their absorption maxima. Canrenone has its maximum at 280 nm but, because of a plateau-like absorption curve, it still shows sufficient absorption at 240 nm to be measured accurately. Of the many steroid compounds tested, megestrolacetate was selected as the internal standard because it has a retention time that did not interfere with spironolactone and its metabolites and with endogenous serum compounds.

Serum standard curves for all compounds were linear over the range 50—400 ng/ml. The coefficients of correlation of the standard curves were 0.9994, 0.9998, 0.999 and 0.998 for 7α-thiomethylspirolactone, spironolactone, canrenone and 6β-hydroxy-7α-thiomethylspirolactone, respectively.

The coefficients of variation at two concentrations and the limits of detection for each compound are listed in Table I. The detection limit was defined as the concentration of the compound yielding a signal-to-noise ratio of 4.

TABLE I

COEFFICIENTS OF VARIATION AND LIMITS OF DETECTION OF THE ASSAY METHOD

Coefficients of variation were obtained at two concentrations for each compound by spiking human blank serum with the appropriate amount of each compound. Analyses were performed in eight-fold. Limits of detection are based on extracting 1-ml samples of serum.

Compound	Coefficient of variation (%)		Limit of detection (ng/ml)
	50 ng/ml	200 ng/ml	
7α-Thiomethylspirolactone	8.7	4.5	5
Spironolactone	7.0	3.6	5
Canrenone	6.8	4.7	10
6β-Hydroxy-7α-thiomethylspirolactone	6.6	5.7	20

TABLE II

EXTRACTION RECOVERIES

Recoveries (mean percentage ± S.D.) were determined by extracting 1 ml of serum with a concentration of 400 ng/ml of each compound ($n = 6$ in all cases) and comparing the peak heights measured with those of unextracted compounds.

Compound	Recovery
Internal standard (megestrolacetate)	94 ± 3
7α-Thiomethylspirolactone	83 ± 3
Spironolactone	89 ± 5
Canrenone	95 ± 2
6β-Hydroxy-7α-thiomethylspirolactone	57 ± 3

The recoveries from serum of each of the compounds tested can be found in Table II.

DISCUSSION

The role of the metabolite canrenone in the metabolism and activity of spironolactone is less important than had previously been assumed [11—16]. Other metabolites, or perhaps spironolactone itself, must contribute considerably to the pharmacological effects of spironolactone [16—18]. The sulphur-containing metabolites of spironolactone have been mentioned particularly as possibly active metabolites [2, 16, 18]. Until now, however, no simple and specific assay for the determination of sulphur-containing metabolites and spironolactone in humans has been described.

Two studies described a quantitative thin-layer chromatographic (TLC) analysis of serum after administration of radioactive labelled spironolactone to volunteers [1, 6]. However, contrasting results were found, which were attributed to methodological differences in work-up procedures. Also, in order to obtain sufficient radioactivity to carry out both extraction and TLC analysis, either serum samples had to be pooled [1], or a relatively high dose of radioactivity had to be administered to volunteers [6].

There is obviously a need for a simple, specific and reproducible assay for the determination of spironolactone and its metabolites in biological material. The HPLC method described in this paper permits a rapid simultaneous determination of serum concentrations of spironolactone and its metabolites 7α-thiomethylspirolactone, 6β-hydroxy-7α-thiomethylspirolactone and canrenone.

Preparation of serum samples prior to chromatography is simple. The analysis time for a single sample is ca. 40 min, but this is shortened considerably if a number of samples are extracted and analysed simultaneously. The sensitivity, reproducibility and limits of detection are satisfactory and no interference of endogenous serum compounds is observed. The method will be valuable in clarifying many unknown pharmacokinetic aspects of spironolactone.

REFERENCES

1 A. Karim, J. Zagarella, J. Hribar and M. Dooley, Clin. Pharmacol. Ther., 19 (1976) 158.
2 L.E. Ramsay, in M.K. Agarwal (Editor), Hormone Antagonists, Walter de Gruyter, Berlin, New York, 1982, p. 335.
3 A. Karim and E.A. Brown, Steroids, 20 (1972) 41.
4 A. Karim, J. Hribar, M. Doherty, W. Aksamit, D. Chappelow, E. Brown, C. Markos, L.J. Chinn, D. Liang and J. Zagarella, Xenobiotica, 7 (1977) 585.
5 W. Stüber, E. Mutschler and D. Steinbach, Arch. Pharm. (Weinheim), 314 (1981) 148.
6 U. Abshagen, H. Rennekamp and G. Luszpinski, Naunyn-Schmiedeberg's Arch. Pharmacol., 296 (1976) 37.
7 A. Melander, K. Danielson, B. Schersten, T. Thulin and E. Wåhlin, Clin. Pharmacol. Ther., 22 (1977) 100.
8 W. Krause, J. Karras and U. Jakobs, J. Chromatogr., 277 (1983) 191.
9 N. Gochman and C.L. Gantt, J. Pharmacol. Exp. Ther., 135 (1962) 312.
10 W. Sadée, M. Dagcioglu and S. Riegelman, J. Pharm. Sci., 61 (1972) 1126.
11 G.B. Neurath and D. Ambrosius, J. Chromatogr., 163 (1979) 230.
12 C.G. Dahlöf, P. Lundborg, B.A. Persson and C.G. Regårdh, Drug Metab. Dispos., 7 (1979) 103.

13 E. Besenfelder and R. Endele, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 419.
14 U. Abshagen, E. Besenfelder, R. Endele, K. Koch and B. Neubert, Eur. J. Clin. Pharmacol., 16 (1979) 255.
15 F.W.H.M. Merkus, J.W.P.M. Overdiek, J. Cilissen and J. Zuidema, Clin. Exp. Hypertens., 5 (1983) 239.
16 L. Ramsay, J. Shelton, I. Harrison, M. Tidd and M. Asbury, Clin. Pharmacol. Ther., 20 (1976) 167.
17 F.W.H.M. Merkus, Clin. Pharmacy, 2 (1983) 209.
18 J.H. Sherry, J.P. O'Donnel and H.D. Colby, Life Sci., 29 (1981) 2727.

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CHROMBIO. 2575

DERIVATIZATION IN AQUEOUS SOLUTION, ISOLATION AND SEPARATION OF TETRAHYDRO-β-CARBOLINES AND THEIR PRECURSORS BY LIQUID CHROMATOGRAPHY

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SUMMARY

Derivatization of indole ethylamines and 1,2,3,4-tetrahydro-β-carbolines in aqueous solution with methyl chloroformate has been used to facilitate the isolation of these compounds. The initial derivatization eliminated the potential for the artifactual formation of these compounds via the condensation of the indole ethylamine with an aldehyde or α-keto acid during the work-up procedure. The derivatized compounds possessed improved chromatographic properties which allowed for their facile separation by reversed-phase liquid chromatography and their fluorescent detection at the nanogram level.

INTRODUCTION

The reaction of an aldehyde or α-keto acid with an indole ethylamine produces a 1,2,3,4-tetrahydro-β-carboline (THBC) via a Pictet—Spengler reaction. A generalized reaction for the formation of THBC compounds is presented in Fig. 1. Such reactions readily occur under physiological conditions [1] and produce compounds which can function as neurotransmitters [2] or neuromodulators via their inhibition of uptake [3] or their inhibition of

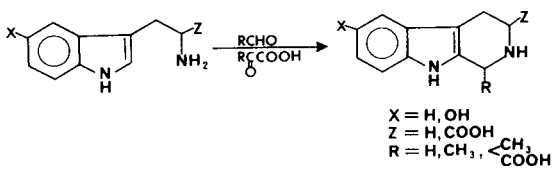

Fig. 1. Formation of 1,2,3,4-tetrahydro-β-carbolines by the condensation of an indoleamine with an aldehyde or α-keto acid.

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monoamine oxidase [4]. Furthermore, these compounds have been increasingly implicated in alcoholism. Acute and chronic administration of select THBC compounds to rats has been reported to significantly alter alcohol consumption [5, 6], and the presence of these compounds in alcoholic beverages and various foods has been confirmed [7—9]. Recently, 6-methoxy-1,2,3,4-tetrahydro-β-carboline has been identified in human pineal and proposed to function as a hormone which modulates the neuronal uptake of serotonin (5-HT) and may play an important role in the pathophysiology of depression [10].

A variety of analytical methods have been employed in attempts to identify and quantitate THBC compounds in vivo [11—18]. All of these methods suffer from limitations of simplicity, selectivity and/or sensitivity. In addition, these methods require considerable sample preparation and manipulation which make them subject to possible artifactual formation of these compounds.

In light of the pharmacological activities of THBC compounds and their possible role in alcoholism and depression, we have developed a simple, sensitive analytical procedure for the isolation, separation and identification of these compounds. In the design of this method three specific objectives were achieved: (1) the development of an analytical method which would allow for the determination of both substrate and products resulting from the reaction of indole ethylamines with aldehydes or α-keto acids; (2) the use of chemical derivatization to fix the sample and eliminate the possibility for artifactual formation; and (3) the use of chemical derivatization to facilitate the isolation of these compounds from tissues or fluids and to improve their chromatographic separation and quantitation.

EXPERIMENTAL

Materials

Tryptamine hydrochloride, α-ethyltryptamine, 5-methoxytryptamine hydrochloride, 5-hydroxytryptamine oxalate, L-tryptophan and Type II porcine liver esterase were purchased from Sigma (St. Louis, MO, U.S.A.). α-Methyl-5-hydroxytryptamine creatinine sulfate was the gift of Upjohn (Kalamazoo, MI, U.S.A.). Tetrahydro-β-carboline and 6-hydroxytetrahydro-β-carboline (6-OHTHBC) were prepared from glyoxylic acid and tryptamine or 5-hydroxytryptamine (5-HT), respectively [19]. 1-Methyltetrahydro-β-carboline (1-MeTHBC) and 6-hydroxy-1-methyltetrahydro-β-carboline (6-OH-1-MeTHBC) were synthesized from acetaldehyde and tryptamine [20] or 5-benzyloxytryptamine [21], respectively. Tetrahydro-β-carboline-3-carboxylic acid (THBC-3-COOH) and 1-methyltetrahydro-β-carboline-3-carboxylic acid (1-MeTHBC-3-COOH) were prepared from L-tryptophan by the procedures of Jacobs and Craig [22] and Snyder et al. [23], respectively. 6-Methoxytetrahydro-β-carboline-1-carboxylic acid (6-OMeTHBC-1-COOH) was prepared from 5-methoxytryptamine and glyoxylic acid under the conditions described by Vejdelek et al. [19]. Methyl chloroformate was purchased from Aldrich (Milwaukee, WI, U.S.A.). Glass-distilled methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and 0.01 *M* sodium acetate, pH 4.6, was prepared from reagent-grade sodium acetate. Solvents were vacuum-

degassed prior to use. Standard solutions (100 μg/ml) were prepared in 0.1 *M* perchloric acid and diluted to the desired concentration.

Instrumentation

Liquid chromatography was performed using a Varian 5020 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a universal loop injector, a 5-cm column guard packed with Vydac reversed-phase hydrocarbon (Separations Group, Hesperia, CA, U.S.A.) and a 5-μm Zorbax ODS, 25 cm × 4.6 mm I.D. column (DuPont, Wilmington, DE, U.S.A.). Samples were eluted at a flow-rate of 0.8 ml/min using one of the following solvent systems: (A) methanol—0.01 *M* sodium acetate (pH 4.6) (65:35); (B) methanol—0.01 *M* sodium acetate (pH 4.6) (45:55). Fluorescence detection was achieved using a Fluorichrom detector (Varian) equipped with a deuterium arc source and using a 200I excitation filter and a Corning 7-60 band filter (360 mm) for emission.

Sample preparation

The sample preparation procedure is outlined in Fig. 2. The sample (tissue homogenate or biological fluid) consisted of 0.5 ml of 0.1 *M* perchloric acid to which were added 25 μl of 1.0 *M* semicarbazide plus the appropriate internal standard. The sample was treated with 0.5 ml of 1.0 *M* dipotassium hydrogen phosphate (pH 7.2), 50 μl of methyl chloroformate, vortexed, and allowed to stand for 5 min. The sample pH was increased to 9.5 by the addition of 0.25 ml of saturated sodium carbonate (pH 11.5), 50 μl of methyl chloroformate were again added, and the sample was vortexed and allowed to stand for 10 min. Extraction of the sample with 6.0 ml of dichloromethane yielded basic precursors and tetrahydro-β-carbolines upon evaporation under nitrogen. The

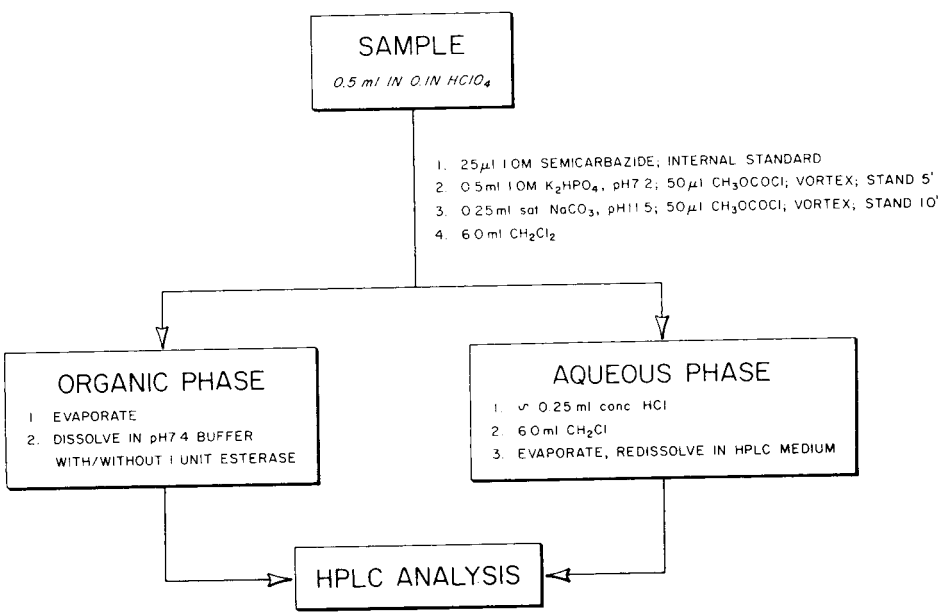


Fig. 2. Flow chart of the sample preparation procedure for the determination of 1,2,3,4-tetrahydro-β-carbolines and their precursors.

residue was dissolved in 0.5 ml of 0.1 *M* disodium hydrogen phosphate buffer (pH 7.4). In samples which were to be analyzed for 5-HT, 6-OHTHBC or 6-OH-1-MeTHBC, the buffer contained 1 U of esterase per 0.5 ml of buffer.

Acidic compounds were isolated from the remaining aqueous phase by careful acidification of the aqueous phase with concentrated hydrochloric acid (ca. 250 μl) followed by extraction with 6.0 ml of dichloromethane and evaporation under nitrogen. The samples were dissolved in solvent system B prior to analysis.

RESULTS AND DISCUSSION

This method is based on the known chemical reactivity of alkyl chloroformates, specifically methyl chloroformate, with amines and phenols in aqueous solution to produce carbamate and carbonate derivatives, respectively [24]. Earlier work by Brooks and Horning [25] had demonstrated the potential utility of acetylation in the isolation of amines from dilute aqueous solutions. By initially derivatizing the aqueous sample with methyl chloroformate, the potential for the artifactual formation of THBC compounds is markedly decreased or eliminated, since the precursor indole ethylamine is no longer free and available to react with an aldehyde or α-keto acid. Indeed, using this method we have demonstrated that under conditions which had previously been shown to produce substantial amounts of artifactual 6-OHTHBC in human platelets [26], initial derivatization with methyl chloroformate completely eliminated this problem and confirmed the absence of 6-OHTHBC in human platelets.

The stable, lipophilic products of this derivatization can be quantitatively extracted into a non-polar organic solvent and isolated. In addition to facilitating the isolation of these compounds from an aqueous medium, the chemical derivatization yields products which possess superior reversed-phase chromatographic properties to those of the parent compounds. The excellent chromatographic separation of tryptamine, THBC, 1-MeTHBC and the internal standard, α-ethyltryptamine, is demonstrated in Fig. 3.

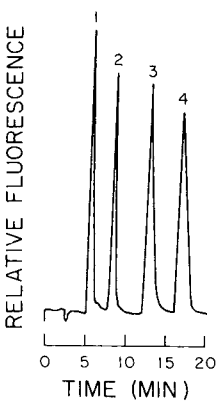


Fig. 3. Chromatogram of tryptamine (1), THBC (3), 1-MeTHBC (4) and the internal standard α-ethyltryptamine (2) eluted with solvent system A.

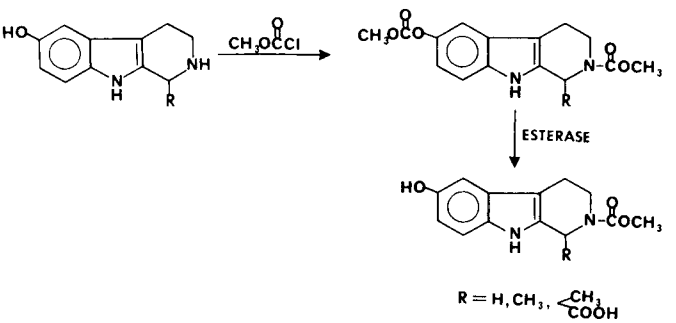
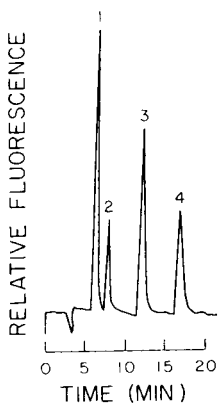


Fig. 4. The chemical derivatization reaction of a 6-OHTHBC compound.

Rapid derivatization of phenols, such as 5-HT, 6-OHTHBC and 6-OH-1-MeTHBC, occurs at low pH and this permits their protection from oxidation prior to shifting to the high pH values required for the derivatization of amines [27]. The acylation of phenolic indoles or phenolic THBC compounds results in the complete loss of their native fluorescence. As a result, this necessitates the selective hydrolysis of the carbonate function in the presence of the carbamate. This can be readily accomplished by treatment of the extraction residue with 1 U of esterase in phosphate buffer for 10 min at room temperature. The derivatization of a 6-OHTHBC compound is shown in Fig. 4. The chromatogram of these phenolic compounds and the internal standard, α-methyl-5-HT, is shown in Fig. 5.

The requirement for the treatment of phenolic indole ethylamines and THBC compounds with esterase can be used to confirm peaks present in the chromatogram of a sample and thereby provide additional structural information. For example, Fig. 6 shows chromatograms of 5-HT and 6-OH-1-MeTHBC in the presence and absence of esterase. When the sample is chromatographed after esterase treatment, a simple chromatogram containing 5-HT and 6-OH-1-MeTHBC is obtained (Fig. 6A); however, the chromatogram obtained in the absence of esterase treatment reveals the absence of these compounds (Fig. 6B).



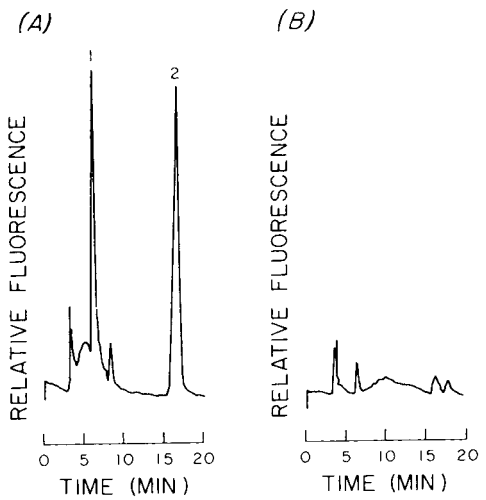


Fig. 5. Chromatogram of 5-HT (1), 6-OHTHBC (3), 6-OH-1-MeTHBC (4) and the internal standard, α-methyl-5-HT (2) eluted with solvent system B.

Fig. 6. Chromatograms of 5-HT (1) and 6-OH-1-MeTHBC (2) with (A) and without (B) esterase treatment.

Using these phenolic compounds the limit of sensitivity and the reproducibility of the method was determined. The limit of sensitivity (three times background) for 5-HT, 6-OHTHBC and 6-OH-1-MeTHBC was found to be less than 1 ng per sample, while the reproducibility of the method (12 ng per sample, n = 8) gave coefficients of variation of 1.63%, 1.75% and 1.46%, respectively.

The chromatogram of the acidic THBC compounds derived from the condensation of tryptophan with formaldehyde and acetaldehyde is shown in

Fig. 7, along with the internal standard, 6-OMeTHBC-1-COOH. The chromatographic data for these compounds are summarized in Table I.

In conclusion, a simple, sensitive procedure for the determination of tetrahydro-β-carbolines and their indolic precursors based on the aqueous derivatization with methyl chloroformate has been described. The utilization of this method, which improves the isolation and chromatographic separation of these compounds and eliminates the potential for artifactual formation, should greatly facilitate the study of THBC compounds and provide an alternate means for examining the in vivo presence of these compounds.

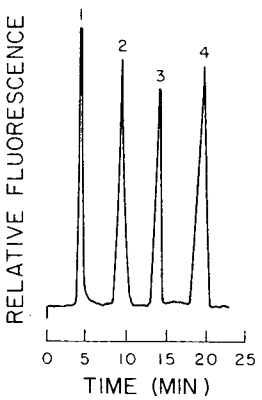


Fig. 7. Chromatogram of tryptophan (1), THBC-3-COOH (3), 1-MeTHBC-3-COOH (4) and the internal standard, 6-OMeTHBC-1-COOH (2) eluted with solvent system B.

TABLE I

CHROMATOGRAPHIC DATA FOR THBC COMPOUNDS AND PRECURSORS

See Experimental for chromatographic conditions. Samples were eluted at a flow-rate of 0.8 ml/min using one of the following solvent systems: (A) methanol—0.01 *M* sodium acetate buffer pH 4.6 (65:35); (B) methanol—0.01 *M* sodium acetate buffer pH 4.6 (45:55).

Compound	Retention time (min)	Solvent system
Tryptamine	6.03	A
α-Ethyltryptamine	8.73	A
THBC	13.59	A
1-MeTHBC	16.86	A
5-HT	6.15	B
α-Me-5-HT	7.73	B
6-OHTHBC	12.01	B
6-OH-1-MeTHBC	16.78	B
Tryptophan	4.84	B
6-OMeTHBC-1-COOH	9.90	B
THBC-3-COOH	14.30	B
1-MeTHBC-3-COOH	19.87	B

REFERENCES

1 W.M. Whaley and T.R. Govindachari, in R. Adams, H. Adkins, H. Blatt and A.M. Cope (Editors), Organic reactions, Vol. 6, Wiley, New York, 1951, p. 151.
2 N.S. Buckholtz, Life Sci., 27 (1980) 893.
3 H. Rommelspacher, S.M. Strauss and K. Rehse, J. Neurochem., 30 (1978) 1573.
4 N.S. Buckholtz and W.O. Boggaan, Biochem. Pharmacol., 25 (1976) 2319.
5 I. Geller, R. Purdy and J.H. Merritt, Ann. N.Y. Acad. Sci., 215 (1973) 54.
6 R.D. Myers and M.M. Oblinger, Drug Alcohol Depend., 2 (1977) 469.
7 O. Beck and B. Holmstedt, Food Cosmet. Toxicol., 19 (1981) 173.
8 O. Beck, T.R. Bosin and A. Lundman, J. Agric. Food Chem., 31 (1983) 288.
9 T.R. Bosin, A. Lundman and O. Beck, J. Agric. Food Chem., 31 (1983) 444.
10 S.Z. Langer, C.R. Lee, A. Segonzac, T. Tateishi, H. Esnaud, H. Schoemaker and B. Winblad, Eur. J. Pharmacol., 102 (1984) 379.
11 D.W. Shoemaker, J.T. Cummins and T.G. Bidder, Neurosciences, 3 (1978) 233.
12 H. Honecker and H. Rommelspacher, Naunyn-Schmiedeberg's Arch. Pharmacol., 305 (1978) 135.
13 H. Rommelspacher, H. Honecker, M. Barbey and B. Meinke, Naunyn-Schmiedeberg's Arch. Pharmacol., 310 (1979) 35.
14 S. Barker, R.E. Harrison, G.B. Brown and S.T. Christian, Biochem. Biophys. Res. Commun., 87 (1979) 146.
15 I. Kari, P. Peura and M.M. Airaksinen, Med. Biol., 57 (1979) 412.
16 S.A. Barker, R.E.W. Harrison, J.A. Monti, G.B. Brown and S.T. Christian, Biochem. Pharmacol., 30 (1981) 9.
17 O. Beck, T.R. Bosin, A. Lundman and S. Borg, Biochem. Pharmacol., 31 (1982) 2517.
18 K.F. Faull, R.B. Holman, G.R. Elliott and J.D. Barchas, in F. Bloom, J. Barchas, M. Sandler and E. Usdin (Editors), Betacarbolines and Tetrahydroisoquinolines, Alan R. Liss, New York, 1982, p. 125.
19 Z.J. Vejdelek, V. Trcka and M. Protiva, J. Med. Pharm. Chem., 3 (1961) 427.
20 S. Akabori and J. Saito, Chem. Ber., 63 (1930) 2245.
21 R.G. Taborsky and W.M. McIsaac, J. Med. Chem., 7 (1964) 135.
22 W.A. Jacobs and L.C. Craig, J. Biol. Chem., 113 (1936) 759.
23 H.R. Snyder, C.H. Hansch, L. Katz, S.M. Parmerter and E.C. Sparth, J. Amer. Chem. Soc., 70 (1948) 219.
24 N.O. Ahnfelt and P. Hartvig, Acta Pharm. Suecica, 17 (1980) 307.
25 C.J.W. Brooks and E.C. Horning, Anal. Chem., 36 (1964) 1540.
26 T.R. Bosin, B. Holmstedt, A. Lundman and O. Beck, Anal. Biochem., 128 (1983) 287.
27 A.P.J.M. de Jong and C.A. Cramers, J. Chromatogr., 276 (1983) 267.

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STUDIES OF FISH ZONA PELLUCIDA BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY ON AGAROSE COLUMNS AND FREE ZONE ELECTROPHORESIS

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SUMMARY

By either free zone electrophoresis or high-performance ion-exchange chromatography on DEAE agarose, zona pellucida from Baltic small herring (*Clupea harengus* L.) was separated into several fractions. These fractions had very similar protein compositions, since on polyacrylamide gel electrophoresis in sodium dodecyl sulphate they all gave the same pattern: chiefly one major and three minor bands corresponding to proteins with the following estimated molecular weights: 78 000, 96 000 (the major component), 115 000, and 125 000. It is likely that these proteins constitute the so-called supramolecular complexes of zona pellucida from Baltic small herring. Only one electrophoretic and one chromatographic fraction gave precipitin arcs on immunodiffusion with rabbit antiserum against zona pellucida from the fish *Aristichthys nobilis* (Richardson).

INTRODUCTION

Zona pellucida, the acellular coat surrounding the mammalian oocyte to which the sperm binds in the initial step of fertilization, is of great interest in reproductive biology research, especially in connection with attempts to devise an immunological contraceptive method. Antibodies against zona pellucida prevent the sperm from binding, and female rabbits immunized with antigens from zona pellucida become temporarily infertile [1]. Some cases of female sterility may be due to autoantibodies against antigens from zona pellucida [2, 3].

For studies of the fertilization process at the molecular level and for the development of a contraceptive vaccine, it is of primary importance to have

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access to highly purified zona proteins and the complexes in which they exist in zona pellucida (called supramolecular complexes by Dunbar et al. [4]. Since zona pellucida can often be prepared in only minute amounts and is relatively labile, methods such as high-performance liquid chromatography (HPLC) and free zone electrophoresis with their high resolution, short run times, and applicability with very small amounts of material, are attractive for the separation of the proteins and the supramolecular complexes from zona pellucida. In this paper we describe the application of these methods to zona pellucida from Baltic small herring (*Clupea harengus* L.). The main reason for the choice of fish eggs as starting material is that these are available in the relatively large amounts that might be required during the development of a purification procedure, which ought to be applicable with zona pellucida from other animal species.

MATERIALS, METHODS AND EQUIPMENT

Rabbit antiserum against zona pellucida from the fish *Aristichthys nobilis* (Richardson) was used for analyses of chromatographic and electrophoretic fractions by the Ouchterlony double-immunodiffusion test.

The following standard proteins from Bio-Rad (Richmond, CA, U.S.A.) were used for estimation of molecular weights of zona pellucida proteins by polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS): ovalbumin (mol. wt. 43 000), bovine serum albumin (67 000), phosphorylase b (94 000), and β-galactosidase (116 000). For estimation of protein molecular weights by pore gradient electrophoresis in the absence of SDS we used the following proteins, available from Pharmacia, Uppsala, Sweden: ovalbumin (43 000), bovine serum albumin (67 000), catalase (232 000), ferritin (440 000), thyroglobulin (669 000). Sephadex G-25 was also obtained from Pharmacia.

Diethylaminoethanol was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.).

G3707, a neutral detergent, was a gift from Dr. D. De Coster (Atlas Chemie, Everberg, Belgium).

Free zone electrophoresis was performed, as previously described, in a quartz tube (390 × 3 mm I.D.) slowly rotating around its long axis to suppress disturbing convection [5, 6].

Preparation of heat-soluble zona pellucida

The method was similar to that described by Wu and co-workers [7, 8]. The fish eggs (ca. 2 g) were suspended in 6 ml of 0.4 *M* Tris—acetic acid (pH 8.7) containing 0.58% (w/v) sodium chloride, 0.29% (w/v) EDTA and 0.2% (w/v) sucrose. Following incubation at 72 °C for 20 min (with stirring) the suspension was cooled to 4 °C. Most of the heavy material was removed by centrifugation at 1500 *g* for 10 min. The supernatant obtained on centrifugation at 23 000 *g* for 30 min contained the zona pellucida and some contaminants, which were removed by further centrifugation under the same experimental conditions. This zona pellucida preparation was used directly in the free zone electrophoresis experiments. However, prior to application onto the HPLC columns, the preparation was passed through a Sephadex G-25 bed equilibrated with 0.05 *M* sodium chloride to remove low-molecular-weight compounds.

Coupling of DEAE groups to agarose

The 15% agarose beads (15 g; 5—15 μm) were prepared as previously described [9] and cross-linked with divinylsulphone [10, 11]; the deactivation step with mannitol was omitted. The beads were washed with water until the supernatant had a pH of 7. These and other washings were made by centrifugation at 600 *g* for 3 min. Diethylaminoethanol (6 ml) was mixed with 14 ml of 2 *M* potassium hydroxide, and the mixture was added dropwise and with stirring to the centrifuged agarose beads. The stirring was continued for 12 h at room temperature to let the alcohol react with vinyl groups in the agarose. The DEAE—agarose beads obtained were then washed five times with 20-ml portions of water, the pH of which had been adjusted to 12 with potassium hydroxide. Finally, the gel was washed with water until pH 7 was reached.

The HPLC equipment (UV-monitor 2158 SD, pump 2150, controller 2152, recorder 2210) was from LKB Produkter (Bromma, Sweden).

RESULTS

Estimation of the molecular weights of the zona pellucida proteins

Electrophoresis of fresh zona pellucida from Baltic small herring in a polyacrylamide gel in the presence of SDS according to the method of Neville [12], as slightly modified by Jergil and Ohlsson [13], gave the pattern shown in Fig. 1a. The electropherogram shows four main protein bands, denoted ZP1, ZP2, ZP3 and ZP4. By means of a calibration curve derived with the standard proteins listed earlier, the molecular weights were estimated at 125 000 (ZP1), 115 000 (ZP2), 96 000 (ZP3), and 78 000 (ZP4).

Zona pellucida material that had been kept at room temperature at pH 8 for 4—5 h gave a more complex pattern (Fig. 1b), probably owing to proteolysis. The proteins were found to be more stable at pH 5.

In an attempt to estimate the molecular weights in the absence of SDS, we employed electrophoresis in a gel gradient of polyacrylamide from $T = 24\%$ $C = 4\%$ to $T = 4\%$ $C = 4\%$ [T is the total concentration (w/v) of acrylamide and N,N-methylenebisacrylamide; C is the cross-linking concentration (w/w), see ref. 14]. The gel had the following dimensions: 6.5 × 6.5 × 0.3 cm. The run was conducted at 250 V (4.8 mA) for 11 h at pH 8.4 in a buffer consisting of 0.09 *M* Tris, 0.08 *M* boric acid and 0.0025 *M* EDTA [15]. Only one major protein band with pronounced tailing was obtained, corresponding to a molecular weight of 220 000 as estimated roughly from a comparison with the standard proteins (Fig. 2a). However, a heavy protein precipitate was also seen at the top of the gel. A sample that had been stored at room temperature (pH 8) for one day gave the pattern shown in Fig. 2b (cf. Fig. 1b). It should be emphasized that all of the molecular weights given above are very approximate, since zona pellucida proteins contain carbohydrate [16, 17] and it is well known that there are large uncertainties in the estimation of molecular weights of glycoproteins by the electrophoresis methods used in this study [18].

Anion-exchange chromatography of zona pellucida

After ultrasonic treatment for 10 min, the DEAE—agarose beads were packed in water to a height of 33 cm in a Plexiglas column (0.6 cm I.D.). Water, adjusted to pH 4 with hydrochloric acid, was passed through the column overnight at a

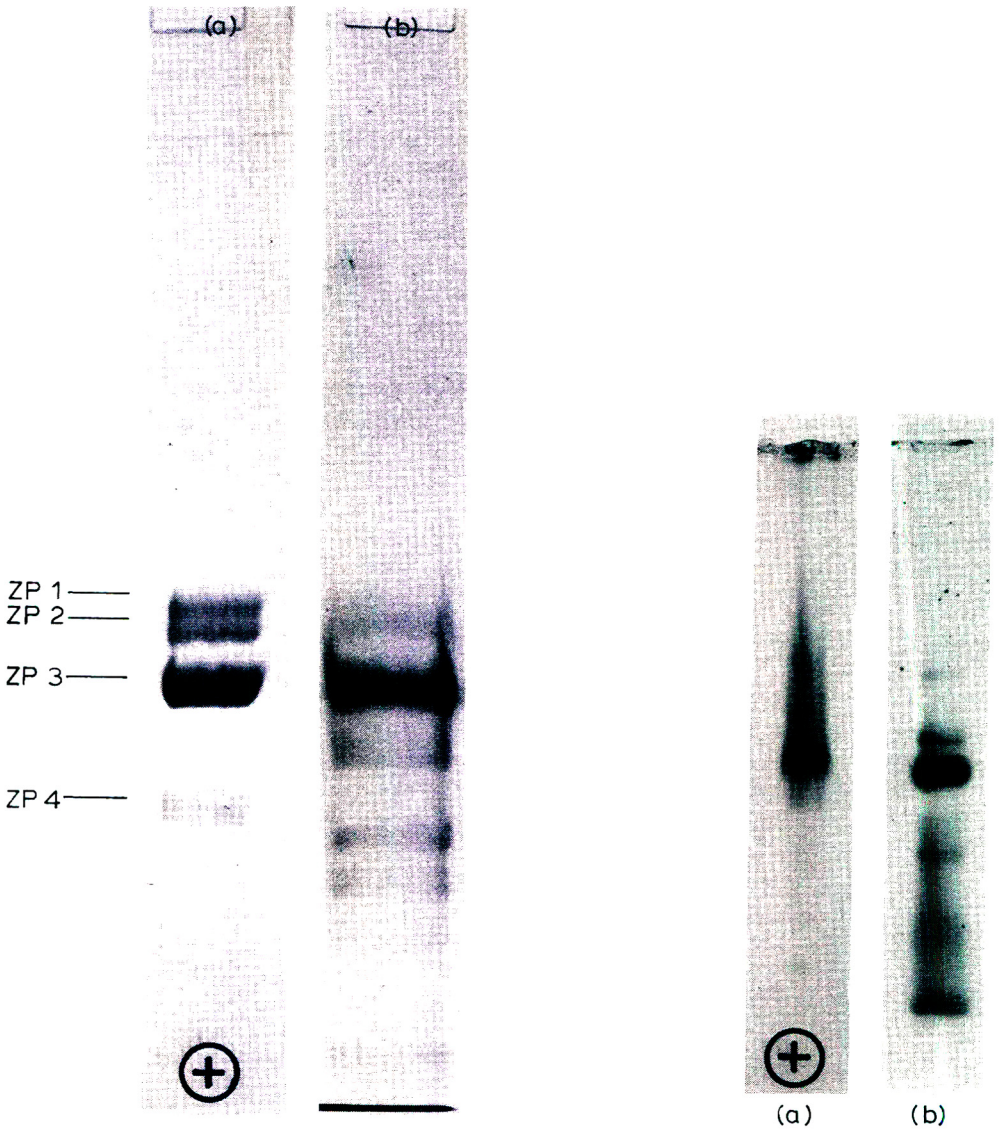

Fig. 1. Polyacrylamide gel electrophoresis in SDS of zona pellucida from Baltic small herring (*Clupea harengus* L.). (a) Fresh material; (b) material stored at room temperature (pH 8) for 4—5 h. The molecular weights of the proteins ZP1, ZP2, ZP3, and ZP4 were estimated roughly at 125 000, 115 000, 96 000, and 78 000, respectively. A comparison between the two protein patterns shows that zona pellucida is easily degraded.

Fig. 2. Pore gradient gel electrophoresis of zona pellucida from Baltic small herring. The run was made in the absence of SDS. (a) Fresh material; (b) material stored at room temperature (pH 8) for one day. The molecular weight of the main protein in fresh material was estimated at 220 000. Much material was retained at the top of the gel.

flow-rate of 0.1 ml/min, followed by 0.05 *M* Tris—acetic acid (pH 7.0) for equilibration of the bed. About 40 µl of a sample of heat-solubilized zona pellucida was applied. A linear gradient from 0.05 *M* Tris—acetic acid (pH 7.0)

to 0.25 *M* Tris—acetic acid (pH 7.0) during 30 min was used for elution at a flow-rate of 1 ml/min and a pressure of 2 bars (Fig. 3a). The experiment was repeated with other compositions of the buffer (see Fig. 3b and c). Upon immunodiffusion, only fractions corresponding to the highest peak in each of the three chromatograms gave a precipitin arc (e.g. Fig. 4), which was visible even when these fractions were strongly diluted (sometimes twenty-fold). No arcs were observed when the chromatographic experiment was performed in buffers of alkaline pH (Tris—acetic acid, pH 8.8; sodium phosphate, pH 8.2; sodium borate, pH 8.0). However, when the Tris—acetic acid buffer (pH 8.8) contained 20% (v/v) ethylene glycol, an immunoprecipitate was again obtained with the material from the highest peak in the chromatogram (ethylene glycol is known to stabilize proteins [19]).

The immunologically active fractions corresponding to the highest peak in Fig. 3a, were rechromatographed on the same column and under the same experimental conditions as the run in Fig. 3a. The chromatogram showed only one peak in the same position as the highest peak in Fig. 3a. The immunologically active material was thus chromatographically homogeneous in this system.

When analyzed by PAGE in the presence of SDS, all of the chromatographic fractions in Fig. 3 (except those corresponding to the first small peaks, which did not contain protein) gave the same electrophoretic pattern as the applied sample, i.e. that shown in Fig. 1a.

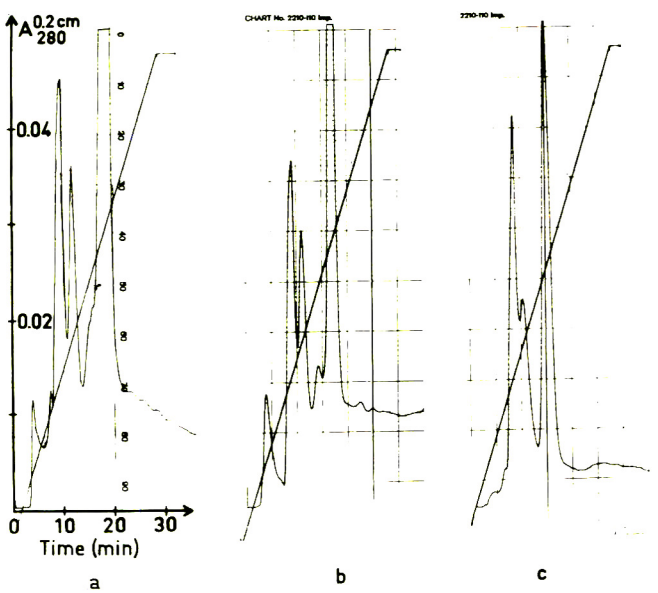

Fig. 3. High-performance ion-exchange chromatogram of zona pellucida from Baltic small herring on DEAE—agarose. (a) Gradient from 0.05 *M* Tris—acetic acid (pH 7.0) to 0.25 *M* Tris—acetic acid (pH 7.0) for 30 min. (b) The same as (a) except that both buffers also contained 1% (w/v) G3707, a neutral detergent [20]. (c) The same as (a) except that both buffers also contained 20% (v/v) ethylene glycol. In all experiments only material corresponding to the highest peak gave a precipitin line on immunodiffusion (Fig. 4). When analyzed by polyacrylamide gel electrophoresis in SDS all peaks gave the same electrophoretic pattern, similar to that shown in Fig. 1a.

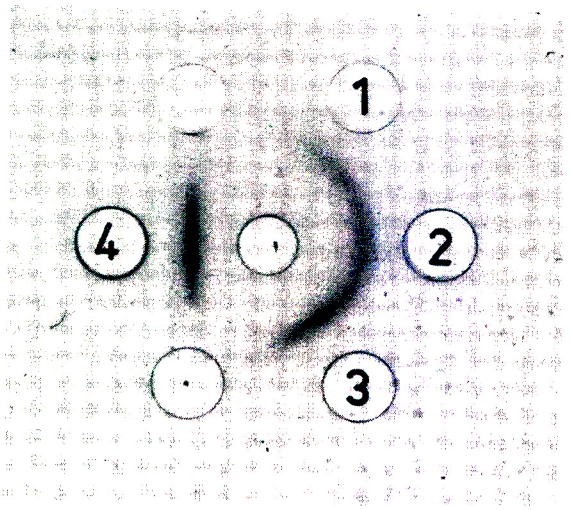

Fig. 4. Immunodiffusion of zona pellucida material. Well 1: heat-solubilized zona pellucida, prepared as described under Materials, methods and equipment, and diluted 1:1 with the buffer used in the extraction of zona pellucida (the same buffer was used in the immunodiffusion experiment). Well 2: the same as well 1, but no dilution of the sample. Well 3: the same as well 1 after "desalting" on Sephadex G-25. Well 4: material corresponding to the highest peak in Fig. 3b.

Free zone electrophoresis of zona pellucida

About 5 µl of heat-solubilized zona pellucida were placed in the rotating electrophoresis tube filled with 0.05 *M* Tris—acetic acid buffer (pH 7.0). The run was conducted at 1580 V (4 mA). Fig. 5a shows the scans obtained after electrophoresis for 0, 10 and 20 min. After the last scan, material corresponding to each of the peaks was withdrawn for analysis by SDS electrophoresis: all peaks gave the same electrophoretic pattern, namely that of the applied sample, i.e. the pattern shown in Fig. 1a. In immunodiffusion, only material corresponding to the highest peak gave a precipitin line.

When the experiment was repeated in a buffer containing 1% (w/v) of the detergent G3707 [20], a similar electropherogram was obtained (Fig. 5b). The analyses by SDS electrophoresis and immunodiffusion gave the same result as the run done without detergent.

DISCUSSION

As the term "free zone electrophoresis" implies, this technique permits separations in a carrier-free medium (in buffer alone) and is therefore suitable for the fractionation of particles that are too large to penetrate the pores in a polyacrylamide gel. The supramolecular complexes of zona pellucida from porcine oocytes seem to be as large as that, to judge from a paper by Dunbar et al. [4]: "Attempts to separate the zona components by electrophoresis in the absence of SDS were totally unsuccessful, as supramolecular aggregates are present which do not enter the gel". Similarly, the corresponding protein complexes in zona pellucida from Baltic small herring were retained at the top

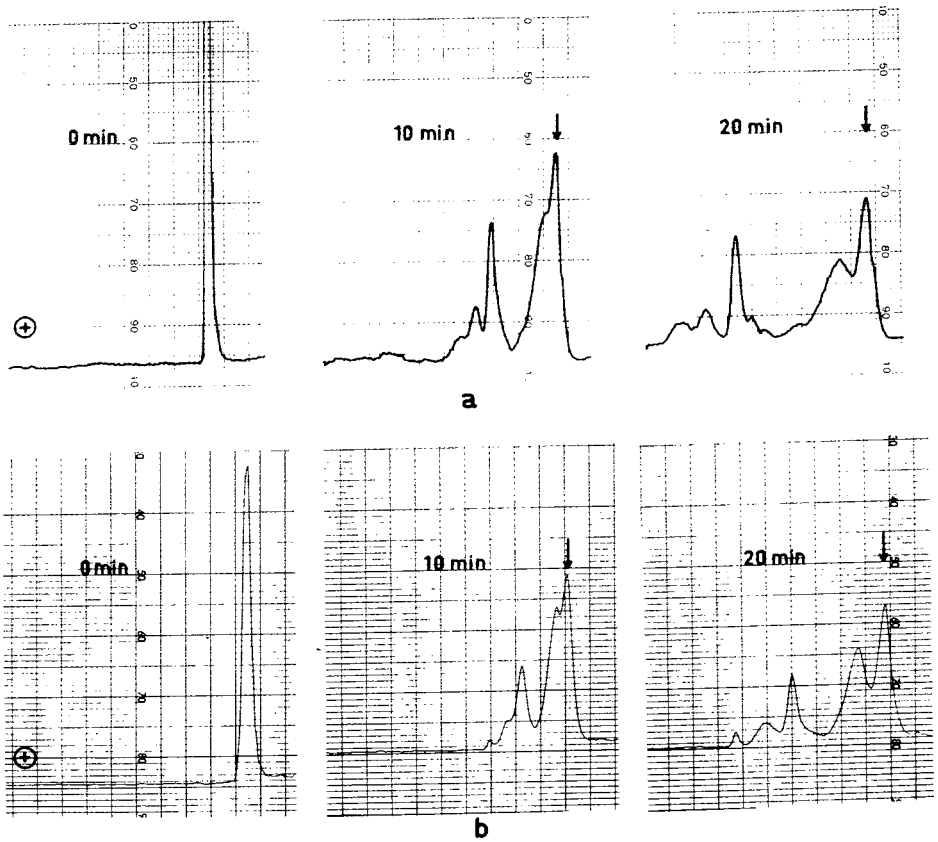

Fig. 5. Free zone electrophoresis of zona pellucida from Baltic small herring. The experiments were performed in (a) 0.05 *M* Tris—acetic acid (pH 7.0) and (b) the same buffer containing 1% (w/v) G3707, a neutral detergent [20]. The arrow indicates the position of the starting zone. When analyzed by immunodiffusion, only fractions corresponding to the highest (stationary) peak gave precipitin arcs. When these fractions were analyzed by polyacrylamide gel electrophoresis in SDS they showed the same pattern as that in Fig. 1a, i.e. that of the applied sample.

of a pore-gradient gel in the absence of SDS: only a complex of a molecular weight ca. 220 000 migrated into the gel (Fig. 2a). It should be emphasized that any supporting medium can induce the formation of protein aggregates and precipitates (see below and ref. 19). Free zone electrophoresis is accordingly a valuable method for analysis and separation of the large supramolecular complexes of zona pellucida.

Both in free zone electrophoresis (Fig. 5) and high-performance ion-exchange chromatography (Fig. 3), zona pellucida from Baltic small herring showed a complex pattern with several peaks. Only one electrophoretic and one chromatographic fraction gave a precipitin arc in the Ouchterlony double-immunodiffusion test, indicating that the peaks do not correspond to identical material. Furthermore, the rechromatography experiment showed that the immunologically active fractions were chromatographically homogeneous. However, when analyzed by PAGE in SDS, all of the electrophoretic and

chromatographic protein-containing peaks in Figs. 3 and 5 gave the same pattern: that of the original, unfractionated material shown in Fig. 1a. This finding indicates that the proteins of zona pellucida, under the conditions used in the free zone electrophoresis and HPLC experiments, are not in free form but are associated into complexes of very similar protein composition. These complexes certainly exist also in the intact zona pellucida, since the chance is very small that complexes formed artificially during the solubilization and the purification of zona pellucida would have the same protein composition as the unfractionated material. However, all experiments on zona pellucida proteins should be interpreted with great caution, since it has been shown recently that membrane proteins have a tendency to form artifactual aggregates on contact with chromatographic and electrophoretic supporting media [19], and zona pellucida proteins resemble membrane proteins in the sense that they have a strong tendency to aggregate [21]. It is therefore an obvious advantage to use separation methods (such as free zone electrophoresis) that do not require the presence of a supporting medium and therefore should not induce the formation of artificial aggregates.

Dunbar et al. [4] state in a study of the zona pellucida from porcine oocytes: "We conclude that the zona pellucida is composed of several glycoprotein macromolecules; interaction of these macromolecules to form supramolecular complexes and the integral zona pellucida is dependent on noncovalent forces". In the above discussion we have put forward several indications that supramolecular complexes also constitute the zona pellucida from Baltic small herring and that these complexes (mainly built up of the protein bands ZP1, ZP2, ZP3, and ZP4 in Fig. 1) have small differences in charge and can thus be (partially) separated by high-performance ion-exchange chromatography (Fig. 3) and free zone electrophoresis (Fig. 5). We have not investigated how these differences in charge among the supramolecular complexes can be explained in molecular terms. It is not unlikely, however, that the proteins in the different complexes have somewhat different structures since it has been stated that the relatively large width of the electrophoretic bands of zona pellucida is caused by microheterogeneity in the carbohydrate moiety [16, 17]. It has been reported that these broad bands can be split into a series of narrow protein zones by two-dimensional electrophoresis [22].

It is interesting to note that the detergent G3707 [20] cannot split the bonds between the proteins in the supramolecular complexes, to judge from analysis of all the chromatographic and electrophoretic fractions in Figs. 3 and 5 by PAGE in SDS, since all of these fractions gave the same gel electrophoresis pattern as unfractionated zona pellucida, independent of whether detergent was used or not in the fractionations. This inability of G3707 to dissociate the zona pellucida proteins is also evident from the observation that addition of G3707 to the buffer did not affect the general appearance of the chromatograms (cf. Fig 3a and b) or the electropherograms (cf. Fig. 5a and b). However, from the PAGE experiments in SDS (Fig. 1a), it is obvious that SDS has the desired bond-splitting property. It has in fact been used already for the isolation of zona proteins [21]. The drawback of SDS is its strongly denaturing power. However, we have recently put forward the hypothesis that hydrophobic proteins should be easier to renature after an SDS treatment than are water-

soluble proteins [19]. Since zona pellucida proteins evidently are hydrophobic there is a good chance that they can be renatured following PAGE in SDS. If this appears to be the case (at least their immunological properties can be restored after removal of SDS [21, 24]), all zona proteins separable by this electrophoresis technique can be purified and recovered in a native state by the methods we have introduced [25—27]. These methods are based on excision of the gel slice containing the protein of interest and recovery of the protein from the gel slice in high yield and without significant dilution of the protein.

The precipitin arcs observed in the immunodiffusion experiments were more distinct and clearly visible following "desalting" (Fig. 4, well 3), chromatography (Fig. 4, well 4) and electrophoresis (not shown) than they were in the original sample (Fig. 4, wells 1 and 2), in spite of the fact the protein concentration was higher in the latter sample. This observation supports the suggestion of Dunbar and Raynor [24] that "there may be some component interfering with the antigen—antibody complexes and that this component can be removed by electrophoretic separation".

A comparison of the electrophoretic patterns presented in Fig. 1a and b (and Fig. 2a and b) show that zona pellucida proteins are degraded rapidly, probably by proteolysis. It is therefore essential to use fresh material and fast separation methods, for instance the high-performance electrophoresis and chromatographic techniques described herein, particularly when the experiments are performed at alkaline pH (ethylene glycol seems to have some stabilizing power). These methods permit fractionation within 20 min. If shorter run times are required one can increase the field strength (voltage) in the electrophoresis experiment and the flow-rate in the HPLC run. An increase in flow-rate will, of course, decrease the resolution, but not very much for flow-rates up to 1.5 ml/min as indicated by experiments not described here. An HPLC separation (molecular sieving) of porcine zona pellucida has recently been published [28]. Unfortunately, few data were given regarding the homogeneity of the fractions obtained.

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REFERENCES

1 R.B.L. Gwatkin and D.T. Williams, Gamete Res., 1 (1978) 19—26.
2 C.A. Shivers and B.S. Dunbar, Science, 197 (1977) 1187—1190
3 T. Mori, T. Nishimoto, M. Kitagawa, Y. Noda, T. Nishimura and T. Oikawa, Experientia, 34 (1978) 797—799.
4 B.S. Dunbar, N.J. Wardrip and J.L. Hedrick, Biochemistry, 19 (1980) 356—365.
5 S. Hjertén, Chromatogr. Rev., 9 (1967) 122—219.
6 S. Hjertén, in D. Glick (Editor), Methods of Biochemical Analysis, Vol. 18, Interscience, New York, 1970, pp. 55—79.
7 B.-l. Wu, Y.-x. Luo and J.-z. Liu, Jinan Liui Xuebao, 6(1) (1982) 1—9.
8 B.-l. Wu, Y.-x. Luo and J.-z. Liu, Jinan Liui Xuebao, 7(1) (1983) 80—90.

9 S. Hjertén, Biochim. Biophys. Acta, 79 (1964) 393—398.
10 J. Porath, T. Låås and J.-C. Jansson, J. Chromatogr., 103 (1975) 49—62.
11 S. Hjertén and B.-l. Wu, in preparation.
12 D.M. Neville, J. Biol. Chem., 246 (1971) 6328—6334.
13 B. Jergil and R. Ohlsson, Eur. J. Biochem., 46 (1974) 13—25.
14 S. Hjertén, Arch. Biochem. Biophys., Suppl. 1 (1962) 147—151.
15 The Pharmacia Gel Electrophoresis System, a brochure available from Pharmacia Fine Chemicals, Uppsala, Sweden.
16 S. Shimizu, M. Ito and J. Dean, Biochem. Biophys. Res. Commun., 109 (1982) 449—454.
17 P.M. Wassarman and J.F. Bleil, in W.A. Frazier (Editor), Cellular Recognition, Alan R. Liss, New York, 1982, pp. 845—863.
18 H. Glossman and D.M. Neville, Jr., J. Biol. Chem., 247 (1971) 5856—5861.
19 S. Hjertén, H. Pan and K. Yao, in H. Peeters (Editors), Protides of the Biological Fluids, Vol. 29, Pergamon Press, Oxford, New York, 1982, pp. 15—25.
20 D.A.W. Grant and S. Hjertén, Biochem. J., 164 (1977) 465—468.
21 Y. Noda, H. Kohda, I. Takai, S. Hayashi, H. Shimada, T. Mori and S. Tojo, J. Repr. Immunol., 5 (1983) 161—172.
22 B.S. Dunbar, C. Liu and D.W. Sammons, Biol. Reprod., 24 (1981) 1111—1124.
23 S. Hjertén, Biochim. Biophys. Acta, 736 (1983) 130—136.
24 B.S. Dunbar and B.D. Raynor, Biol. Reprod., 22 (1980) 941—954.
25 S. Hjertén, Biochim. Biophys. Acta, 237 (1971) 395—403.
26 L.-G. Öfverstedt, G. Johansson, G. Fröman and S. Hjertén, Electrophoresis, 2 (1981) 168—173.
27 S. Hjertén, Z.-q. Liu and S.-l. Zhao, J. Biochem. Biophys. Methods, 7 (1983) 101—113.
28 J. Dietl. A.B. Czuppon and W. Königsmann, J. Chromatogr., 275 (1983) 423—427.

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CHROMBIO. 2587

DETERMINATION OF PLASMA PROTEIN BINDING OF PROPAFENONE IN RATS, DOGS AND HUMANS BY HIGHLY SENSITIVE GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A highly sensitive method for the determination of propafenone in plasma has been established using gas chromatography—mass spectrometry with the deuterium-labelled compound as an internal standard. Plasma levels as low as 1 ng/ml were measured using 0.5-ml plasma samples. Plasma protein binding of the drug in rats, dogs and humans in vitro and in vivo was determined by the proposed method. About 90% of the drug was bound to the plasma protein in all species in vitro (20—500 ng/ml) and 69—88% in rats, 90—95% in dogs and 77—89% in humans after oral administration of the drug at doses of 50 mg/kg, 5 mg/kg and 200 mg per person, respectively.

INTRODUCTION

Propafenone hydrochloride is an antiarrhythmic drug with a structure common to β-blocking agents. It has been reported that the drug is effective in suppressing supraventricular and ventricular arrhythmias [1, 2].

Many methods have been reported for the determination of β-blocking agents in biological fluids using fluorimetry, high-performance liquid chromatography (HPLC), gas chromatography with electron-capture detection (GC—ECD), gas chromatography—mass spectrometry (GC—MS) and radioimmunoassay methods [3, 4].

For the determination of propafenone in plasma, HPLC [5, 6] and GC—ECD methods [7] have been reported. These methods had sufficient sensitivity (5—10 ng/ml) for the determination of the drug in plasma after administration of therapeutic dosages to humans. However, the values represent the sum of the

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drug unbound and bound to the plasma protein. To analyse the pharmacokinetics of the drug in more detail, it is necessary to determine the binding of the drug to the plasma protein. In our preliminary experiments, the drug was bound strongly to the plasma protein, so that the unbound drug concentration in plasma was considered to be very low.

Therefore, in this study, a more sensitive method has been established using GC—MS, and the protein binding in vitro and in vivo were determined in rats, dogs and humans.

EXPERIMENTAL

Chemicals

Propafenone hydrochloride (Fig. 1) was supplied by Helopharm W (Berlin, F.R.G.). Deuterium-labelled propafenone hydrochloride (Fig. 1) was prepared by the previously reported method [8], except that epichlorohydrin labelled with five deuterium atoms (d_5-epichlorohydrin) (MSD Isotopes, Montreal, Canada) was substituted for unlabelled epichlorohydrin, and the reaction of the d_5-epichlorohydrin was conducted at 100°C for 40 min in dimethylformamide (DMF)—dioxane (1:1). The chemical and isotopic purity of the d_5-propafenone hydrochloride, which was checked by thin-layer chromatography and mass and nuclear magnetic resonance spectrometry, was > 99%. Other chemicals and reagents were obtained commercially and were of analytical-reagent grade.

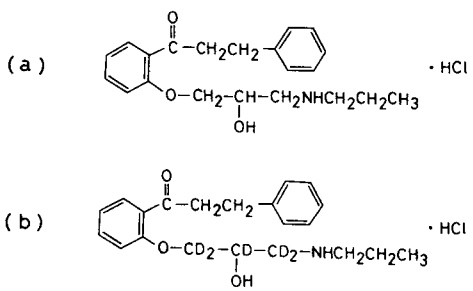

Fig. 1. Structures of (a) propafenone and (b) d_5-propafenone hydrochloride.

Gas chromatography—mass spectrometry

A JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) and a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) were used. Separations were carried out on a glass column (1.8 m × 1.8 mm I.D.) packed with 3% OV-22 on Chromosorb W (80—100 mesh). The column temperature was maintained isothermally at 260°C. The injector, separator and ion-source temperatures were 300°C, 250°C and 200°C, respectively. The flow-rate of the carrier gas (helium) was 30 ml/min. The ionization potential and emission current were 70 eV and 300 μA, respectively. The multiplier voltage supply was set at 1.6—2.2 kV.

Determination of unchanged drug in plasma

To each plasma sample (0.1—0.5 ml), d_5-propafenone hydrochloride (30—100 ng) was added as an internal standard (I.S.). After addition of 0.5 ml

of 2 *M* sodium hydroxide solution, the mixture (pH > 12) was extracted with benzene (2 × 2 ml). The benzene layer was evaporated to dryness under reduced pressure and pentafluoropropionic anhydride (PFPA) (Pierce, Rockford, IL, U.S.A.) (50 μl) and ethyl acetate (50 μl) were added to the residue. The mixture was kept at room temperature for 10 min and then evaporated to dryness under a stream of nitrogen. Aliquots (2—5 μl) of the solution were injected into the column of the GC—MS system. Fragment ions at *m/z* 408 and 413 were used to monitor the pentafluoropropionic (PFP) derivatives of propafenone and the I.S., respectively. The peak height ratio was used to calculate the amount of propafenone in each sample by referring to a calibration graph.

Extraction recoveries

Control plasma (0.5 ml) containing propafenone hydrochloride (15 or 150 ng) was carried through the above procedure without addition of I.S. The I.S., dissolved in benzene (15 or 150 ng), was added to the benzene solution, extracted and the benzene solution was evaporated to dryness under reduced pressure. The subsequent procedure was carried out as described above. Recoveries were calculated by comparing the peak height ratios with those obtained when the drug and I.S., dissolved in benzene, were processed without the extraction procedure.

Determination of plasma protein binding

Plasma protein precipitation. The plasma protein was precipitated by ultracentrifugation (Beckman L 8-70) at 170 000 *g* for 18 h at 4°C. Protein in the supernatant was determined by the Lowry method [9] and the ratio of the precipitated protein was calculated.

In vitro study of plasma protein binding. The drug solution (0.8—20 μg/ml) was prepared in 0.01 *M* phosphate buffer (pH 7.4) and 0.05 ml of this solution was added to 1.95 ml of rat, dog or human plasma to prepare plasma samples (20—500 ng/ml). The plasma samples were incubated at 37°C for 1 h, then centrifuged at 170 000 *g* for 18 h at 4°C. The drug concentration in the supernatant was determined by the GC—MS method as described above. The drug bound to plasma protein (*B*) was calculated according to the equation

$$B\,(\%) = \frac{A - C}{A} \cdot 100$$

where *A* and *C* are the total drug concentration in plasma and the drug concentration in the supernatant after centrifugation, respectively.

In vivo study of plasma protein binding. Male Sprague—Dawley rats (170—200 g) and male beagle dogs (11—14 kg) were used. They were maintained with free access to food and water and the drug was administered orally in aqueous solution (50 mg/kg in rats and 5 mg/kg in dogs) after overnight fasting. Blood samples were obtained from the inferior cava in rats and the antecubital vein in dogs with a heparinized syringe. Plasma samples were stored at −20°C until required for assay.

We were requested to determine the plasma concentration in healthy male volunteers (52—82 kg, age 30—41 years) who had been administered

propafenone hydrochloride in gelatin capsules (200 mg) by clinicians [10]. Samples from these subjects were used for human studies.

The total drug concentration in plasma and the unbound concentration in plasma were determined by the method used for the in vitro study.

RESULTS AND DISCUSSION

Because of the low plasma concentration of β-blocking agents, many sensitive methods have been developed [3, 4]. Among them, the GC method currently in general use is based on the method used for the determination of propranolol [11], the first used β-blocker in therapy. Propranolol [11],

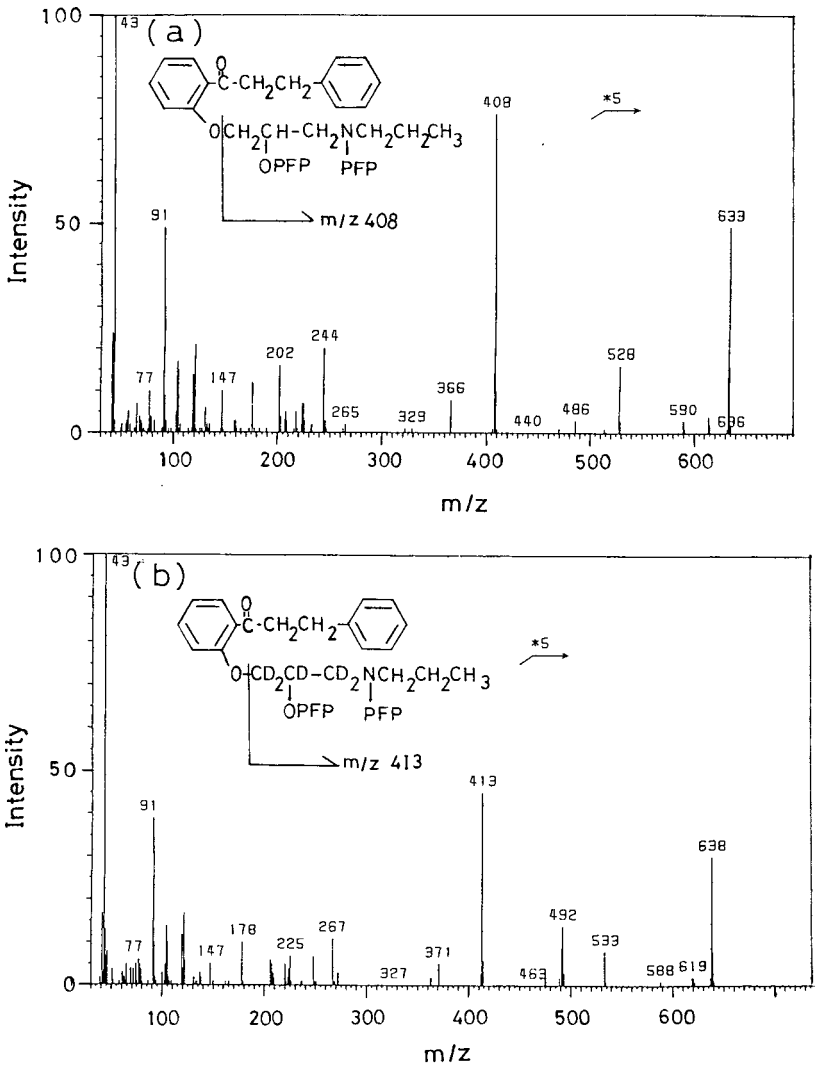

Fig. 2. Mass spectra of PFP derivatives of (a) propafenone and (b) d_5-propafenone.

alprenolol [11], metoprolol [12] and oxprenolol [11—13] were determined as the trifluoroacetyl (TFA) derivatives and timolol [14] and atenolol [15] as the heptafluorobutyric (HFB) derivatives. In the GC—ECD method used for the determination of propafenone [7], the drug was determined as the TFA derivative. The reaction of propafenone with TFAA was completed at 45°C after 45 min [7]. In the present method, we used PFPA instead of TFAA, because propafenone was completely derivatized with PFPA within 10 min at room temperature.

As shown in Fig. 2, the mass spectrum of the PFP derivative showed the molecular ion at *m/z* 633, indicating that the two PFP groups were introduced into the hydroxy and amino moieties in the structure. Another intense peak (base peak) was observed at *m/z* 408, which was used to monitor the drug. This peak shows the fragment ion caused by the cleavage of the side-chain, as shown in Fig. 2.

These derivatizations and fragmentations were similar to those of β-blocking agents with a common structure. For propranolol, two TFA groups were introduced into the hydroxy and amino moieties of the structure, showing the molecular ion at *m/z* 451, and the base peak at *m/z* 308 showed the fragment ion caused by the cleavage of the side-chain [16].

In this study, the deuterium-labelled compound was synthesized and used as an internal standard. Because the mass spectrum of the PFP derivative of propafenone showed an intense fragment ion at *m/s* 408, the deuterium atoms were introduced into this fragment (Fig. 2). The labelled propafenone was

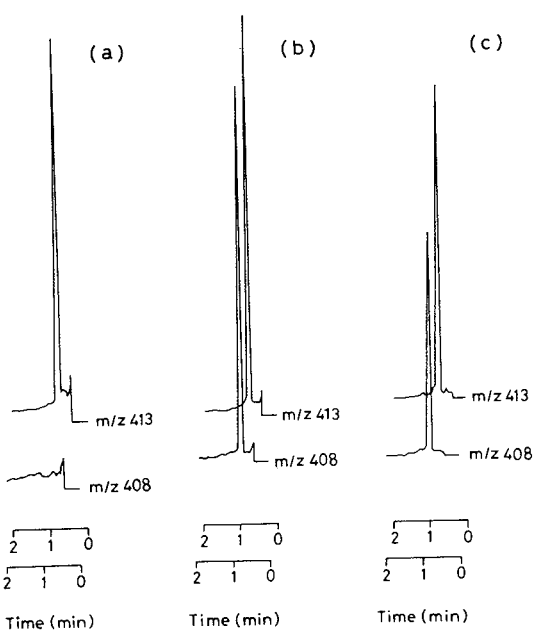

Fig. 3. Chromatograms obtained from human plasma samples. (a) Control plasma to which I.S. (100 ng/ml) was added; (b) control plasma to which propafenone · HCl (100 ng/ml) and the I.S. (100 ng/ml) were added; (c) plasma obtained 1 h after oral administration of propafenone · HCl (200 mg) to a human.

prepared by the procedure reported previously [8], except that d_5-epichlorohydrin was used instead of unlabelled epichlorohydrin. In the reported synthetic method, a large amount of epichlorohydrin was used both as a reagent and as a solvent. However, the amount of the labelled compound used was limited. Therefore, we modified the conditions of the reaction with d_5-epichlorohydrin. The reaction gave a high yield after 1 h at 100°C in DMF—dioxane (1:1).

The chromatograms of propafenone and I.S. extracted from plasma are shown in Fig. 3. The drug-free control plasma gave no interfering peaks.

A calibration graph obtained by the above procedure showed a linear response in the range 1—1000 ng/ml. The extraction recoveries for 30 and 300 ng/ml were 78.4 ± 0.8% (n = 6, mean ± standard error) and 84.4 ± 1.2% (n = 6), respectively. In this study, benzene was used as the extraction solvent, because endogenous substances that interfere in the assay were removed using this solvent, although the extraction recovery was lower than those obtained with other polar solvents.

The limit of determination, which was 1 ng/ml using 0.5-ml plasma samples, was five to ten times higher than those of the previous HPLC and GC—MS methods [4—6].

Plasma protein binding of the drug in vitro and in vivo were determined in rats, dogs and humans. Plasma protein precipitation was conducted by ultracentrifugation. To confirm the precipitation of plasma protein under the ultracentrifugation conditions used, the protein in the supernatant was determined by the Lowry method [9]. The protein was completely precipitated by centrifugation at 170 000 g for 18 h at 4°C (99.5 ± 0.03%; n = 4).

TABLE I

PLASMA PROTEIN BINDING OF PROPAFENONE IN VITRO

Propafenone · HCl concentration (ng/ml)	Propafenone · HCl bound* (%)		
	Rat	Dog	Human
20	—	92.4 ± 2.0	—
100	91.4 ± 0.6	91.4 ± 1.7	92.2 ± 0.7
500	90.5 ± 3.0	90.9 ± 0.9	91.1 ± 0.8

*Results are means ± standard errors (rat and dog, n = 4; human, n = 5).

The concentration of the drug in protein-free plasma was determined by the proposed method. The in vitro results are shown in Table I. The drug bound to plasma protein was about 90% in all species over the concentration range examined. In this study, the control plasma containing the drug was incubated at physiological temperature (37°C). However, we had to carry out the centrifugation at a lower temperature in order to prevent an increase in temperature caused by ultracentrifugation for a long period (18 h). Therefore, the protein binding determined in this study might be different from that under physiological conditions.

The in vivo results are shown in Table II. The drug was administered orally

TABLE II

TOTAL AND FREE PLASMA CONCENTRATIONS AND PROPAFENONE BOUND TO PLASMA PROTEIN AFTER ORAL ADMINISTRATION OF PROPAFENONE TO RATS, DOGS AND HUMANS

The drug was administered orally to rats (50 mg/kg, n = 4), dogs (5 mg/kg, n = 3) and humans (200 mg, n = 3). The results are means ± standard errors.

Time (h)	Total plasma concentration of propafenone · HCl (ng/ml)			Free plasma concentration of propafenone · HCl (ng/ml)			Propafenone · HCl bound (%)		
	Rat	Dog	Human	Rat	Dog	Human	Rat	Dog	Human
0.5	662 ± 88	520 ± 214	51 ± 13	200 ± 34	23 ± 8	9 ± 1	68.9 ± 4.9	95.3 ± 1.2	80.6 ± 4.7
1	391 ± 54	283 ± 99	199 ± 37	69 ± 7	14 ± 5	31 ± 5	80.6 ± 4.2	95.2 ± 0.7	84.6 ± 0.3
2	304 ± 126	80 ± 22	123 ± 40	34 ± 10	4 ± 1	15 ± 8	87.5 ± 1.7	94.3 ± 1.4	88.7 ± 2.7
4	71 ± 6	15 ± 4	56 ± 12	10 ± 1	0.7 ± 0.2	11 ± 2	85.9 ± 1.6	94.7 ± 1.2	80.8 ± 0.6
6	20 ± 15	3 ± 3	29 ± 10	3 ± 2	0.3 ± 0.3	4 ± 2	86.0 (n=2)	90.1 (n=1)	76.5 (n=2)

to rats (50 mg/kg), dogs (5 mg/kg) and humans (200 mg). Although these doses differed among the species, the total drug concentration levels were similar (Fig. 2). The drug bound to the plasma protein was 69—88% in rats, 94—95% in dogs and 76—89% in humans. The values in rats and in humans were lower than those in vitro, whereas in dogs there was no difference between the in vitro and in vivo values.

REFERENCES

1 O.A. Beck, K.D. Kramer, R. Wolf, A. Muller and H. Hochrein, Med. Klin., 70 (1975) 95.
2 H. Hochrein, H.J. Hapke and O.A. Beck (Editors), Fortschritte in der Pharmakotherapie von Herzrhythmusstorungen. 1. Internationales Propafenone-Symposium, Gustav Fischer Verlag, Stuttgart, New York, 1977.
3 W. Sadee and G.C.M. Beelen, Drug Level Monitoring, Wiley, New York, 1980, p. 400.
4 E. Vervloet and P. Vermeig, in F.W.H.M. Merkus (Editor), The Serum Concentration of Drugs, Excerpta Medica, Amsterdam, 1980, Abstract No. 155.
5 E. Brode, R. Sachse and H.D. Hoffman, Arzneim.-Forsch., 32 (1982) 1.
6 S.R. Harapat and R.E. Kates, J. Chromatogr., 230 (1982) 448.
7 B. Marchesini, S. Boschi and M.B. Mantovani, J. Chromatogr., 232 (1982) 435.
8 Helopharm, W. Petrik and Co., Deut. Patentschr., 2 001 431, 1970.
9 D.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 263.
10 T. Onodera, Y. Kobayashi, M. Kou, H. Saito, T. Ino, H. Atarashi, K. Munakata, T. Kato, T. Aramaki, H. Hayakawa, S. Motomura and K. Hashimoto, Abstracts of 4th Annual Meeting of the Japanese Society of Clinical Pharmacology, 1983, Abstract No. 66.
11 T. Walle, J. Pharm. Sci., 63 (1974) 1885.
12 P.H. Degen and W. Riess, J. Chromatogr., 121 (1976) 72.
13 O.B. Jack and W. Riess, J. Chromatogr., 88 (1974) 173.
14 D.J. Tocco, F.A. deLuna and A.E.W. Duncan, J. Pharm. Sci., 64 (1975) 1879.
15 B. Scales and P.B. Copsey, J. Pharm. Pharmacol., 27 (1975) 430.
16 T. Walle, J. Morrison, K. Walle and E. Conradi, J. Chromatogr., 114 (1975) 351.

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CHROMBIO. 2579

DETERMINATION OF DICLOFENSINE, AN ANTIDEPRESSANT AGENT, AND ITS MAJOR METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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SUMMARY

A sensitive and selective high-performance liquid chromatographic assay was developed for the determination of diclofensine (I) and its key metabolites in human plasma. The assay involves deproteinization of plasma, overnight glusulase incubation to hydrolyze the major metabolite (I-B-glucuronide), extraction of the parent compound and its deconjugated metabolites (I-A, I-B and I-C) from the alkalinized aqueous phase into diethyl ether—ethanol (95:5), the residue of which (containing compounds I, I-A, I-B and I-C) is alkylated with 2-iodopropane dissolved in acetone, using solid potassium hydroxide as a catalyst. The compounds are extracted from the reaction mixture into diethyl ether, after adding ethanol—water—acetic acid (55:40:5), the residue of which is dissolved in 0.05 *M* sulfuric acid, and reacted with mercuric acetate at 100°C, which oxidizes tertiary tetrahydroisoquinolines to their 3,4-dihydroisoquinoline derivatives, followed by a photochemical reaction in the same solution to form intensely fluorescent isoquinolinium derivatives.

An aliquot of this reaction mixture is injected onto a reversed-phase high-performance liquid chromatography column (5-μm Nova-Pac C_{18} phase in a radial compression cartridge, 10 cm × 8 mm), using the mobile phase 0.25 *M* triethylammonium phosphate (pH 2.5)—0.25 *M* acetic acid—methanol—acetonitrile—tetrahydrofuran (150:350:125:375:25). The void volume (V_0) is approximately 1.4 min and the retention times (t_R) of the respective isoquinolium derivatives of diclofensine (I) are ca. 3.5 min, internal standard (II) ca. 4.2 min, nordiclofensine (I-A) ca. 5 min, while the phenolic metabolites I-B and I-C give peaks at 6.4 min and 10.4 min, respectively. The derivatives are detected by fluorescence.

The method was used to determine plasma concentrations of the parent drug (I) and its major phenolic metabolite I-B (aglycone) in plasma in two normal volunteers following a single oral 45-mg dose and following seven consecutive days of oral dosing of 45 mg three times a day as part of a multiple ascending dose tolerance study.

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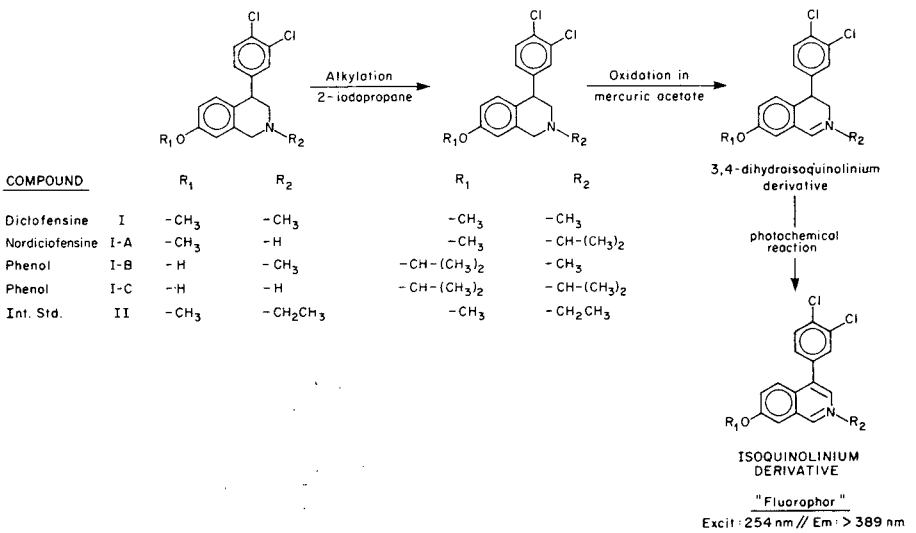
INTRODUCTION

Diclofensine, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-methylisoquinoline (I, Fig. 1), is an antidepressant drug with an unusual profile of pharmacologic activity and a novel chemical structure [1, 2]. It differs chemically from tricyclic antidepressants, such as imipramine, and tetracyclic antidepressants, such as mianserin. Nomifensine (Merital®) is the only antidepressant currently available [3, 4] which contains the basic isoquinoline moiety of diclofensine for which a high-performance liquid chromatographic (HPLC) method using UV detection was recently published [5]. However, diclofensine differs from nomifensine in its central and peripheral nervous system pharmacological properties [6].



COMPOUND		R_1	R_2	R_1	R_2
Diclofensine	I	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
Nordiclofensine	I-A	$-CH_3$	$-H$	$-CH_3$	$-CH-(CH_3)_2$
Phenol	I-B	$-H$	$-CH_3$	$-CH-(CH_3)_2$	$-CH_3$
Phenol	I-C	$-H$	$-H$	$-CH-(CH_3)_2$	$-CH-(CH_3)_2$
Int. Std.	II	$-CH_3$	$-CH_2CH_3$	$-CH_3$	$-CH_2CH_3$

Fig. 1. Chemical structures and reactions of the compounds referred to in the text.

Preliminary studies on the pharmacokinetics and pharmacodynamics of diclofensine in man following 25- and 50-mg oral doses [7] showed that the compound was rapidly absorbed but also extensively metabolized by N- and O-demethylation to nordiclofensine (I-A) and the phenolic metabolites O-desmethyldiclofensine (I-B) and O-desmethylnordiclofensine (I-C), respectively (Fig. 1). The half-life ($t_{1/2}$ β) of the parent drug (I) was of the order of 14.9 h (range 8.2—20.2 h). Oral absorption was complete based on the recovery of the major metabolite in urine, viz. O-desmethyldiclofensine (I-B) [7—9].

Plasma concentrations of diclofensine and nordiclofensine are in the low nanogram concentration range as determined by gas chromatography—mass spectrometry (GC—MS) [10] and in metabolic studies in man using ^{14}C-labeled drug [11]. The major metabolite (I-B) accounted for over 30% of the dose in urine as a glucuronide conjugate, therefore it was expected that this metabolite could also be determined in plasma after enzymatic deconjugation.

A sensitive and selective HPLC assay was developed for the determination of diclofensine (I) and its key metabolites in human plasma (Fig. 1). The assay involves deproteinization of plasma, overnight glusulase incubation to

hydrolyze the major metabolite, I-B-glucuronide, and extraction of the parent compound and its metabolites I-A, I-B and I-C. Following evaporation of the organic phase, compounds I-A, I-B and I-C in the residue are alkylated with 2-iodopropane dissolved in acetone. The compounds are extracted from the reaction mixture into diethyl ether after adding ethanol—water—acetic acid (55:40:5), which is then evaporated to dryness. The residue is dissolved in 0.05 *M* sulfuric acid and reacted with mercuric acetate at 100°C to oxidize the tertiary tetrahydroisoquinolines to their 3,4-dihydroisoquinoline derivatives, followed by a photochemical reaction in the same solution to form intensely fluorescent isoquinolinium derivatives [12] (Fig. 1).

An aliquot of this reaction mixture is injected onto a reversed-phase HPLC column in a radial compression cartridge. The derivatives are detected by fluorescence. Diclofensine (I) per se is determined separately following selective extraction, as its isoquinolinium derivative (omitting the alkylation step) with pre-concentration prior to HPLC analysis.

Plasma concentrations of the parent drug (I) and its major phenolic metabolite I-B (aglycone) were determined by the separate procedures in two normal volunteers following a single oral 45-mg dose and following seven consecutive days of oral dosing of 45 mg three times a day (t.i.d.), as part of a multiple ascending dose tolerance study.

EXPERIMENTAL

Reagents

All inorganic reagents were analytical-reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered through a 0.2-μm filter (Type DS System, Hydro-Service and Supplies, Durham, NC, U.S.A.). The inorganic reagents include: 0.1 *M* and 10 *M* sodium hydroxide, solid potassium hydroxide pellets, 0.05 *M* sulfuric acid, and mercuric acetate reagent [dissolve 32.8 g of sodium acetate, anhydrous (0.4 mol), 1.5 g of mercuric acetate and 3 ml of glacial acetic acid in 100 ml of distilled water (final pH ca. 6); this solution should be made fresh weekly]. Molar (pH 5.4) phosphate buffer was prepared by mixing 1.0 *M* potassium dihydrogen phosphate (136.1 g/l) and 1.0 *M* dipotassium hydrogen phosphate (174.2 g/l), then titrating the former solution to pH 5.4 with the latter.

The following organic solvents and reagents were also used: ethanol (200 proof, Pharmco, Publicker Industries, Philadelphia, PA, U.S.A.), diethyl ether [anhydrous, analytical reagent (ACS), Mallinckrodt, Paris, KY, U.S.A. washed with 0.05 *M* sulfuric acid immediately before use], acetone (ACS, Fisher, Fair Lawn, NJ, U.S.A.), 2-iodopropane, 97% (Aldrich, Milwaukee, WI, U.S.A.), glacial acetic acid (ACS), Glusulase® (an enzyme preparation containing 100 000 U of glucuronidase and 50 000 U of sulfatase per ml, available from Endo Labs., Garden City, NY, U.S.A.), and 0.25 *M* (pH 2.5) triethylammonium phosphate (TEAP) (Regis Chemical, Morton Grove, IL, U.S.A.).

Analytical standards

The following compounds, all of pharmaceutical-grade purity (>99%), were used as the analytical standards: compound I · HCl, diclofensine, (±)-4-(3,4-

TABLE I

PREPARATION OF WORKING SOLUTIONS 1—10

Working solution No.	Aliquots (μl) of standard solution									Final concentration (ng/ml of solution)				
	A-2	A-3	B-2	B-3	C-2	C-3	D-2	D-3	E-2	I	I-A	I-B	I-C	II
1	1000	—	500	—	200	—	2000	—	200	1000	500	200	2000	200
2	500	—	1000	—	—	—	200	—	200	500	1000	—	200	200
3	200	—	200	—	1000	—	—	—	200	200	200	1000	—	200
4	100	—	—	—	—	—	1000	—	200	100	—	—	1000	200
5	—	—	—	500	500	—	100	—	200	—	50	500	100	200
6	—	500	100	—	100	—	500	—	200	50	100	100	500	200
7	—	200	—	100	—	500	—	500	200	20	10	50	50	200
8	—	100	—	200	—	100	—	200	200	10	20	10	20	200
9	—	50	—	50	—	200	—	100	200	5	5	20	10	200
10	—	—	—	—	—	—	—	—	200	—	—	—	—	200

dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-methylisoquinoline · hydrochloride, $C_{17}H_{17}ONCl_2$ · HCl, molecular weight (MW) = 358.70, m.p. = 273—275°C; compound I-A · HCl, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxyisoquinoline · hydrochloride, MW = 344.68, m.p. = 240—242°C; compound I-B · HBr, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-2-methyl-7-isoquinolinol · hydrobromide, MW = 389.14, m.p. = 284—285°C; compound I-C · HCl, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-isoquinolinol · hydrochloride, MW = 330.66, m.p. = 273—275°C; and compound II · HCl, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-ethylisoquinoline · hydrochloride (internal standard), MW = 372.72, m.p. = 247—249°C.

Stock solutions of I · HCl, I-A · HCl, I-B · HBr, I-C · HCl and II · HCl were prepared as follows (amberized 10.0-ml volumetric flasks were used for all standard solutions).

Solution A-1. Dissolve 1.11 mg of I · HCl (equivalent to 1.0 mg of I, free base) in 10.0 ml of absolute ethanol in a 10-ml amberized volumetric flask to yield 111 μg of I · HCl per ml.

Solution B-1. Dissolve 1.12 mg of I-A · HCl (equivalent to 1.0 mg of I-A, free base) in 10.0 ml of absolute ethanol.

Solution C-1. Dissolve 1.26 mg of I-B · HBr (equivalent to 1.0 mg of I-B, free base) in 10.0 ml of absolute ethanol.

Solution D-1. Dissolve 1.12 mg of I-C · HCl (equivalent to 1.0 mg of I-C, free base) in 10.0 ml of ethanol.

Prepare separate 1:10 serial dilutions of each stock solution to yield standard solutions A-2, A-3, B-2, B-3, C-2, C-3 and D-2, D-3 containing 10 μg/ml or 1.0 μg/ml, respectively.

Solution E-1. Dissolve 1.11 mg of II · HCl (equivalent to 1.0 mg of II, free base) in 10.0 ml of ethanol (internal standard for HPLC assay).

Solution E-2. Dilute 1.0 ml of solution E-1 to 10.0 ml in ethanol to yield a 10 μg/ml solution.

Working standard solutions 1—10 were prepared by diluting various aliquots of the standard solutions above to 10.0 ml in ethanol as given in Table I. These working solutions (1—10) were used for the determination of total (free and bound) compounds in plasma (procedure A).

Analysis of parent drug (I) per se

A separate set of working solutions a—g were prepared by diluting various aliquots of the standard solutions of I and II to 10 ml of ethanol as given in Table II. These working solutions (a—g) were used for the determination of free (unconjugated) I in plasma (procedure B).

The primary stock solutions and mixed standard solutions are stable for two months when stored at 5°C.

Equipment and instrument parameters

The photolytic reaction was conducted with a PyroLux R-57 lamp (Luxor, NY, U.S.A.). A Model PR-J refrigerated centrifuge with a No. 253 rotor (Damon/IEC Division, Needham, MA, U.S.A.) was used for all centrifugations.

Column. The column used for reversed-phase HPLC was a pre-packed, 10 cm

TABLE II

PREPARATION OF WORKING SOLUTIONS a—g

Working solution	Aliquots (μl) of standard solution			Final concentration (ng per 20 μl of solution)	
	A-2	A3	E2	I	II
a	1000	—	200	20	4
b	500	—	200	10	4
c	200	—	200	4	4
d	100	—	200	2	4
e	—	500	200	1	4
f	—	200	200	0.4	4
g	—	—	200	—	4

× 8 mm, Radial-Pak cartridge containing Nova-Pak® C_{18} (a C_{18} phase bonded to 5-μm spherical silica particles) (Waters Assoc., Milford, MA, U.S.A.).

Instrument. The HPLC system consisted of a Model 6000A reciprocating piston pump (Waters Assoc.), a Waters Intelligent Sample Processor (WISP™) Model 710B, a Waters Z-module™ radial compression system (to compress and hold the Radial-Pak cartridge) and a Schoeffel Model FS-970 LC fluorometer operated at 254 nm for excitation and emission at wavelengths greater than 389 nm (Kratos Analytical Instruments, Westwood, NJ, U.S.A.). The fluorescence detector range was set at 0.2 μA full scale and the photomultiplier sensitivity was 580. The chart speed on the 10-mV recorder, Model 7132A (Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP auto-injector was programmed for a 15-min run time per sample using methanol as the rinse solvent.

The isocratic mobile phase consisted of 0.25 *M* triethylammonium phosphate (pH 2.5)—0.25 *M* acetic acid—methanol—acetonitrile—tetrahydrofuran (150:350:125:375:25), operated at a constant flow-rate of 1.8 ml/min which resulted in a pressure of ca. 3 MPa (ca. 500 p.s.i.).

The void volume, V_0, was ca. 1.4 min, and the retention times (t_R) of the respective isoquinolinium derivatives of diclofensine (I) were ca. 3.5 min, internal standard (II) ca. 4.2 min, nordiclofensine (I-A) ca. 5 min, while the phenolic metabolites I-B and I-C gave peaks at 6.4 min and 10.4 min, respectively (Fig. 2).

Procedure A: analysis for total (free and bound) I and metabolites in plasma

Extraction. For each unknown, a 300-μl aliquot of working solution 10 (equivalent to 60 ng of II, the internal standard) was pipetted into a separate 100 × 13 mm disposable borosilicate culture tube. The organic solvent was then evaporated at 20—30°C in an N-EVAP. A 0.1-ml aliquot of unknown plasma was then added to each tube.

A calibration curve of ten standards was processed with each set of unknowns. Aliquots (300 μl) of solutions 1—9 were evaporated separately in 100 × 13 mm culture tubes and 0.1 ml of control plasma was added to each. The tenth standard is a 0.1-ml sample of control plasma without any compound added.

To each culture tube containing 0.1 ml of plasma were added 2 ml of 1 *M*

phosphate buffer pH 5.4. The contents were mixed, then heated at 90°C for 3—5 min (with mixing, every 30 sec) to denature and precipitate the proteins. The samples were centrifuged at 1100 *g* (2100 rpm) for 10 min at 10—15°C. The clear supernate was transferred to a 50-ml round-bottom glass-stoppered centrifuge tube. The precipitate was re-eluted, as above, with 1 ml of 1 *M*

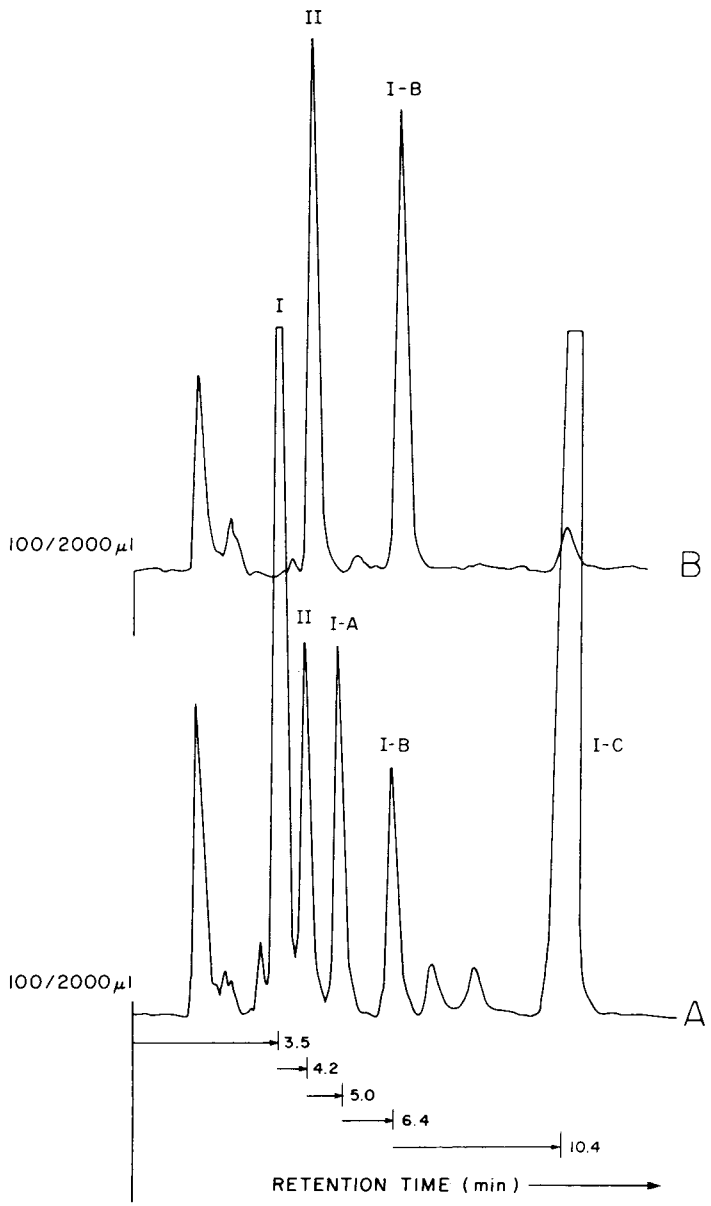


Fig. 2. Chromatograms showing (A) the analysis of compounds I, I-A, I-B, I-C and the internal standard (II) recovered from plasma as their respective alkylated and/or isoquinolinium derivatives; (B) the analysis of I-B (aglycone), the major metabolite in human plasma following the oral administration of a 45-mg dose of I · HCl.

phosphate buffer, pH 5.4, centrifuged, and the supernates were combined. To each sample, 150 μl of Glusulase were added using a 1-ml glass hypodermic syringe fitted with a stainless-steel needle. The samples were then placed in a rack, loosely stoppered with cotton and incubated overnight (about 16 h) at 37°C in a mechanical incubation shaker (Dubnoff, Precision Scientific, Chicago, IL, U.S.A.).

Following incubation, the samples were removed from the water bath, adjusted to alkaline pH by the addition of 0.4 ml of 10 *M* sodium hydroxide, mixed well, and allowed to equilibrate to room temperature. The samples were then extracted twice with 5-ml portions of a freshly prepared mixture of diethyl ether—absolute ethanol (950:50) (sealing the tubes using PTFE stoppers) by shaking for 5 min on a reciprocating shaker, and then centrifuging for 10 min at 1100 *g* (2100 rpm) at 15—20°C. Following each centrifugation, the organic extract was transferred to a 125 × 16 mm culture tube with a PTFE-lined screw cap. If an emulsion occurred, then 0.5 ml of additional ethanol was added to each sample, mixed by manual inversion, then centrifuged again. The organic extracts were evaporated to dryness at 40°C under a gentle stream of nitrogen in an N-EVAP (Organomation Assoc., Worchester, MA, U.S.A.) water bath.

Alkylation. The residues were reconstituted in 1 ml acetone and 0.1 ml of 2-iodopropane was added to each tube. Three pellets (approx. 300 mg) of solid potassium hydroxide (washed with 2 vols. of acetone just prior to use) were added and the tubes were tightly capped. The tubes were placed in a rack in a 70—75°C bath for 30 min, then taken out and cooled to room temperature.

After alkylation, the samples were first diluted with 0.5 ml of a mixture of ethanol—water—glacial acetic acid (55:40:5) and then extracted twice with 4.5-ml portions of acid-washed diethyl ether by mixing on a vortex mixer, and centrifuging at 20°C for 5 min at 1100 *g* (2100 rpm). Following each centrifugation the clear supernate was transferred to a clean 125 × 16 mm culture tube and the organic extracts were evaporated to dryness at 40°C under a gentle stream of nitrogen in an N-EVAP water bath. The residues were vacuum dried (over Drierite) in a vacuum desiccator for 10 min to insure the removal of less volatile organic solvents which would otherwise contaminate the sample.

The samples were then processed for oxidation and photolysis as described below.

Oxidation and photolysis. To the residue in each tube 0.3 ml of 0.05 *M* sulfuric acid was added and the contents were mixed on a vortex mixer. Then 0.3 ml of the mercuric acetate reagent was added and the culture tubes were tightly capped with PTFE-lined screw caps. The culture tubes were placed in a rack in a boiling water bath (100°C) and allowed to react for 30 min to complete the oxidation to the intermediate. The samples were cooled to room temperature and then exposed directly to high-intensity light from a Pyro-Lux R-57 lamp contained in a 75 cm × 75 cm × 60 cm (approximately) wooden box lined with aluminum foil. The samples must be arranged in a single row in a suitable rack and placed 30 cm from the light source. The samples were exposed for 20 min to complete the photochemical reaction to the final fluorophore.

HPLC procedure. The tubes were cooled to room temperature and 1.4 ml of

mobile phase added and the solution was vortex-mixed. The solution was transferred to a standard Waters Assoc. 4-ml glass vial (Part No. 72711) and each vial was sealed with a cap with a self-seal septum (Part No. 73010). The vials were placed in an autoinjector (WISP 710B) which was programmed to inject 100 μl (out of a total volume of 2.0 ml) for HPLC analysis using the chromatographic parameters previously described.

Procedure B: analysis for parent drug, diclofensine (I) per se

For each unknown to be analyzed, a 20-μl aliquot of standard solution g (equivalent to 4 ng of II, the internal standard) was pipetted into a separate 100 × 13 mm disposable borosilicate culture tube (Cat. No. 14-962-10C, Fisher). These aliquots were then evaporated at 20—30°C in an N-EVAP evaporator under a stream of clean, dry nitrogen. A 1.0-ml aliquot of unknown plasma sample was then added to each culture tube containing the internal standard (II).

A calibration curve of seven recovered standards was processed with each set of unknowns. Aliquots of 20 μl of the appropriate solutions a—f were evaporated separately in 100 × 13 mm culture tubes and 1.0 ml of control plasma was added to each to yield plasma standards containing 20, 10, 4, 2, 1, 0.4 or 0 ng/ml I, each containing 4 ng/ml II (internal standard). Metabolites I-A, I-B and I-C in the unconjugated form are not quantitated in this procedure due to the extremely low concentrations present in vivo [11].

To each culture tube containing plasma were added 1.0 ml of 0.1 *M* sodium hydroxide and 4.5 ml of acid-washed diethyl ether, then the tubes were stoppered with polyethylene caps (Plugtite Cat. No. 127-0019-100, Elkay Products, 800 Boston Turnpike, Shrewsbury, MA, U.S.A.) and mixed for 10—15 sec on a vortex mixer. The caps were loosened momentarily to release the ether vapor pressure, stoppered, and shaken on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 50—80 strokes per min for 5 min. The samples were centrifuged at 1100 *g* (2100 rpm) for 10 min at 10—15°C in a refrigerated centrifuge and the ether phase was transferred into a 125 × 16 mm borosilicate culture tube with a PTFE-lined screw cap (Corning 9826-16X). The aqueous mixture was extracted again with an additional 4.0 ml of diethyl ether. The ether extracts were combined as before and evaporated at 20—30°C in an N-EVAP evaporator under a stream of clean, dry nitrogen. (Note: the alkylation step is omitted.)

The residues are processed for chemical/photochemical oxidation as previously described to yield the final isoquinolinium derivatives, which are extracted once into 5 ml of methylene chloride. After 10 min of shaking and 5 min of centrifugation, the lower methylene chloride phase was transferred by pipet to a clean 100 × 13 mm disposable culture tube and evaporated to dryness at 25°C under a stream of nitrogen. The samples were re-dissolved in 400 μl of mobile phase (using a vortex mixer) and sonicated in an ultrasonic cleaner bath (to insure complete dissolution), then capped and centrifuged for 1—2 min (to precipitate solids). An aliquot of 300—350 μl was transferred into a Waters plastic low-volume insert (Part No. 72030) held in a standard 4-ml glass vial (Waters Part No. 72711) by a compression spring (Part No. 72708). Each vial was sealed with a cap containing self-seal septum (Part No.

73010) and the vial was tapped to remove any air bubbles trapped at the bottom of the low-volume insert. The autoinjector (WISP 710B) was programmed to inject up to 200 μl (50% of the total sample) for HPLC analysis (Fig. 3).

Calculations

The major component quantitated in procedure A was metabolite I-B whose concentrations far exceeded those of the parent drug (I) and metabolites I-A and I-C which were diluted out in order to keep the peak for I-B on scale. The parent drug (I) per se was quantitated by procedure B. The concentration of either I or I-B in the unknowns was determined by interpolation from a least-squares regression equation (weighted linear equation: $y = ax + b$) of the calibration data (processed by a Hewlett-Packard Model 3357 Laboratory Automation System) of the respective recovered standards processed along with the unknowns using peak height ratios [peak height of either compound I or I-B to peak height of internal standard (II)] versus concentration of either I (0.4—20 ng/ml) using a 1.0-ml specimen per assay or I-B (0.06—3.0 μg/ml) using a 0.1-ml specimen per assay.

RESULTS AND DISCUSSION

Sensitive and selective reversed-phase HPLC assays with fluorescence detection were developed for the determination of directly extractable diclofensine (I), I-A, I-C, and the major metabolite (I-B, aglycone) in human plasma. Compound II was chosen as the internal standard due to its structural similarity to compound I.

The major UV absorption bands of diclofensine (I), of its metabolites (I-A, I-B, I-C) and of the internal standard (II) occur at about 210—215 nm, with shoulders at 230 ± 3 nm while the minor UV absorption bands occur at 270—290 nm. The Waters Model 440 absorbance detector, used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp, permitted quantitation of all of these compounds in the μg/ml concentration range. The use of an extended-wavelength module (EWM) (Waters Assoc.) with a 229-nm deuterium lamp and wavelength kit permitted quantitation of these compounds in the approx. 100 ng/ml concentration range. These underivatized compounds did not exhibit intrinsic fluorescence in aqueous buffers or in the HPLC mobile phase used. Due to the complex biotransformation of I, chromatographic separation was essential to ensure specificity of analysis. Initially, the intact (underivatized) compounds were chromatographed on a Waters Nova-Pak (5 μm, C_{18}) Rad Pak cartridge in the Z-module with a mobile phase consisting of 0.25 *M* triethylammonium phosphate (pH 2.5)—water—methanol—acetonitrile (100:400:150:350), pumped at a constant flow-rate of 2.0 ml/min. The void volume was 1.3—1.4 min and the respective retention times were as follows: diclofensine (I), ca. 5.5 min, I-A ca. 5.3 min, I-B, ca. 3.1 min, I-C ca. 3.2 min and II (internal standard) ca. 6.4 min. The extraction recovery of I, I-A and II from plasma was shown to be quantitative after extraction into diethyl ether at alkaline pH, using the above system. Extractions from higher salt concentrations showed lower recoveries of I-A. Ethanol was necessary to prevent foaming in the deconjugated samples. UV detection was not sensitive enough for the

determination of the low concentrations expected.

Derivatization to yield a fluorescent product was examined as a means for sensitive detection. The intact compounds (Fig. 1) are substituted tetrahydroisoquinolines and were expected to undergo a previously published two-step reaction to form fluorescent derivatives [12]. The first step is an oxidative dehydrogenation reaction in aqueous mercuric acetate solution at 100°C to form moderately fluorescent 3,4-dihydroisoquinoline derivatives. The second step is a photochemical oxidation (dehydrogenation) which inserts a second double bond to form the highly fluorescent isoquinolinium derivatives.

The HPLC system used above for the intact compounds was also used for the optimization of the mercuric acetate oxidation reaction to the fluorescent intermediate. The mercuric acetate oxidation (at 100°C) yielded maximum conversion of intact I and II when conducted for 30—45 min. Nordiclofensine (I-A) did not produce significant fluorescent peaks. The retention times of the major derivatives of the initial oxidation were: I ca. 4.8 min, II ca. 5.6 min. The reaction mixture was diluted with mobile phase and injected directly. The fluorescence detector was set at λ_{ex} = 254 nm, and λ_{em} = > 389 nm (UV cut-off filter).

The HPLC system developed for the determination of the intact compounds was also used for the optimization of the photolysis reaction after chemical oxidation. The optimum photolysis time was determined to range between 15 and 25 min. The final products were identified by the disappearance of the

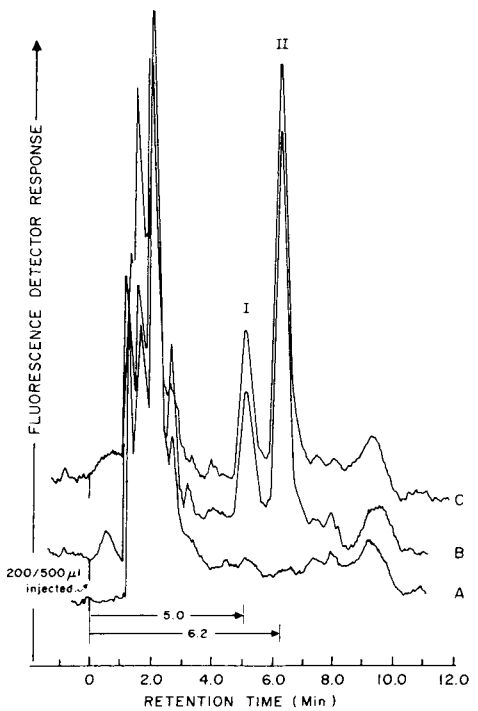


Fig. 3. Chromatograms of (A) control human plasma; (B) analysis of I and II (internal standard) recovered from human plasma; and (C) analysis of diclofensine (I) in human plasma following the oral administration of a 45-mg dose (three 15-mg capsules) of I • HCl.

3,4-dihydroisoquinolinium derivative peaks from the chemical oxidation (100°C) and the appearance of the fully aromatized isoquinolinium derivative peaks due to photolysis. The reaction products were chromatographed at 2.0 ml/min and gave retention times of ca. 5.7 min for I and ca. 7.2 min for II. The metabolites I-A, I-B and I-C, however, did not form strongly fluorescent derivatives in these initial experiments.

Alkylation of metabolites I-A, I-B, and I-C

Since the alkylated compounds I and II yielded the best results it was apparent that the O- and N-dealkylated metabolites should be alkylated to favor the overall derivatization reactions.

Initial experiments with alkylhalide (C_2H_5I) catalyzed by quaternary ammonium salts as suggested by several workers [13, 14] yielded inconsistent results although corroborating the feasibility of the reaction. Other workers [14, 15] used strongly basic inorganic salts as catalysts under anhydrous conditions to provide more vigorous reaction conditions, thus our investigation of the use of potassium hydroxide pellets in acetone heated at 70—75°C (water bath) gave encouraging yields of the alkylated products.

Several alkyl halides, including 1-iodopropane, 2-iodopropane, 1-iodobutane, 2-iodobutane and 1-iodo-2-methylpropane, were investigated as alkylation reagents. The highest conversion efficiency and optimal retention times were obtained with 2-iodopropane. The primary advantage of this reagent was that all the alkylated compounds, viz. I-A, I-B and I-C were chromatographically resolved from I and II (internal standard) after the oxidation steps. Although ethyliodide yielded better alkylation of I-A and I-B than did 2-iodopropane, I-A, however, reacted to yield II (internal standard). An internal standard suitable for use with ethyliodide as the alkylation reagent was not available.

The 2-iodopropane derivatives of I-A, I-B and I-C had retention times of 8.6, 12.3 and 11.7 min, respectively, in this HPLC system.

Extraction of the alkylated products from the reaction mixture also presented problems with respect for recovery, chromatographic cleanliness and overall yield of the respective fluorophors.

When the alkylation mixture was eluted with anhydrous solvents, small particles of potassium hydroxide and condensation products from acetone under alkaline conditions were carried over into the culture tubes used for the mercuric acetate oxidation reaction. This resulted in a final solution (after photolysis) which sometimes was yellow to tan in color, probably due to the precipitation of mercuric salts, which gave low and non-reproducible reaction efficiencies to the respective fluorophores. Addition of ethanol—acetic acid—water to the elution solvents resulted in an effective extraction procedure which yielded clean oxidation reactions with good recoveries. Washing the diethyl ether with 0.05 *M* sulfuric acid and rinsing the potassium hydroxide pellets with acetone immediately prior to use significantly improved the reproducibility of quantitation and the cleanliness of the chromatograms. The alkylation procedure is essential for the sensitive determination of the metabolites. However, the sensitivity for the determination of I was reduced to 2 ng/ml of plasma with the alkylation procedure, whereas without the alkylation the sensitivity for I was about 0.4 ng/ml, following extraction and pre-concentration of the isoquinolinium derivatives (see Table IV).

Luminescence spectral characteristics

Individual (100 μg/ml) solutions of each compound were taken through the total derivatization steps of alkylation (where applicable), chemical and photochemical reactions to yield the final alkylated isoquinolinium derivatives. The reaction mixtures were diluted to 1 μg/ml concentrations with 80% ethanol—water and scanned in this solution for their excitation/emission spectral characteristics. The corrected spectra of each compound determined at ambient temperature show very similar characteristics (Fig. 4) with the major excitation maximum at 250—254 nm and a minor one at 365 nm, whereas the emission spectrum is broad with a maximum at 425 nm. Alkylation is essential, not only to enhance the efficiency of fluorophor formation of the isoquinolinium derivative but also to yield products with similar spectral characteristics, i.e. excitation/emission maxima and fluorescence quantum yield. The non-alkylated derivatives of I-A, I-B and I-C not only gave lower fluorescence quantum yields but also exhibited widely different emission maxima, the derivatives of the phenolic compounds I-B and I-C having emission maxima at 580 nm instead of 425 nm for their respective alkylated products.

The two-step nature of the oxidation reaction was demonstrated by reacting I and II at 100°C in the mercuric acetate oxidation reaction per se, diluting the reaction mixture as described above, and obtaining corrected fluorescence spectra. In this case, the non-photolyzed oxidation products of I and II (3,4-dihydroisoquinolinium derivatives) each exhibited major excitation maxima at 250 and 280 nm with a minor peak at 365 nm. The fluorescence emission

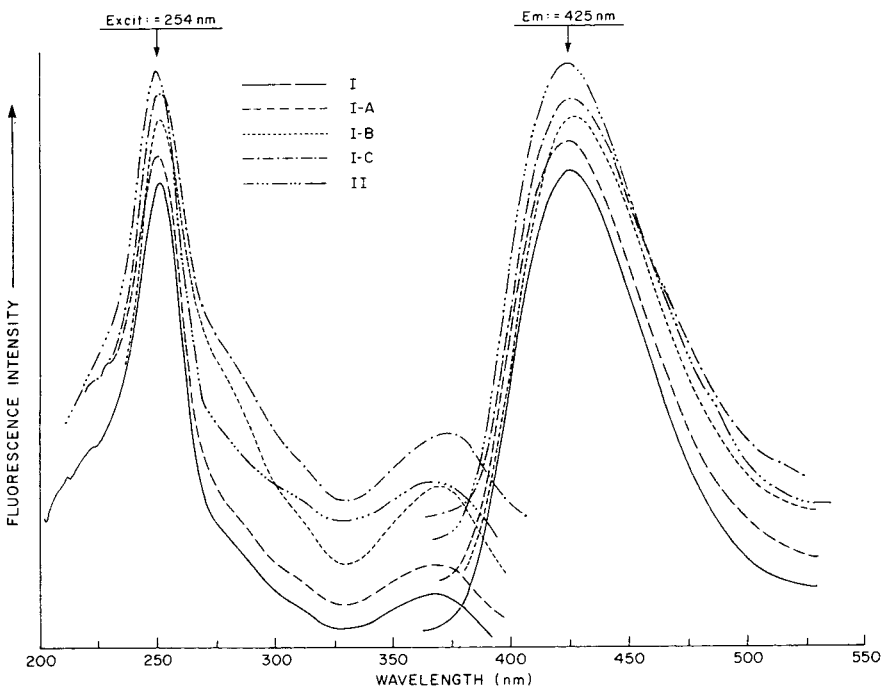


Fig. 4. Corrected ambient temperature excitation/emission spectra of the isoquinolinium derivatives of the respective alkylated compounds I, I-A, I-B, I-C and II (internal standard) in ethanol—0.05 *M* sulfuric acid.

maxima were very broad, with a peak at 475 nm. The fluorescence intensity of these derivatives were one third to one tenth that of the completely derivatized compounds. The alkylated isoquinolinium derivatives exhibited a major excitation maximum at 250—254 nm, disappearance of the 280-nm peak, retention of the minor peak at 365 nm, while the fluorescence emission spectrum showed a hypsochromic (blue) shift from 475 to 425 nm owing to the significant increase in the aromaticity of the compound.

Chromatographic behavior of the alkylated isoquinolinium derivatives

The fully derivatized products of I-A, I-B and I-C exhibited broad, tailing peaks when chromatographed in the HPLC mobile phase developed for the intact, non-derivatized parent compounds. Modifications to this mobile phase resulted in a mixed phase composed of 0.25 *M* triethylammonium phosphate (pH 2.5)—0.25 *M* acetic acid—methanol—acetonitrile—tetrahydrofuran (150:350:125:375:25) pumped at a flow-rate of 1.8 ml/min which improved the peak symmetry, shortened the analysis time, and resulted in the overall resolution of the derivatives (Fig. 2). The volume of tetrahydrofuran may be adjusted to ca. 15 ml to optimize resolution/analysis time.

The HPLC system is flushed initially with methanol, followed by methanol—water (50:50), to remove deposits from the column accumulated from previous use which can have adverse effects on the chromatography of the derivatives. The analytical mobile phase is allowed to recycle through the system for at least 2 h at a flow-rate of 1.8 ml/min to equilibrate the system. Non-equilibration will result in changes in the capacity factor (k') during chromatography.

Studies on the feasibility of the assay

Initial studies for the determination of the parent drug (I) per se without the use of either the alkylation step or the extraction of the isoquinolinium derivative yielded sensitivity and linearity over the concentration range 0.50—20.0 ng/ml of plasma. Although the precision at 0.50 ng/ml was poor (20%), the precision over the concentration range 1.0—20.0 ng/ml was significantly better than 10%. Plasma concentrations of I in man following a 45-mg oral dose (three 15-mg capsules) indicated very low concentrations, in the range 0.6—0.9 ng/ml over the 0.5—2.0 h post-dosing period. Extraction of the isoquinolinium derivatives of I and II (internal standard) from the reaction mixture into methylene chloride and their pre-concentration prior to HPLC analysis enabled their quantitation down to 0.4 ng/ml (400 pg/ml) with acceptable precision and reproducibility (Table IV). Analysis of I and I-A (nordiclofensine) using the alkylation step and without the extraction of the derivatives reduced the overall sensitivity of the assay for both compounds to 2.0 ng/ml with an overall inter-assay coefficient of variation of 14% for I and 16% for I-A. Based on plasma concentrations reported for I using a GC—MS assay with selected-ion monitoring [10, 11], it appeared very probable that I could also be quantitated using the HPLC assay.

Attempts at quantitating the directly extractable metabolite fraction using Procedure A but with alkylation, extraction of the isoquinolinium derivatives and pre-concentration prior to HPLC analysis was unsuccessful due to the carry-over of impurities from the alkylation step which interfered significantly with the chromatographic resolution of these compounds, hence was

abandoned. Thus the parent drug (I) and its major metabolite (I-B) were the two compounds quantitated in the clinical study reported herein.

Procedure A

Statistical validation of the assay. The quantitation of the major metabolite (I-B) was validated over the concentration range 0.06—3.0 μg/ml using weighted ($1/y$) linear regression analysis of the calibration data. Typical calibration curves over the above concentration range were linear ($y = 1.583x + 0.0035$). The correlation coefficient (r) was 0.9969 and the average deviation from the line was 9.6%.

Inter-assay validation data over the linear concentration range of I-B yielded a mean coefficient of variation of 18% (Table III). The poor precision is probably due to the absence of a suitable internal standard to monitor the variation incurred in the alkylation step.

TABLE III

STATISTICAL VALIDATION OF PROCEDURE A (INTER-ASSAY VARIABILITY) OF THE HPLC ASSAY FOR I-B (AGLYCONE)

Number of replicates	Concentration added (μg/ml)	Concentration found (± S.D.) (μg/ml)	Coefficient of variation (%)
5	3.00	2.77 ± 0.40	14.4
7	1.50	1.54 ± 0.18	11.9
4	0.60	0.68 ± 0.10	14.5
4	0.30	0.38 ± 0.07	19.3
3	0.15	0.13 ± 0.03	19.1
3	0.06	0.05 ± 0.01	26.8
Mean			17.7

Percentage recovery and sensitivity limits. The overall recovery of I-B from plasma was 86 ± 18% over the concentration range 0.06—3.0 μg/ml.

The sensitivity limit of the assay for the deconjugated compounds was approx. 30—60 ng/ml for diclofensine (I), 300 ng/ml for nordiclofensine (I-A) and I-C and 60 ng/ml for I-B (major metabolite) using 0.1 ml of plasma and 60 ng of II added as internal standard.

Procedure B

Statistical validation of the assay. The quantitation of diclofensine (I) was validated over the concentration rang 0.4—20 ng/ml of plasma using weighted ($1/y$) linear regression analysis of the calibration data. Typical calibration curves were linear over the above concentration range ($y = 0.1813x + 0.0145$). The correlation coefficient (r) was 0.9961 and the average deviation from the line was 9.6%. Intra- and inter-assay validation data over the linear concentration range of I yielded mean coefficients of variation of 6.2% and 9.1%, respectively (Table IV).

Percentage recovery and sensitivity limits. The overall recovery of I from plasma was 80 ± 13%. The sensitivity limit of the assay for I was 0.4 ng/ml,

TABLE IV

STATISTICAL VALIDATION OF PROCEDURE B OF THE HPLC ASSAY FOR FREE I (DICLOFENSINE) ONLY (NO ALKYLATION)

Number of samples	Concentration added (ng/ml)	Concentration found (± S.D.) (ng/ml)	Coefficient of variation (%)
A. Intra-assay variability			
4	20.0	21.05 ± 0.64	3.0
4	10.0	9.46 ± 0.75	8.0
4	4.0	3.92 ± 0.09	2.2
4	2.0	1.89 ± 0.06	3.5
4	1.0	0.98 ± 0.05	5.3
4	0.4	0.51 ± 0.08	15.1
Mean			6.20
B. Inter-assay variability			
8	20.0	21.02 ± 1.47	7.0
10	10.0	9.95 ± 1.22	12.3
11	4.0	3.65 ± 0.26	7.1
12	2.0	1.97 ± 0.24	12.4
8	1.0	0.99 ± 0.04	4.1
7	0.4	0.53 ± 0.06	11.6
Mean			9.1

following extraction and pre-concentration of the isoquinolinium derivative of I.

Application of the method to biological specimens

Plasma concentrations of diclofensine (I) and its major phenolic metabolite (I-B) were determined in two normal volunteers following the administration of a 45-mg (three 15-mg capsules) oral dose t.i.d. at 0 h, 8 h and 16 h (total daily dose = 135 mg) over seven consecutive days, with a final 45-mg dose at 0 h on day 8. The wash-out of the drug was monitored through days 8, 9 and 10. Blood samples were collected at 0 h, 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 24 h on day 1, and at 24 h thereafter on days 2—8. On day 8 blood samples were drawn at the same time points as on day 1 with the addition of a 12-h sample. Plasma was separated and stored frozen at −20°C until analyzed.

Plasma concentration—time profiles of I and I-B in two subjects from the above study are shown in Fig. 5A and B and Fig. 6A and B, respectively. Plasma concentrations of I in both subjects peaked at 0.95 and 2.65 ng/ml on day 1, between 45 and 60 min after the first 45-mg dose and declined rapidly thereafter to 0.4 ng/ml at 6 h before the second 45-mg dose was administered. The plasma concentrations showed a progressive increase thereafter over the seven days of t.i.d. treatment reaching apparent steady state by day 3, peaked at 3.4 and 7.8 ng/ml, 1 h after the last 45-mg dose on day 8, declining rapidly thereafter to 1.5 and 0.9 ng/ml, respectively at 24 h. The relatively low plasma concentrations of I following total daily dose of 135 mg (45 mg, three times)

suggested either extensive tissue distribution and/or extensive biotransformation and elimination of the drug.

Quantitation of the major metabolite (I-B) present in plasma and urine as a glucuronide conjugate [7, 11] was feasible due to the relatively high concentrations present. Procedure A which describes the quantitation of I-B after enzymatic deconjugation was used to determine plasma concentrations of I-B in the same two subjects.

Plasma concentrations of I-B were sufficiently high as to dilute out the quantitation of any minor components present in the sample, viz. I, I-A and I-C.

Plasma concentrations of I-B following the first 45-mg dose of diclofensine in both subjects peak between 1 and 2.5 μg/ml, indicating rapid biotransforma-

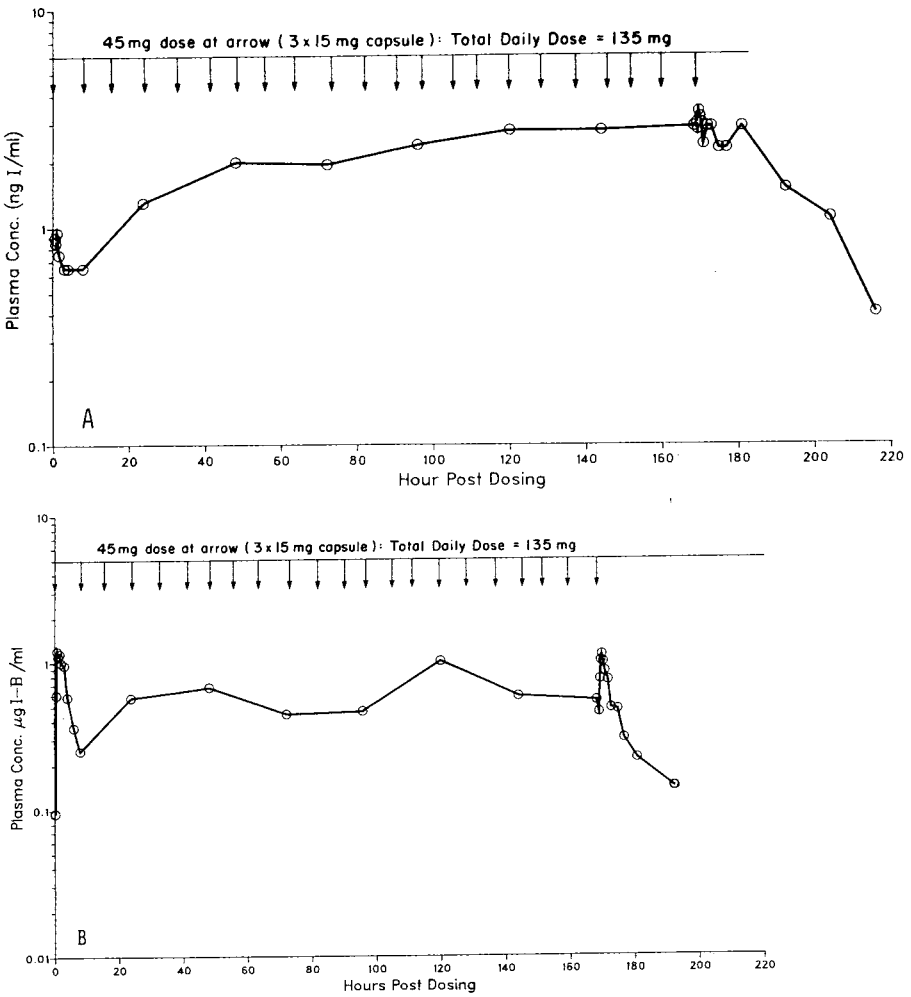


Fig. 5. Plasma concentration—time profiles of (A) diclofensine (I) in subject A following a 45-mg t.i.d. regimen for seven consecutive days followed by a single 45-mg dose on day 8; (B) metabolite I-B (aglycone) in subject A over seven consecutive days of a 45-mg t.i.d. dosing regimen and a final 45-mg single dose on day 8.

tion, decline gradually thereafter and are measurable at 24 h post-dose (100—200 ng/ml). The plasma profiles over the eight-day period in both subjects are shown in Fig. 5B and 6B, respectively. The data suggest that steady-state concentrations of I-B-glucuronide are maintained over the seven-day period of multiple daily dosing (45 mg t.i.d.), and that on day 8 after the last 45-mg dose the plasma concentrations of I-B decline rapidly from a peak of 1.0—2.0 μg/ml

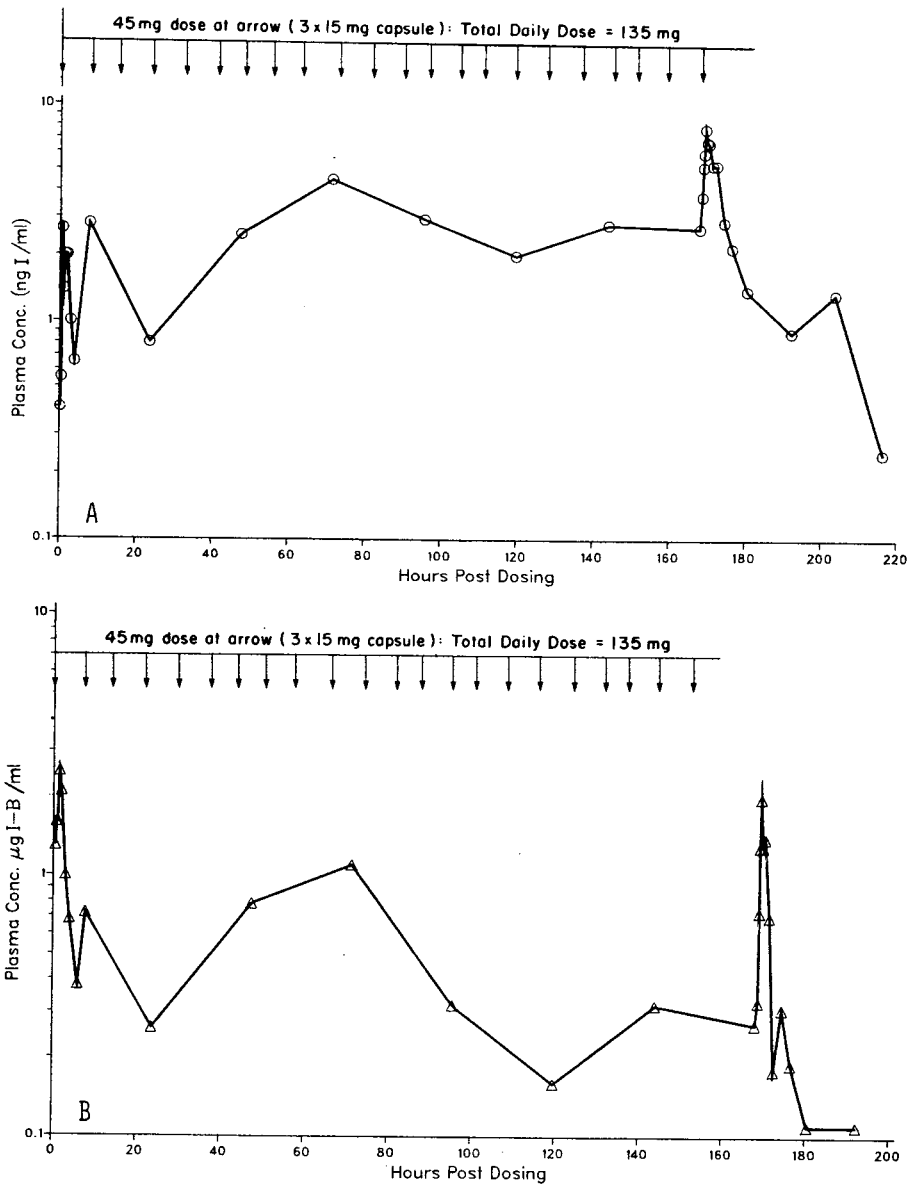


Fig. 6. Plasma concentration—time profiles of (A) diclofensine (I) in subject B following a 45-mg t.i.d. regimen for seven consecutive days followed by a single 45-mg dose on day 8; (B) metabolite I-B (aglycone) in subject B over seven consecutive days of a 45-mg t.i.d. dosing regimen and a final 45-mg single dose on day 8.

at 1 h to around 0.15 μg/ml at 24 h in a parallel manner to that seen on day 1 after the first 45-mg dose. The data suggest that the plasma profile of the major metabolite appears to mimic that of the parent drug in each subject and that, although steady-state concentrations of the metabolite were maintained during therapy, both the parent drug and the metabolite were cleared rapidly upon cessation of therapy.

ACKNOWLEDGEMENTS

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REFERENCES

1 L.M.O. Omer, Int. J. Clin. Pharmacol. Toxicol., 20 (1982) 320.
2 H.H. Keller, R. Schaffner, M.O. Carruba, W.P. Burkard, M. Pieri, E.P. Bonetti, R. Scherschlicht, M. Da Prada and W.E. Haefely, Adv. Biochem. Psychopharmacol., 31 (1982) 249.
3 I. Hoffmann, Arzneim.-Forsch., 23 (1973) 45.
4 R.N. Brodgen, R.C. Heel, T.M. Speight and G.S. Avery, Drugs, 18 (1979) 1.
5 R.L.P. Lindberg, J.S. Salonen and E.I. Iisalo, J. Chromatogr., 276 (1983) 85.
6 M.O. Carruba, W.P. Burkard and M. Da Prada, Experientia, 36 (1980) 705.
7 L.M.O. Omer and J. Raaflaub, Clin. Pharmacol. Ther., 33 (1983) 259 (Abstract No. C-6).
8 P. Gentili, F. de Maria, M. de Vanna, F. Drago, L.M.O. Omer and S. Ismail, Curr. Ther. Res. Clin. Exp., 35 (1984) 386.
9 L.M.O. Omer, J. Raaflaub, I. Forgo, D. Hartman, U.B. Ranalder and U.C. Dubach, Clin. Pharmacol. Ther., 35 (1984) 265.
10 U.B. Ranalder and J. Raaflaub, Hoffmann-La Roche, Basle, unpublished data on file, 1982.
11 R. Jauch, G. Osterhelt and W. Arnold, Hoffmann-La Roche, Basle, unpublished data on file, 1982.
12 J.A.F. de Silva, N. Strojny and N. Munno, J. Pharm. Sci., 62 (1973) 1066.
13 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
14 M. Ervik and K. Gustavii, Anal. Chem. 46 (1974) 39.
15 P. Kovac and D. Anderle, in K. Blau and G.S. King (Editors), Handbook of Derivatives for Chromatography, Heyden and Son, Bellmawr, NJ, 1977, pp. 201—233.

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CHROMBIO. 2577

SELECTIVE AND SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE METABOLITES OF NOMIFENSINE IN HUMAN PLASMA

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SUMMARY

A selective high-performance liquid chromatographic method for the determination of the three metabolites of nomifensine in human plasma is described. All metabolites and the internal standard, mexiletine, are extracted with diethyl ether and then back-extracted into an acidic aqueous phase. After subsequent extraction into diethyl ether the metabolites are analysed by high-performance liquid chromatography. A reversed-phase C_{18} column is used with a mobile phase of dioxane—methanol—potassium phosphate buffer (pH 2.25). The sensitivity of the method is 0.007 μmol/l for all metabolites. Extraction efficiencies are 84.6%, 75.8%, and 78.2% for 4′-hydroxynomifensine, 4′-hydroxy-3′-methoxynomifensine and 3′-hydroxy-4′-methoxynomifensine, respectively. The reproducibility of the method is good, the coefficients of variation (%) varying between 2.1% and 9.9% in the concentration range 0.05—1.00 μmol/l. The procedure was applied to human plasma samples from a volunteer who had received a single oral dose of nomifensine. The method is accurate and sensitive for pharmacokinetic studies on the metabolites of nomifensine.

INTRODUCTION

Nomifensine is a psychotropic agent with demonstrated antidepressant properties [1]. The chemical structure of nomifensine is 8-amino-1,2,3,4-tetrahydro-2-methyl-4-phenylisoquinoline (Fig. 1). The first step in the metabolism of nomifensine is hydroxylation; the phenyl ring is hydroxylated in the 3′ and 4′ positions. So the first and probably the most important metabolite is 4′-hydroxynomifensine (M_1) [2]. In the next phase the hydroxyl groups are methylated, resulting in the formation of 4′-hydroxy-3′-methoxynomifensine (M_2) and 3′-hydroxy-4′-methoxynomifensine (M_3) [2]. Besides these three main metabolites (M_1, M_2 and M_3) four further metabolites are formed, but in

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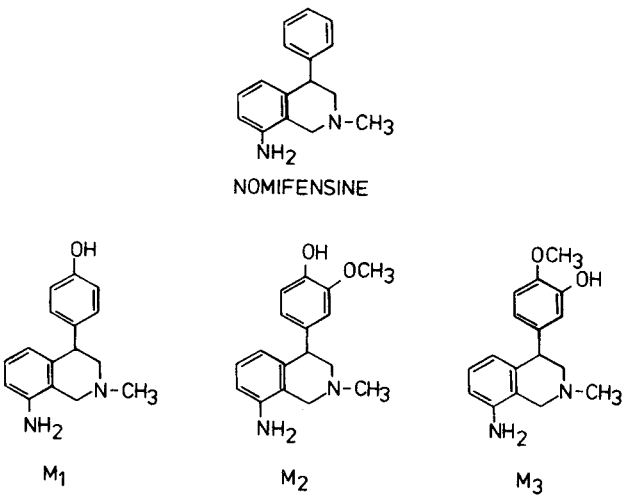

Fig. 1. Structures of nomifensine and its three main metabolites M_1, M_2 and M_3.

negligible amounts [3]. All metabolites are conjugated in the last phase of the metabolism [4].

An accurate and selective methodology for the measurement of the parent drug and its metabolites in human body fluids is a prerequisite for detailed pharmacokinetic studies. A number of methods for the determination of nomifensine have been published [5–9]. Uihlein and Hajdú [10] have described a liquid chromatographic method for the analysis of nomifensine, metabolite M_1 and the sum of metabolites M_2 and M_3. The sensitivity and selectivity of this procedure are insufficient for pharmacokinetic studies. A quantitative thin-layer chromatographic method for the measurement of nomifensine and its three metabolites in human urine has also been published [11]. However, no method for the quantitation of all three main metabolites of nomifensine, M_1, M_2 and M_3, in human plasma has been described.

This paper describes a selective, sensitive and accurate high-performance liquid chromatographic (HPLC) procedure for the detection of all three principal metabolites of nomifensine, M_1, M_2 and M_3, in non-conjugated form in human plasma. The procedure was used to monitor plasma concentrations of these metabolites in a volunteer who had received a single oral dose of nomifensine.

EXPERIMENTAL

Reagents and chemicals

The following reagents were used: 4-hydroxynomifensine hydrogen maleate (M_1), 4-hydroxy-3-methoxynomifensine (M_2) and 3-hydroxy-4-methoxy-nomifensine (M_3) were gifts from Hoechst (Frankfurt am Main, F.R.G.). Mexiletine hydrochloride was obtained from Boehringer (Mannheim, F.R.G.). Diethyl ether, dioxane and methanol were of analytical-reagent grade (Merck, Darmstadt, F.R.G.).

A solution of mexiletine in distilled water (20 μmol/l) was used as the internal standard.

Working metabolite solutions contained 10 or 100 μmol/l in 50% methanol. Drug plasma standards were prepared by spiking blank control plasma with appropriate microlitre volumes of each working metabolite solution to obtain seven plasma standards with the following concentrations of each metabolite: 0.025, 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 μmol/l.

Extraction procedure

To a 1.0-ml plamsa sample, 125 μl of mexiletine solution (20 μmol/l) were added. The plasma was made alkaline by adding 1 ml of 0.1 *M* sodium tetraborate buffer (pH 9.0). The metabolites M_1, M_2 and M_3 and the internal standard were extracted with 5 ml of diethyl ether by shaking for 20 min. After centrifugation (1200 *g*) the diethyl ether layer was transferred to a new clean tube containing 1 ml of 0.2 *M* hydrochloric acid. The mixture was shaken for 20 min. The diethyl ether phase was separated by centrifugation (1200 *g*) and aspirated. The acidic layer was made alkaline with 3 ml of 0.1 *M* sodium tetraborate buffer (pH 9.0). The metabolites M_1, M_2 and M_3 and the internal standard were extracted with 5 ml of diethyl ether by shaking for 20 min. The organic phase was evaporated at 40°C under a gentle stream of pure nitrogen. The residue was reconstituted in 100 μl of phosphoric acid (0.05 *M*), and 50 μl were injected into the chromatograph. The concentrations of all three metabolites in the plasma samples were determined from a calibration curve of peak height ratio (drug/internal standard) versus drug concentration in plasma standards carried through this procedure.

Chromatographic system

The determinations were carried out using the following chromatographic system: SP 8700 solvent-delivery system with SP 8750 organizer (Spectra-Physics, Santa Clara, CA, U.S.A.); Rheodyne injector with 50-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.); variable-wavelength UV detector SF 773 set at 210 nm (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). The reversed-phase column was a 10-μm μBondapak C_{18}, 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.). The solvent used was methanol—dioxane—0.01 *M* potassium phosphate buffer (pH 2.25) (6.5:7:86.5) and the flow-rate was 2.2 ml/min. Chromatograms were recorded with a laboratory potentiometric recorder.

Application of the method

The formation of the three metabolites of nomifensine, M_1, M_2 and M_3, was studied in a healthy volunteer who had taken 100 mg of nomifensine in capsule form orally. The volunteer fasted overnight and received a breakfast 3 h after taking the drug. Seventeen blood samples were taken, up to 24 h after administration of the drug. Plasma was promptly separated and frozen at −60°C until analysis.

RESULTS AND DISCUSSION

Chromatograms of extracts from blank plasma, the plasma sample of a volunteer after a single dose of nomifensine and blank plasma spiked with

0.50 μmol/l of metabolites M_1, M_2 and M_3 are illustrated in Fig. 2A, B and C, respectively. The metabolites M_1, M_2 and M_3 and the internal standard, mexiletine, were well separated with retention times of 4.00, 4.50, 5.60 and 6.60 min, respectively. The HPLC method showed a linear increase in response over the concentration range 0.01—1.00 μmol/l in plasma for all three metabolites (Fig. 3). A plot of peak height ratio against metabolite concentration gave a linear calibration curve for each metabolite as well. The equations of the

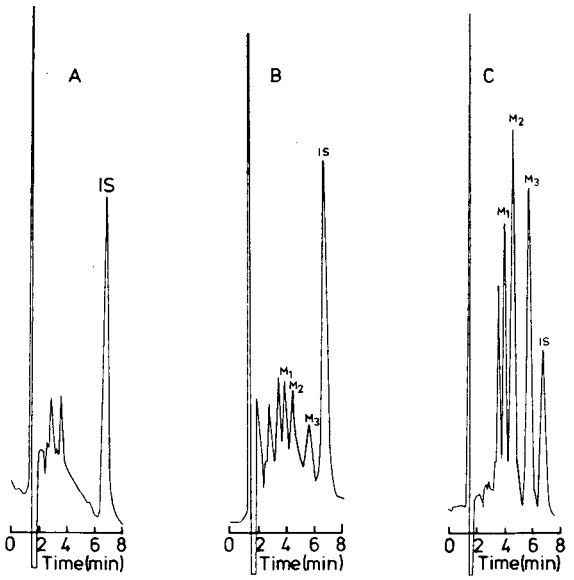

Fig. 2. Liquid chromatograms of the extracts from blank plasma (A), a plasma sample of a volunteer 50 min after a single 100-mg dose of nomifensine (B) and blank plasma spiked with 0.50 μM metabolites M_1, M_2 and M_3 (C). The concentrations of the metabolites M_1, M_2 and M_3 are 0.070, 0.050 and 0.045 μmol/l, respectively. IS = internal standard, mexiletine. For chromatographic conditions, see text.

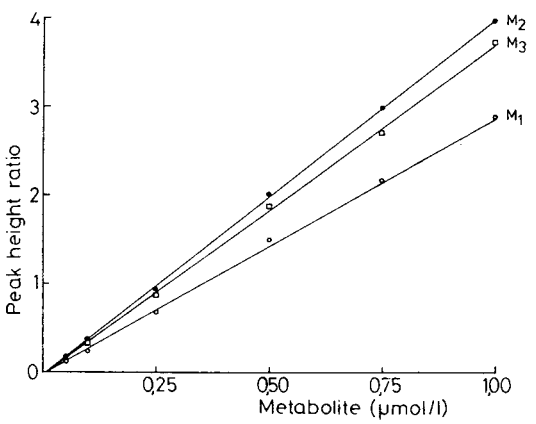

Fig. 3. Calibration graphs for metabolites M_1, M_2 and M_3. Peak height ratios of metabolites to those of the internal standard are plotted against metabolite concentration in plasma. The correlation coefficients of the lines are $r > 0.999$, 0.999 and 0.998 for M_1, M_2, and M_3, respectively.

calibration curves were $y = 2.93x - 0.04$, $y = 4.12x - 0.06$ and $y = 3.79x - 0.06$ (y = peak height ratio, metabolite/internal standard and x = metabolite concentration) for M_1, M_2, M_3, respectively. When defined as a signal level exceeding three times the background noise, the detection limit of the present HPLC method is 0.007 μmol/l for all three metabolites.

The precision was assessed by multiple analyses of six standard plasma pools in the concentration range 0.05—1.00 μmol/l for all three metabolites. The coefficients of variation for intra-assay variability of metabolites are given in Table I: they varied from 2.1% to 9.9% in the concentration range studied. The results demonstrate the high accuracy and reproducibility of the method.

From a comparison of metabolite peak heights obtained from direct injection of aqueous solutions and from samples carried through the assay procedure, the extraction efficiencies were estimated as 84.6 ± 1.0%, 75.8 ± 1.4% and 78.2 ± 1.4% (± S.E., $n = 8$) for M_1, M_2 and M_3, respectively (Table II). The recoveries were calculated over the concentration range 0.05—1.00 μmol/l.

To extract nomifensine metabolites M_1, M_2 and M_3, four different extraction solvents were tried: ethyl acetate, diethyl ether, hexane and dichloromethane. The highest recovery was found with diethyl ether for all three metabolites. The optimum pH value for the extraction of all three metabolites was found to be 9. This is supported by the maximum partition coefficients in octanol—water

TABLE I

INTRA-ASSAY REPRODUCIBILITY ($n = 8$) OF HPLC FOR THE DETERMINATION OF NOMIFENSINE METABOLITES IN HUMAN PLASMA

Concentration (μmol/l)	Coefficient of variation (%)		
	M_1	M_2	M_3
0.05	4.5	7.5	9.9
0.10	7.3	6.8	8.3
0.25	5.2	6.1	8.0
0.50	5.5	4.7	6.9
0.75	2.2	2.4	2.1
1.00	6.4	3.8	4.7

TABLE II

DETERMINATION OF NOMIFENSINE METABOLITES IN HUMAN PLASMA ($n = 8$)

Concentration (μmol/l)	Extraction efficiency (mean ± S.E. %)		
	M_1	M_2	M_3
0.05	85.5 ± 1.4	69.7 ± 4.1	79.7 ± 3.4
0.10	83.1 ± 3.1	61.3 ± 2.0	65.5 ± 3.5
0.25	86.5 ± 0.8	81.2 ± 1.1	81.2 ± 2.2
0.50	78.3 ± 1.7	81.3 ± 1.2	87.1 ± 1.9
0.75	82.7 ± 1.2	75.7 ± 0.8	74.0 ± 0.7
1.00	91.6 ± 2.7	85.6 ± 1.4	81.5 ± 2.0
Mean	84.6 ± 1.0	75.8 ± 1.4	78.2 ± 1.4

of 79 ± 9 for nomifensine, 50 for M_1 and 26 for M_2 at pH 8—10, as estimated by Sistovaris [11]. Back-extraction of the metabolites into an acidic aqueous phase almost purified the extract from the endogenous compound eluting just before M_1 (Fig. 2). Mexiletine was chosen as the internal standard because it was extracted well in the procedure used here and eluted later than all three metabolites, with a retention time of 6.60 min.

Several mobile phases were investigated before the final selection of the chromatographic conditions was made. Acetonitrile, tetrahydrofuran, methanol and dioxane were evaluated as organic components of the eluent. Acetonitrile in the mobile phase diminished the UV absorption of all metabolites, probably by a chemical reaction with the metabolites, resulting in poor detection. Acetonitrile, tetrahydrofuran and methanol did not sufficiently separate the metabolites M_1 and M_2. Dioxane was the only organic component that separated all three metabolites from each other. Replacing a part of dioxane by methanol in the mobile phase diminished the background noise markedly.

The interference of several psychotropic drugs with the present HPLC method was checked by injecting concentrated solutions of these compounds into the chromatographic system. From the tested substances chlordiazepoxide, perphenazine, desmethyldoxepin, thioridazine, sulpiride, mianserin, doxepin and oxazepam did not interfere. Caffeine was also tested and it eluted just before the metabolite M_1, so it might be the endogenous compound seen in the chromatograms of extracted human plasma (Fig. 2).

The parent drug, nomifensine, eluted after the metabolites and the internal standard with a retention time of 9.80 min. Nomifensine did not, therefore, interfere with the analysis of metabolites, but lengthened the total time for each chromatographic run. In principle it is possible to determine the parent drug, nomifensine, simultaneously with the metabolites, but a more accurate and sensitive method published earlier [9] is recommended for the analysis of nomifensine. Plasma concentrations of free metabolites M_1, M_2 and M_3 in a male volunteer who had received a single oral dose of 100 mg of nomifensine are shown in Fig. 4. The results clearly demonstrate that the sensitivity of the method is sufficient for pharmacokinetic studies.

In conclusion, the HPLC method described here is demonstrated to be selective and sensitive, and therefore suitable for pharmacokinetic studies on the metabolites M_1, M_2 and M_3. In preliminary experiments the method proved to be applicable to an urine analysis of nomifensine metabolites M_1, M_2 and

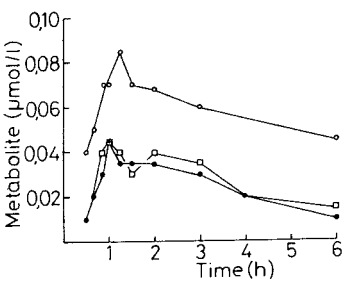

Fig. 4. Plasma profile of three non-conjugated metabolites M_1 (○), M_2 (●) and M_3 (□) in a volunteer after a single oral dose of 100 mg of nomifensine.

M_3, too. The method is currently in use for the determination of nomifensine metabolites in human plasma and urine after intravenous administration of the drug.

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REFERENCES

1 R.N. Brogden, R.C. Heel, T.M. Speight and G.S. Avery, Drugs, 18 (1979) 1.
2 I. Hoffmann, Int. Pharmacopsychiatry, 17 (1982) 4.
3 W. Heptner, I. Hornke, F. Cavagna, W. Fehlhaber, W. Rupp and H.P. Neubauer, Arzneim.-Forsch., 28 (1978) 58.
4 I. Hornke, H.W. Fehlhaber, M. Girg and H. Jantz, Brit. J. Clin Pharmacol., 9 (1980) 255.
5 L. Vereczkey, G. Bianchetti, V. Rovei and A. Frigerio, J. Chromatogr., 116 (1976) 451.
6 E. Bailey, M. Fenoughty and L. Richardson, J. Chromatogr., 131 (1977) 347.
7 J. Chamberlain and H.M. Hill, Brit. J. Clin. Pharmacol., 4 (1977) 117.
8 W. Heptner, M.J. Badian, S. Baudner, O.E. Christ, H.M. Fraser, W. Rupp, K.E. Weimer and H. Wissmann, Brit. J. Clin. Pharmacol., 4 (1977) 123.
9 R.L.P. Lindberg, J.S. Salonen and E.I. Iisalo, J. Chromatogr., 276 (1983) 85.
10 M. Uihlein and P. Hajdú, Arzneim.-Forsch., 27 (1977) 98.
11 N. Sistovaris, J. Chromatogr., 276 (1983) 139.

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CHROMBIO. 2583

DETERMINATION OF NIFEDIPINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid, accurate and sensitive high-performance liquid chromatographic assay was developed for the determination of nifedipine in human plasma. A toluene extract of an alkalinized plasma sample was chromatographed on a reversed-phase column with electrochemical detection at +0.95 V. The recovery of nifedipine from plasma was about 100%. The detection limit for nifedipine in plasma was 2 ng/ml using 0.5 ml of sample. The assay gave a linear response over the concentration range 5—400 ng/ml in plasma. The coefficients of variation from 9.6 to 191.0 ng/ml varied between 5.2 to 1.0% and the accuracy did not exceed 3.0%. Photodegradation products and metabolites of nifedipine did not interfere in the analysis. This method allowed the behaviour of nifedipine in humans to be studied.

INTRODUCTION

Nifedipine belongs to a group of calcium channel antagonists widely used as coronary vasodilators. The major therapeutic application is for angina pectoris [1] and hypertension [2]. Nifedipine is more than 90% absorbed from oral doses and almost completely metabolized before it is excreted [3—5]. As shown in Fig. 1, dimethylpyridinecarboxylic acid (M-I), hydroxymethylpyridinecarboxylic acid (M-II) and the corresponding lactone (M-III) are reported as metabolites [3—6]. Also, the pyridine derivative (P-I) is postulated as a precursor of known metabolites of nifedipine [7—10]. Nifedipine is very sensitive to light and the 2-nitroso derivative (P-II) and pyridine derivative (P-I) have been detected as its light degradation products [11]. These metabolites and photodegradation products are pharmacologically inactive [3, 5].

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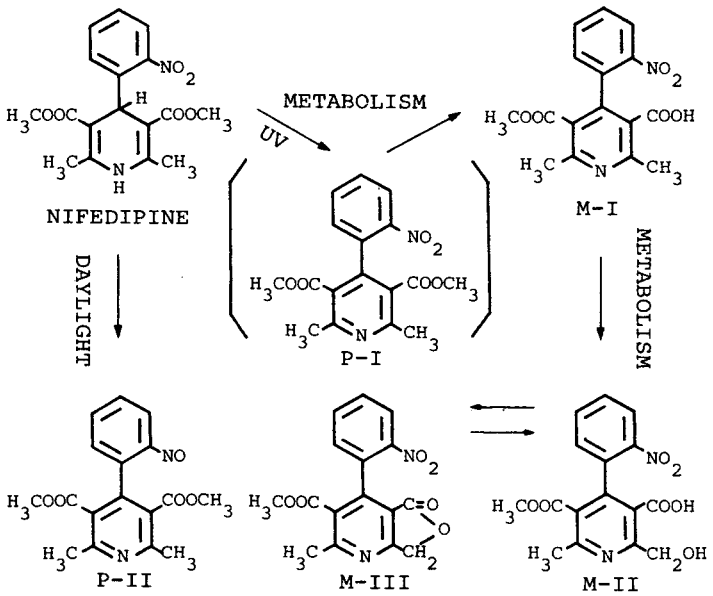


Fig. 1. Structures of nifedipine, its metabolites and photodegradation products.

Many methods for the determination of nifedipine in plasma have been described. Recently, novel gas chromatographic (GC) [6—14], high-performance liquid chromatographic (HPLC) [15—18] and radio-receptor assays [19] have been developed. However, the GC determination of nifedipine suffers from a serious problem as high temperatures are employed and the non-reproducible degradation of nifedipine to P-I during chromatography cannot be avoided. Therefore, nifedipine is oxidized to its more stable pyridine derivative (P-I) prior to analysis [6, 12]. The specificity of the method, however, may be reduced when considerable amounts of P-I are present in the original sample. The HPLC methods are highly accurate and selective, but the sensitivity of most of them is low owing to the use of a UV detector, and they therefore require a large amount of plasma [16, 17] and/or complex sample preparation [15].

The purpose of this study was to develop a simple, sensitive and selective HPLC method using an electrochemical detector for the determination of nifedipine in human plasma.

EXPERIMENTAL

Materials

The nifedipine supplied (Kanebo, Osaka, Japan) was used without further purification. Diethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate, used as an internal standard, was synthesized according to the reported procedure [6]. HPLC-grade methanol, tetrahydrofuran and toluene were purchased from Wako (Osaka, Japan). Other reagents and chemicals were of analytical-reagent grade.

Chromatography

The chromatographic system consisted of a Model 510 solvent delivery

system, a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and a Model VMD-501 electrochemical detector (Yanagimoto Seisakusho, Kyoto, Japan). The potential of the detector was set at +0.95 V versus an Ag/AgCl reference electrode. Chromatographic experiments were performed on a Unisil Pack 5C18-150A column (octadecylsilica, 15 cm × 4.6 mm I.D., particle size 5 μm) (Gasukuro Kogyo, Tokyo, Japan). Methanol—tetrahydrofuran—0.05 *M* phosphate buffer (pH 3.0) (660:10:330) was employed as the mobile phase at a flow-rate of 0.8 ml/min. The mobile phase was degassed by vacuum plus sonication prior to use. The chromatography was carried out at 20°C.

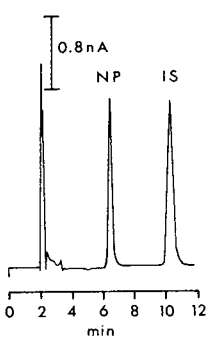
Sample preparation

To 0.5 ml of plasma in a 10-ml conical extraction tube fitted with a glass cap were added 50 μl of the internal standard solution (1 μg/ml in methanol). Then 0.5 ml of 0.1 *M* borate buffer (pH 9, adjusted with sodium hydroxide solution) was added and the mixture was vortex-mixed for 10 sec. The extraction was carried out on a shaking board for 15 min with 6 ml of toluene followed by centrifugation at 2000 *g* for 10 min. A 5-ml volume of the organic layer was transferred into a second tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase and 10-μl aliquots were injected into the chromatograph. All steps were carried out in a dark room and samples were shielded from exposure to direct lighting to prevent light degradation of nifedipine [11].

RESULTS AND DISCUSSION

Electrochemistry

The electrochemical detector used was equipped with dual electrodes, but we used only a single electrode. The chromatogram shown in Fig. 2 was obtained from a standard solution of nifedipine and the internal standard at a detector potential of +0.95 V vs. Ag/AgCl. Hydrodynamic voltammograms observed for the oxidation of nifedipine and the internal standard under the HPLC conditions used are illustrated in Fig. 3. Based on these curves, +1.15 V



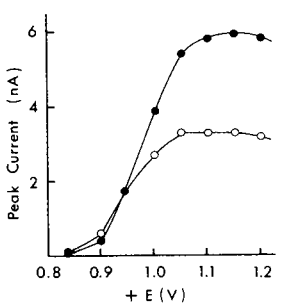


Fig. 2. Chromatogram of a standard mixture of nifedipine (NP) and the internal standard (IS). All amounts injected were 7.5 ng.

Fig. 3. Hydrodynamic voltammograms of nifedipine (•) and the internal standard (○). All amounts injected were 7.5 ng.

was the most sensitive potential. However, at high potentials (> +1.0 V) the background current becomes high, the baseline drifts owing to the oxidation of water, oxygen and other mobile-phase components and the performance of the electrode decreases rapidly [20]. Therefore, an applied electrode potential of +0.95 V was chosen. The detection limit (signal-to-noise ratio = 2) for a standard solution was approximately 40 pg injected at +0.95 V. This detection limit could be lowered by increasing the detector potential.

Calibration

Calibration samples were prepared by using drug-free plasma. Plasma samples (0.5 ml) spiked with nifedipine at known concentrations (0, 5, 10, 30, 60, 100, 200 and 400 ng/ml) were assayed as described above. A least-squares linear regression evaluation of the peak height ratio (y) versus concentration (x) relationship gave $y = 0.0114x - 0.0241$, with a correlation coefficient of 0.9998.

Reproducibility and accuracy

Reproducibility and accuracy were determined for five or six spiked plasma samples with respect to a calibration graph (Table I). The within-day coefficients of variation were 1.0—5.2%. The day-to-day coefficients of variation for analyses of the same plasma samples on three days over a period of one week were 3.2% at 41.8 ng/ml ($n = 6$) and 2.9% at 83.1 ng/ml ($n = 5$). The accuracy of the method expressed as the mean deviation of all concentrations from the theoretical value ranged from —2.9% to 2.3%.

TABLE I

REPRODUCIBILITY AND ACCURACY FOR NIFEDIPINE

Spiked value (ng/ml)	Number of samples	Assay value (ng/ml) (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%)
Within-day				
9.6	6	9.5 ± 0.5	5.2	—0.2
47.8	6	46.4 ± 1.3	2.8	—2.9
95.5	6	96.8 ± 1.0	1.0	1.4
191.0	6	195.1 ± 4.4	2.2	2.2
Day-to-day				
41.8	6	41.7 ± 1.3	3.2	—0.3
83.1	5	81.6 ± 2.3	2.9	2.3

Recovery

The recovery of nifedipine was estimated as follows. Control plasma samples spiked with 10, 50 and 100 ng/ml nifedipine were extracted as described above without addition of the internal standard solution. Before evaporation the internal standard solution was added to each extract and the subsequent procedure was carried out as described above. The recovery was calculated by comparing the peak height ratios of control plasma samples with those of non-extraction standards.

TABLE II

EXTRACTION YIELD OF NIFEDIPINE FROM SPIKED PLASMA SAMPLES (n = 3)

Spiked value (ng/ml)	Extraction yield (%)	Coefficient of variation (%)
10	101.2	5.1
50	101.7	2.0
100	102.6	1.4

As shown in Table II, nifedipine was recovered quantitatively, with a range of 101.2—102.6%.

Chromatography

In reversed-phase HPLC, the mobile phase is commonly a binary mixture of solvents. In this study, various combinations of methanol and buffer solution were examined, but a small peak in the blank plasma was not well separated from the internal standard. A ternary solvent system was then investigated using combinations of methanol, acetonitrile and tetrahydrofuran [21]. As a result, methanol—tetrahydrofuran—0.05 *M* phosphate buffer (660:10:330) was selected for their assay.

Typical chromatograms of a blank plasma and a plasma sample taken 20 min after oral administration of 10 mg of nifedipine to a healthy volunteer are shown in Fig. 4. The retention times of nifedipine and the internal standard were approximately 6.5 and 10.6 min, respectively. No interfering peaks were found in several blank plasma samples examined. The concentration of nifedipine in this sample was about 120 ng/ml. Based on a signal-to-noise ratio of 2, the detection limit of the assay for a plasma sample (0.5 ml) was ca. 2 ng/ml, which is well below the drug concentration expected in biological specimens from patients given therapeutic doses of nifedipine.

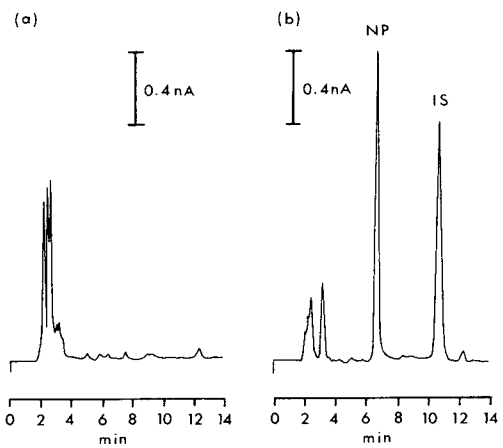


Fig. 4. Chromatograms of extracts from (a) human blank plasma and (b) human plasma collected 20 min after oral administration of 10 mg of nifedipine preparation. Peaks: NP = nifedipine; IS = internal standard.

Selectivity

The metabolites and photodegradation products of nifedipine did not interfere as they did not have electrochemical activity. Nifedipine may be administered in combination with other drugs such as pindolol, carteolol, acebutolol, trichlormethiazide, furosemide, methyldopa, reserpine, diazepam, oxazepam, aspirin, warfarin and trapidil. Among the drugs tested, pindolol, methyldopa and resperine had electrochemical activity, but they did not interfere in the assay because they were well resolved from nifedipine and the internal standard.

Application to biological samples

The proposed method was applied to the determination of nifedipine in plasma samples obtained from three healthy, fasting volunteers who received orally fine granules containing 10 mg of nifedipine (Sepamit; Kanebo, Japan). Blood samples were drawn 0, 20, 40, 60, 120, 240 and 420 min after administration. After immediate centrifugation the plasma was stored at −20°C until taken for assay.

Fig. 5 shows the mean plasma concentration curve of the three subjects and Table III gives the parameters calculated from the data in Fig. 5. Nifedipine was rapidly absorbed from this preparation with maximum concentrations of ca. 150 ng/ml 20 min after administration. These values are in agreement with a previous report [5].

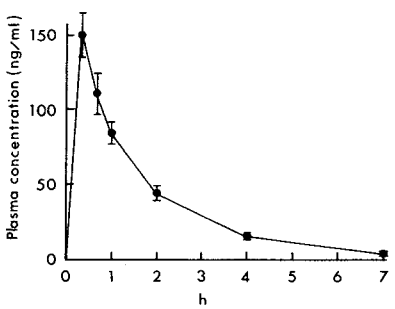


Fig. 5. Mean plasma concentrations of nifedipine in three healthy volunteers following a single oral administration of fine granules containing 10 mg of nifedipine (Sepamit). Each point represents the mean ± standard error for three subjects.

TABLE III

INDIVIDUAL PHARMACOKINETIC PARAMETERS AFTER ORAL ADMINISTRATION OF 10 mg NIFEDIPINE (SEPAMIT)

Subject	T_{max} (min)	C_{max} (ng/ml)	$AUC_{0-\infty}$* (ng · h/ml)	$t_{1/2}$ (h)
A	20	174.3	316.3	1.6
B	20	119.4	212.7	1.3
C	20	157.9	287.2	1.9
Mean	20	150.5	272.1	1.6

*AUC = Area under the curve.

CONCLUSION

The method described is sufficiently simple, sensitive, specific and rapid for the determination of nifedipine at therapeutic concentrations in human plasma. It can be used for routine clinical monitoring and in pharmacokinetic studies of small animals.

REFERENCES

1 P. Lynch, H. Dargie, S. Krikler and D. Krikler, Brit. Med. J., 281 (1980) 184.
2 M.T. Olivali, C. Bartorelli, A. Polese, C. Fiorentini, P. Moruzzi and M.D. Guazzi, Circulation, 59 (1979) 1056.
3 F.A. Horster, B. Duhm, W. Maul, H. Medenwald, K. Patzschke and L.A. Wegner, Arzneim.-Forsch., 22 (1972) 330.
4 H. Medenwald, K. Schlossman and C. Wunsche, Arzneim.-Forsch., 22 (1972) 53.
5 K.D. Raemsch and J. Sommer, Hypertension, 5 (1983) 18.
6 S. Kondo, A. Kuchiki, K. Yamamoto, K. Akimoto, K. Takahashi, N. Awata and I. Sugimoto, Chem. Pharm. Bull., 28 (1980) 1.
7 J. Dokladalova, J.A. Tykal, S.J. Coco, P.E. Durkee, G.T. Quercia and J.J. Korst, J. Chromatogr., 231 (1982) 451.
8 S.R. Hamann and R.G. McAllister, Clin. Chem., 29 (1983) 158.
9 P. Jakobsen, O.L. Pedersen and E. Mikkelsen, J. Chromatogr., 162 (1979) 81.
10 M.T. Rosseel and M.G. Bogaert, J. Chromatogr., 279 (1983) 675.
11 R. Testa, E. Dolfini, C. Reschiotto, C. Secchi and P.A. Biondi, Farmaco Ed. Prat., 34 (1979) 463.
12 S. Higuchi and Y. Shiobara, Biomed. Mass Spectrom., 5 (1978) 220.
13 L.J. Lesko, A.K. Miller, R.L. Yeager and D.C. Chatterji, J. Chromatogr. Sci., 21 (1983) 415.
14 N. Kurosawa, S. Morishima, E. Owada, K. Ito, K. Ueda, A. Takahashi and T. Kikuiri, Yakugaku Zasshi, 104 (1984) 775.
15 P.R. Bach, Clin. Chem., 29 (1983) 1344.
16 T. Sadanaga, K. Hikida, K. Tameto, Y. Matsushima and Y. Ohkura, Chem. Pharm. Bull., 30 (1982) 3807.
17 P. Pietta, A. Rava and P. Biondi, J. Chromatogr., 210 (1981) 516.
18 C.H. Kleinbloesem, J. van Harten, P. van Brummelen and D.D. Breimer, J. Chromatogr., 308 (1984) 209.
19 R.J. Gould, K.M.M. Murply and S.H. Snyder, Life Sci., 33 (1983) 2665.
20 D.A. Roston, R.E. Shoup and P.T. Kissinger, Anal. Chem., 54 (1982) 1417A.
21 J.L. Glajch and J.J. Kirkland, Anal. Chem., 55 (1983) 319A.

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ISOLATION AND ANALYSIS OF N-OXIDE METABOLITES OF TERTIARY AMINES: QUANTITATION OF NICOTINE-1′-N-OXIDE FORMATION IN MICE

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SUMMARY

To investigate the formation and elimination of nicotine-1′-N-oxide (NNO) in mice treated with a single injection of nicotine, sensitive and selective methods were developed to quantitate this polar and heat-labile metabolite. The compound was isolated from tissue homogenates as a dodecyl sulfate ion pair with C_{18} extraction cartridges and analyzed on an amino bonded-phase high-performance liquid chromatographic column with a mobile phase consisting of isopropanol—water. Overall recoveries of NNO were 64—76% from biological media. Several methods of detection were evaluated; radiolabeling was necessary to achieve the sensitivity required for pharmacokinetic studies in mice. The *cis* and *trans* isomers of NNO were separated on a Partisil PAC column and enzymatic selectivity was evaluated for the formation of these isomers in mice.

INTRODUCTION

Many compounds containing tertiary aliphatic or aromatic amino groups and those with heteroaromatic nitrogens can be metabolized to N-oxides in the liver [1]. Examples of substances that undergo N-oxidation by the microsomal flavin-containing monooxygenase system are trimethylamine, N,N-dimethylaniline and chlorpromazine [2, 3]. Several compounds containing a pyridyl group have been reported to undergo N-oxidation by the cytochrome P-450 monooxygenases [4]. Nicotine, with both pyridyl and tertiary amino groups, is oxidized preferentially at the latter position to produce nicotine-1′-N-oxide (NNO) (Fig. 1). This oxidation occurs in liver microsomes from several animal species [5, 6] and the metabolite has been found in urine from cats [7] and humans [8] treated with nicotine or tobacco smoke. Another important path-

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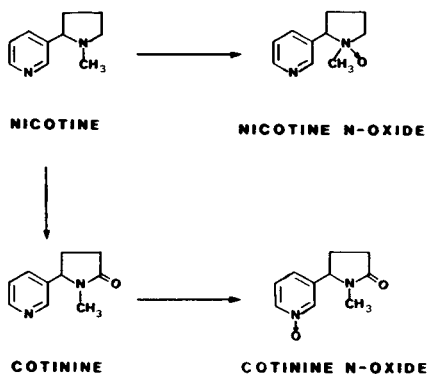


Fig. 1. Structures of nicotine and its metabolites discussed in the text.

way of nicotine metabolism is the conversion to cotinine (Fig. 1) [5, 6], and the subsequent N-oxidation of the pyridyl group to cotinine N-oxide has also been reported [9].

Our laboratory has been investigating the effects of genetic differences on the disposition of nicotine by determining the pharmacokinetic properties of this pharmacologically important constituent of tobacco and its primary metabolites in several inbred strains of mice [10, 11]. Because only small doses are tolerated, highly sensitive and selective analytical techniques were necessary to quantitate these compounds in blood and tissues of single animals. NNO and N-oxides in general are thermally labile, and the inherent polarity of the N-oxide group results in low extraction efficiencies by organic solvents. These characteristics, combined with low concentrations in complex biological matrices, complicate quantitative analyses. In studies not requiring such high sensitivity, analyses have been conducted by thin-layer chromatography (TLC) and paper chromatography of NNO produced from radiolabeled nicotine [7]. An alternative quantitative method has involved removal of unmetabolized nicotine from the sample followed by reduction of NNO with titanium trichloride; the nicotine produced is then analyzed by gas chromatography (GC) [8]. Polarographic methods have been developed for chloropromazine N-oxide [12] and indicine N-oxide [13], but these methods require extensive sample preparation and sensitivities extending to the low nanogram range have not been demonstrated. The N-oxides of nicotinamide and pyridine have been analyzed by high-performance liquid chromatography (HPLC) with a C_{18} column [14]; these polar compounds have low affinities for the C_{18} stationary phase, and elute relatively quickly along with other polar compounds in the samples.

The present paper summarizes the results of our efforts to develop procedures for the efficient and selective isolation of NNO from blood and tissue homogenates. Extracts were analyzed by HPLC with an amino bonded-phase column, which produced good retention, peak shape and selectivity, and several methods for detecting NNO were evaluated. The techniques were applied to a pharmacokinetic study of NNO formation in mice, including a determination of enzyme selectivity in the formation of the *cis* and *trans* isomers of NNO. With slight modifications, these methods should be useful to study a wide range of N-oxides in biological samples.

EXPERIMENTAL

Materials

Nicotine (Sigma, St. Louis, MO, U.S.A.) was purified by fractional distillation under reduced pressure and stored at −20°C. Cotinine was prepared from nicotine as described [15]. NNO was synthesized by oxidation of nicotine with 10% hydrogen peroxide in methanol at 25°C for 48 h. The product was purified by preparative TLC on silica gel G with ethyl acetate—methanol—ammonium hydroxide (5:4:1). Nicotine dihydrochloride (methyl-^{14}C), specific activity 10.1 mCi/mmol, was obtained from ICN (Irvine, CA, U.S.A.). Radiolabeled NNO was prepared by oxidizing [^{14}C] nicotine and purifying the product as described above. Sodium dodecyl sulfate and sodium octyl sulfate were obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and sodium heptane sulfonate was from Alltech (Deerfield, IL, U.S.A.). Solvents were HPLC grade from Fisher (St. Louis, MO, U.S.A.). Silica and C_{18} Sep Pak extraction cartridges (Waters Assoc., Milford, MA, U.S.A.) were attached to luer-tipped syringes and eluted by gravity flow. The C_{18} cartridges were prepared by washing successively with 2-ml volumes of methanol and water.

Instrumentation

Radioactivity was determined with a Beckman Model LS 8000 liquid scintillation counter and counting efficiency measured with standard [^{14}C] toluene (Amersham, Arlington Heights, IL, U.S.A.). HPLC was performed with a Beckman Model 110A pump and Hitachi Model 100-10 UV detector fitted with an 8-μl HPLC flow cell. Chromatograms were recorded on a Houston Instruments omniscribe strip chart recorder. The analytical columns, Alltech NH_2, Whatman (Clifton, NJ, U.S.A.) Partisil-10 PAC and Brownlee (Santa Clara, CA, U.S.A.) RP-2 were 250 × 4.6 mm with 10-μm packings. A 46 × 3.2 mm guard column packed with Whatman Co:Pell PAC was employed with the former two columns. Electrochemical detection was performed with a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model LC-9 detector employing a TL-6A gold—mercury thin-layer cell and an $Ag/AgCl_2$ reference electrode. Mass spectrometry (MS) was carried out with a Hewlett-Packard Model 5984A instrument operated in the chemical ionization (CI) mode with isobutane. Samples were introduced with a heated probe.

Animal experiments

Male DBA mice, 60—75 days of age, were injected intraperitoneally with a 1.0 mg/kg dose of [^{14}C] nicotine (specific activity 2.9 μCi/μmol) in 0.25 ml of normal saline. Animals were killed by cervical dislocation at specific times after injection, livers were removed immediately, weighed and homogenized with a Potter—Elvehiem apparatus in 4 ml of ice-cold distilled water containing 5.0 μg of unlabeled NNO as carrier. Liver weights ranged from 0.90—1.5 g. To develop the extraction techniques, livers, brains and blood were removed from untreated mice and spiked with various amounts of [^{14}C] NNO and 5.0 μg of unlabeled NNO. The N-oxide was then isolated and analyzed as described under Results and discussion.

RESULTS AND DISCUSSION

Isolation from biological samples

Solid phase methods were investigated for extracting NNO from aqueous media. Liver homogenates were spiked with [^{14}C] NNO and prepared for extraction by first preparing a tissue-free supernatant. This procedure was facilitated by the addition of acids or organic solvents; the latter produced higher and more consistent recoveries of NNO in the supernatants (Table I). Solvent was then evaporated under a stream of nitrogen at low temperatures (35—40°C) to avoid decomposition of this thermally labile compound. Lipophilic substances were removed by extraction with ethyl acetate under basic (pH 11—12) and acidic (pH 1—2) conditions, and the aqueous samples were applied to Sep-Pak extraction cartridges containing an octadecyl-bonded stationary phase. The polar N-oxide was not retained on this hydrophobic phase but was efficiently adsorbed on cartridges packed with underivatized silica. Removal of NNO from silica could not be accomplished with methanol, acetonitrile or isopropanol, but a mixture of methanol—isopropyl amine (50:50) was effective. Other polar compounds found to be present in the fraction of NNO isolated by this method interfered with HPLC analysis. In order to prepare purer extracts of the metabolite, C_{18} Sep-Pak cartridges were reinvestigated in conjuction with ion-pairing reagents so that polar compounds not forming extractable ion pairs could be separated from the metabolite.

The retention of NNO on C_{18} cartridges in the presence of sodium octyl sulfate was dependent on the pH and the concentration of ion-pairing reagent (Table II). The optimal pH was 2.0—2.1, which can be rationalized based on the existence of two basic sites within NNO. At a sufficiently low pH, both the 1′-oxygen and the pyridyl nitrogen would be protonated and the resulting dication would associate with two octylsulfate anions. If the medium is less acidic only a single ion pair would form, and if the medium is too acidic the reagent would be protonated. The effects of alkyl chain length of the reagent on retention of NNO by C_{18} cartridges are shown in Fig. 2. Increasing the number of carbon atoms from 7 to 12 resulted in ion pairs with increasing affinity for the resin. Dodecylsulfate—NNO ion pairs were employed in subsequent extractions because this lipophilic species permitted washing the

TABLE I

RECOVERIES OF NICOTINE N-OXIDE IN THE SUPERNATANT AFTER REMOVING TISSUE FROM LIVER HOMOGENATES ($n = 4$)

Each sample contained 1 g of liver homogenate in 4 ml of water with 0.50 μg of [^{14}C]NNO. Radioactivity was measured in the supernatant after adding the agent and removing the tissue by centrifugation.

Addition	Percentage recovery (mean ± S.D.)
Perchloric acid	57 ± 11
Hydrochloric acid	71 ± 10
Methanol	82 ± 4
Acetonitrile	81 ± 5

TABLE II

RETENTION CHARACTERISTICS OF NICOTINE N-OXIDE ON C_{18} EXTRACTION CARTRIDGES WITH SODIUM OCTYLSULFATE

Samples (4 ml) containing [^{14}C]NNO and the ion-pairing reagent were applied to the cartridges and radioactivity measured in the initial effluent and after eluting with 2 ml of each solvent. The pH experiment contained 8 mg of reagent and the concentration experiment was conducted at pH 2.0.

Cartridge eluent	Radioactivity (dpm) measured in cartridge effluents				
	Effect of pH		Effect of octylsulfate concentration		
	pH 2.5	pH 2.0	20 μg	1.0 mg	8.0 mg
Aqueous solution of NNO	1984	74	—	—	—
Water	196	80	3289	640	79
Methanol—water (20:80)	100	55	5276	100	63
Methanol	2150	5750	812	8915	9565

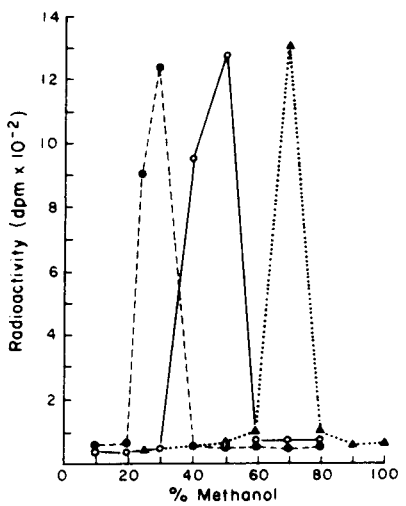


Fig. 2. The effects of alkyl chain length of ion-pairing reagents on the retention of NNO by C_{18} Sep-Pak cartridges. Aqueous solutions at pH 2.0 containing [^{14}C]NNO and an ion-pairing reagent were applied to the cartridges, eluted with 2-ml volumes of aqueous solvents. containing increasing percentages of methanol, and radioactivity measured in the effluents. The ion-pairing reagents were heptanesulfonate (— — —), octylsulfate (——) and dodecylsulfate (• • • •).

cartridges with methanol—water mixtures containing up to 50% methanol to remove polar substances before NNO was finally eluted with 100% methanol.

The complete procedure for isolating NNO from tissue homogenates is summarized in Fig. 3. Blood samples (1 ml) were first diluted with 2 ml of water and the protein was removed by addition of acetonitrile (2 ml), cooling to 5 °C and centrifugation. Biological samples containing known quantities of NNO were extracted and overall recoveries determined after chromatographic analysis as discussed below.

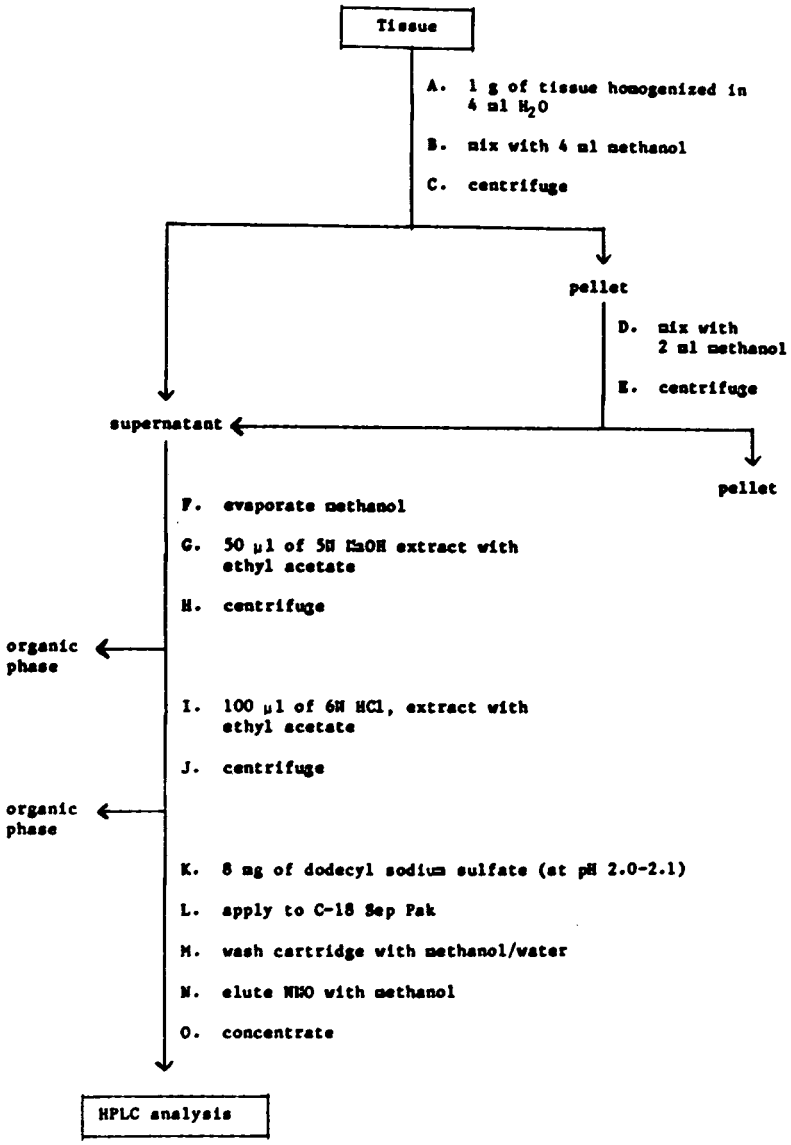
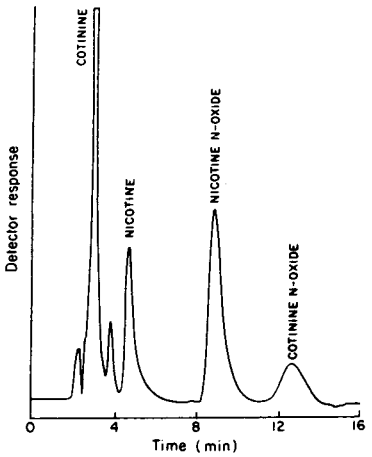


Fig. 3. Complete extraction scheme for isolating NNO from mouse tissues.

HPLC analysis

NNO was incompatible with columns containing hydrophobic stationary phases (C_8 and C_{18}) because the compound was not adequately retained to permit separation from other polar substances in the extracts. Moderately polar amino (NH_2) and aminocyano (PAC) bonded phases, however, exhibited good retention and selectivity characteristics for NNO. The former was employed for routine analyses with isopropanol—water mobile phases. A chromatogram illustrating the separation of NNO from nicotine, cotinine and cotinine N-oxide is shown in Fig. 4. The elution order is consistent with a normal-phase separation



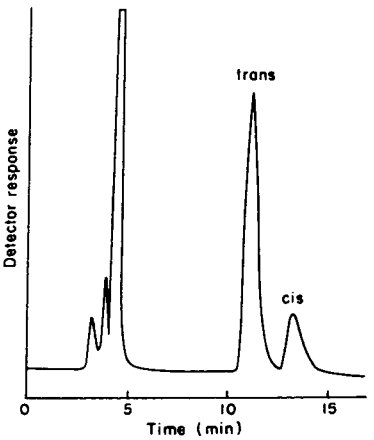


Fig. 4. Chromatogram from the HPLC analysis of standard compounds on an amino bonded phase column with isopropanol—water (75:25) as the mobile phase at 1.0 ml/min and detection at 254 nm.

Fig. 5. HPLC separation of *cis*- and *trans*-NNO on a partisil PAC column with methanol—water (95:5) as the mobile phase.

mechanism except for the short retention time of cotinine, which is more polar than nicotine on a reversed-phase column [16]. It was advantageous to eliminate the residual ion-pairing reagent from NNO extracts to avoid rapid degradation of the HPLC columns. This removal was accomplished by passing the extract [in 2 ml of isopropanol—water (12:88)] through another C_{18} Sep-Pak cartridge. The reagent was retained and the NNO analyzed in the effluent after concentration.

The oxygen atom of NNO can be introduced so that the N-methyl group is in a *cis* or *trans* relationship to the pyridyl group about the pyrrolidine ring. These isomers could be separated readily on a PAC column with methanol—water (Fig. 5). The peak assignments were based on data showing that the hydrogen peroxide oxidation of nicotine produces a larger quantity of *trans*- compared to *cis*-NNO [17]. It has been reported that the *trans* form also predominates when other oxidizing agents are employed, and that the isomers of NNO can be separated on a silica HPLC column, although no chromatograms were presented to demonstrate the separation and peak shape obtained [18].

The limit of detection for NNO with a UV monitor at 260 nm was in the range 20—25 ng, which was not sufficiently low for our pharmacokinetic experiments. Because N-oxides can be reduced electrochemically [12, 13], an amperometric HPLC detector with a gold—mercury thin-layer cell was investigated. Several chromatographic conditions were employed in searching for the optimal detector response. The most successful procedure involved a C_2 column with a thoroughly deoxygenated mobile phase containing a high percentage of water [isopropanol—0.1 *M* chloroacetic acid (10:90)] and 1 m*M* EDTA. The signal obtained for NNO increased with increasingly negative electrode potentials to a practical limit (because of high background signals) of —0.90 V. The sensitivity limit of approximately

25—30 ng did not represent an improvement over UV detection due to the relatively high potentials necessary to reduce NNO under HPLC conditions.

MS detection was also investigated. The column effluent corresponding to the elution time of NNO was collected, the solvent evaporated and the sample analyzed by direct probe CI-MS. Due to the thermal instability of NNO, the most successful method involved loading a few microliters of a methanol solution of NNO into a shallow (4 mm) quartz capillary tube, inserting the sample with the ion source at a relatively cool temperature (100°C) and then heating the probe tip rapidly with isobutane as the reagent gas. Under these conditions, the most prominent ions were *m/z* 179 (MH^+, 30%), 163 ($MH^+ - O$, 100%) and 161 ($MH^+ - H_2O$, 82%). Analysis of methanol solutions containing various amounts of NNO indicated that 1.0 ng could be detected by selected-ion monitoring, but the response was non-linear over the range 1—100 ng. When tissues extracts were analyzed by this technique, there were signals which interfered with the low-level detection of NNO.

To circumvent the problems discussed above for detection of low nanogram amounts of NNO in biological media, radiolabeling was necessary. Water samples and several biological samples were mixed with various amounts of [^{14}C]NNO together with 5 μg of unlabeled NNO as carrier. The samples were processed as described (Fig. 3) and the column effluent corresponding to NNO was collected from the HPLC columns and radioactivity measured. The results are summarized in Fig. 6. Overall recoveries were not dependent on the concentration of [^{14}C]NNO and the mean values were 64—76% for the biological

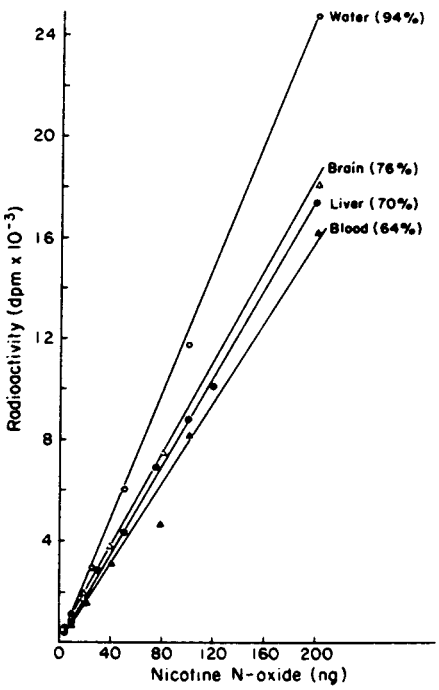


Fig. 6. Calibration curves and recoveries of [^{14}C]NNO obtained from water and biological media by application of the extraction and chromatographic methods. The mean recovery for NNO from each type of sample is shown in parentheses.

samples and 94% for the water samples. These plots were used subsequently to quantitate metabolically generated NNO in mouse tissues.

Applications of the method

Mice were injected with [^{14}C]nicotine at a dose of 1.0 mg/kg, killed at various times, and livers removed, homogenized and spiked with unlabeled NNO. These samples were extracted and analyzed for [^{14}C]NNO. The reconstructed chromatogram from a representative liver sample is compared to the chromatogram obtained with a UV detector in Fig. 7. The radioactive peak corresponds to the elution of NNO. No significant amounts of other metabolites were present in the extract. The concentrations of NNO in liver were plotted against time after injection with nicotine (Fig. 8). The maximum concentration, 80 mg/g of liver, was attained in 15 min and an elimination half-life of 15.9 min was calculated from the data. Several tissue concentrations on the elimination phase of the pharmacokinetic profile were below 20 ng/g, which emphasizes the need for a highly sensitive method of quantitation. As determined in our previous work, levels in blood and brain tissue of mice never exceeded 20 ng/g during the entire time course of the study [11]. Urine and feces were collected from six mice during a period of 20 h after nicotine administration; approximately 2.0—2.5% of the dose was excreted as NNO in urine and 0.03% in feces.

The relative amounts of *cis*- and *trans*-NNO formed from [^{14}C]nicotine in mice were determined by separation of the isomers on a PAC column. The results presented in Table III demonstrate that the amount of the *trans* isomer formed at three time points after nicotine administration was approximately two-fold greated than the *cis* isomer. The selectivity for

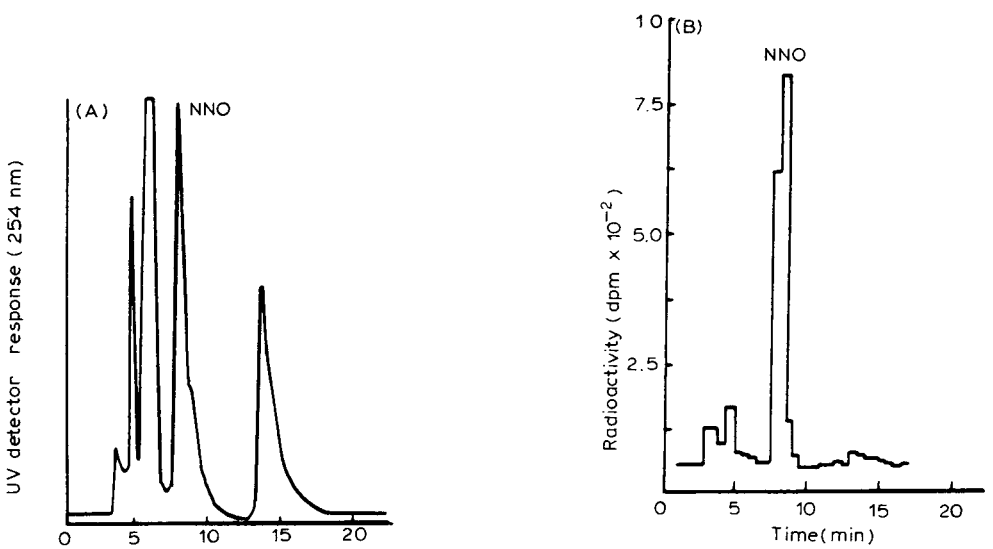


Fig. 7. (A) Chromatogram obtained by HPLC analysis with UV detection of the liver extract from a mouse injected with [^{14}C]nicotine. (B) Reconstructed chromatogram obtained by HPLC separation of the same sample with radioactivity measured in samples collected every 30 sec.

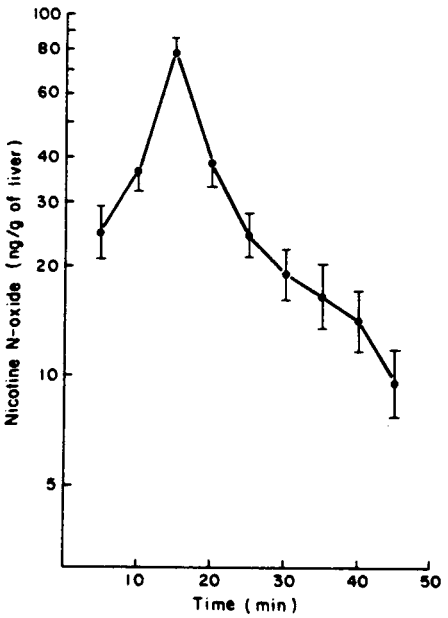


Fig. 8. Concentration of NNO in livers of DBA mice injected with nicotine versus time. Each point is the mean ± S.E. of determinations of three animals.

TABLE III

ISOMERS OF NICOTINE N-OXIDE FORMED IN MICE ($n = 3$)

Mice were injected intraperitoneally with [^{14}C] nicotine, livers removed at the times shown, and the isomers of NNO quantitated.

Time after injection (min)	Ratio of *trans/cis*-N-oxides (mean ± S.D.)
15	2.01 ± 0.08
25	1.94 ± 0.10
35	1.87 ± 0.07

formation of these products has been determined in vitro with 10 000*g* supernatant fractions of liver from several species by paper chromatography [6]. The results show substantial species variation in the *cis/trans* ratio, but there are several factors that complicate the comparison of in vivo and in vitro data. These include the finding that the isomers of NNO are partially reduced back to nicotine at different rates by a liver supernatant fraction in the absence of oxygen [19]. The ratio determined in vivo, therefore, probably represents the net result of both the stereoselective oxidation of nicotine and the selective reduction of *cis*- and *trans*-NNO.

CONCLUSION

A method has been developed for the efficient and selective isolation of NNO from biological media. Recoveries averaged about 70% and the extracts were sufficiently pure to be analyzed by HPLC with minimal interference from

contaminants. Columns containing amino- or aminocyano-bonded stationary phases produced good chromatographic results with NNO. The method is an alternative to those used previously, which include paper chromatography [7] and chemical reduction to nicotine followed by GC [8]. With radiolabeled nicotine, the NNO produced could be quantitated at very low levels, which were limited only by the specific activity of nicotine. NNO was quantitated in mouse tissues at concentrations ranging from 5.0 to 80 ng/g of tissue [11]. For levels above approximately 30 ng/g, it should be possible to obtain accurate data by UV or electrochemical detection due to the high purity of the extracts. The data reported here and in the previous paper [11] represent the only comprehensive description of NNO pharmacokinetics in any mammalian species. Urinary excretion following nicotine administration to human subjects has been reported, but plasma levels of NNO were too low to be measured accurately [16]. The method we have developed, with minor modifications, should be readily adaptable to the analyses of other hydrophilic amine N-oxides in biological media.

ACKNOWLEDGEMENTS

The authors thank Dr. Karl Bratin of Bioanalytical Systems for assistance in the design of the electrochemical experiments. This work was supported by Grant No. 1243 from the Council for Tobacco Research, U.S.A.

REFERENCES

1 L.A. Damani, in W.B. Jakoby, J.R. Bend and J. Caldwell (Editors), Metabolic Basis of Detoxication, Academic Press, New York, 1982, pp. 135—143.
2 L.L. Poulsen and D.M. Ziegler, J. Biol. Chem., 254 (1979) 6449.
3 S.S. Sofer and D.M. Ziegler, Drug Metab. Dispos., 6 (1978) 232.
4 J.W. Gorrod and L.A. Damani, Xenobiotica, 9 (1979) 219.
5 P. Jenner, J.W. Gorrod and A.H. Beckett, Xenobiotica, 3 (1973) 563.
6 P. Jenner, J.W. Gorrod and A.H. Beckett, Xenobiotica, 3 (1973) 573.
7 D.M. Turner, Biochem. J., 115 (1969) 889.
8 A.H. Beckett, J.W. Gorrod and P. Jenner, J. Pharm. Pharmacol., 23 Suppl. (1971) 55S—61S.
9 E. Dagne and N. Castagnoli, J. Med. Chem., 15 (1972) 840.
10 J.A. Thompson, M.-S. Ho and D.R. Petersen, J. Chromatogr., 231 (1982) 53.
11 D.R. Petersen, K.J. Norris and J.A. Thompson, Drug Metab. Dispos., 12 (1984).
12 D.A. Cowan, in E. Reid (Editor), Methodological Developments in Biochemistry, Vol. 5, North-Holland, New York, 1976, pp. 193—201.
13 M. McCornish, I. Bodek and A.R. Branfman, J. Pharm. Sci., 69 (1980) 727.
14 B.J. Blaauboer and A.J. Paine, Xenobiotica, 10 (1980) 655.
15 E.R. Bowman and H. McKennis, Biochem. Prep., 10 (1963) 36.
16 G.A. Kyerematen, M.D. Damiano, B.H. Dvorchik and E.S. Vesell, Clin. Pharmacol. Ther., 32 (1982) 769.
17 A.H. Beckett, P. Jenner and J.W. Gorrod, Xenobiotica, 3 (1973) 557.
18 S. Brandange, L. Lindblom and D. Samuelsson, Acta Chem. Scand. B, 31 (1977) 907.
19 J. Booth and E. Boyland, Biochem. Pharmacol., 20 (1971) 407.

Journal of Chromatography, 341 (1985) 361—371
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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SENSITIVE LIQUID CHROMATOGRAPHIC METHOD FOR PHYSOSTIGMINE IN BIOLOGICAL FLUIDS USING DUAL-ELECTRODE ELECTROCHEMICAL DETECTION

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SUMMARY

A liquid chromatographic method using dual-electrode detection has been developed for determination of physostigmine in biological fluids. The limit of detection is in the order of 25—50 pg mol^{-1} of plasma. A high sample throughput is obtained by a single solvent extraction step and autoinjection into the chromatograph. Data following oral doses of physostigmine are presented.

INTRODUCTION

Physostigmine, an alkaloid of the Calabar bean, is a potent inhibitor of cholinesterase. It is a lipophilic tertiary amine which is absorbed from the gastrointestinal tract and enters the central nervous system. Its short duration of action suggests that it has a short elimination half-time but pharmacokinetic analysis has not been possible due to the lack of a suitable plasma assay. Liquid chromatography (LC) with ultraviolet (UV) detection [1, 2] is too insensitive for determining plasma concentrations in man. Using electrochemical detection an LC method sensitive to approximately 0.5 ng ml^{-1} has been reported [3] and, although plasma concentrations following a single subcutaneous dose of 1 mg physostigmine salicylate were determined, it was obvious that greater sensitivity was required. The electrochemical method has been modified to give approximately a ten-fold improvement in sensitivity.

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EXPERIMENTAL

Materials and apparatus

HPLC-grade methanol and acetonitrile were purchased from Fisons Scientific (Loughborough, U.K.). Spherisorb 3-μm columns, 150 × 4.6 mm I.D., were from Phase Separations (Queensferry, U.K.). Physostigmine, physostigmine sulphate and neostigmine bromide were from Sigma (Poole, U.K.). Other reagents were analytical-reagent grade.

Chromatographic eluents were prepared by mixing 1 vol. of 0.1 *M* ammonium nitrate buffer with either 9 vols. of methanol or 9 vols. of methanol—acetonitrile (1:1). The eluents were pumped with an Applied Chromatography Systems Series 300 pump and samples introduced manually via a Rheodyne valve fitted with a 50-μl sample loop. Alternatively, a Kontron MSI 660 autosampler was used. Detection was either by a Laboratory Data Control variable-wavelength detector Model 1204A, or an Environmental Science Association (ESA) Model 5100A Coulochem detector.

Effect of pH on electrochemistry of physostigmine

Solutions of physostigmine (1 μg ml^{-1}) in 50% methanol: Britton—Robinson buffers (0.1 *M*) were pumped through the electrochemical detector cell at a flow-rate of 1 ml min^{-1}. Current—voltage curves were obtained using the scanning facility on the detector. Subtraction of the signals obtained using physostigmine-free solvents gave typical sigmoidal polarographic waves. The experiment was performed using buffers at pH 3, 5, 7 and 9.

Preparation and evaluation of compounds as internal standards

Eseroline was prepared by hydrolysing physostigmine sulphate as described by Ellis [4]. Crystallization from benzene—light petroleum gave pale buff needles, m.p. 128—129°C.

Ethyl and propyl carbamate homologues of physostigmine were prepared by treating eseroline with the appropriate alkyl isocyanate. Eseroline was dissolved in diethyl ether in the presence of a speck of sodium and the alkyl isocyanate added [5]. The N,N-dimethylcarbamate analogue was prepared by dissolving eseroline in ethyl acetate to which a few drops of pyridine had been added and treating with N,N-dimethylcarbamyl chloride. The required products were separated by thin-layer chromatography (SiO_2; chloroform—ethanol—0.88 SG ammonium hydroxide, 80:10:1). The spots were located as shadows under UV light, removed from the plates and the compounds eluted with methanol. The solutions were examined by UV spectroscopy, LC and mass spectroscopy.

pH-Controlled solvent extraction

Britton—Robinson buffers (0.1 *M*) were prepared to cover the range pH 4 to pH 12. Solutions of physostigmine and its analogues were prepared in diethyl ether at approximately 2 μg ml^{-1}. Aliquots of the ether solutions (2 ml) were shaken with buffer solutions (1 ml) for 15 min. After centrifugation, 1 ml of the ether layer was transferred to a clean tube, evaporated under nitrogen and the residue dissolved in methanol (1 ml). Samples were assayed by LC at 254 nm. The results were plotted as percentage present in ether layer versus pH.

Loss of physostigmine in vitro

Samples of blood bank plasma (19.9 ml) either with or without neostigmine bromide (50 μg ml^{-1}) were brought to temperature (4 °C, 22 °C or 37 °C) and physostigmine solution (0.1 ml) was added to give an initial concentration of 5 ng ml^{-1}. Aliquots (2 ml) were withdrawn at intervals over 4 h for physostigmine assay as described below. The percentage remaining at each time was calculated.

Extraction from plasma, blood and urine

Calibration curves for plasma or blood assays were prepared using blood bank plasma containing neostigmine bromide (50 μg ml^{-1}). Samples (2 ml) were pipetted into screw-cap extraction tubes and 0.1 ml internal standard solution, the N,N-dimethylcarbamyl ester of eseroline (approximately 40 ng ml^{-1} in methanol) was added. Ammonium hydroxide solution (1 ml, 0.1 *M*) and freshly distilled diethyl ether (5 ml) were added. The tubes were shaken mechanically for 15 min, centrifuged to separate the layers and the organic layers (4 ml) transferred to clean pointed tubes. The diethyl ether was evaporated under a gentle stream of nitrogen and the residue dissolved in methanol (0.1 ml). Aliquots of methanol were either injected into the liquid chromatograph or transferred to glass autosampler vials. Routinely, calibration curves were prepared between 10 and 0.1 ng ml^{-1}. For low concentrations, e.g. those expected after oral dosing, larger samples (up to 4 ml) were taken and the final volume of methanol was 0.06 ml. Calibration standards were prepared between 2 and 0.025 ng ml^{-1}.

Urine samples (2 ml) were extracted as for blood or plasma using benzene in place of diethyl ether. The calibration range and amount of internal standard were adjusted to suit the samples being analyzed.

An estimate of precision was obtained by assaying replicate samples. Inter-assay precision was estimated by assaying six blood and plasma samples containing 3 ng ml^{-1} physostigmine. Samples were stored at −20 °C and assayed on separate days over a three-week period.

Chromatographic conditions

For the pH partition studies methanol—pH 8.6 buffer was used as eluent which was pumped at a flow-rate of 0.5 ml min^{-1}. For biological extracts the pH of the ammonium nitrate buffer was increased to 8.9 and a mixture of methanol—acetonitrile used rather than methanol. Two columns were connected in series. The flow-rate was 1 ml min^{-1}.

The guard cell of the Coulochem detector was placed between the column exit and the analytical cell. The guard cell voltage was + 0.4 V. The analytical cell electrode potentials were + 0.7 V and − 0.2 V.

Biological samples

The data presented are from a healthy female volunteer, age 38 years, weight 59 kg, who received oral doses of physostigmine on three separate occasions. Physostigmine salicylate (1, 2 or 4 mg) was given with 100 ml of water to drink. Blood samples (10 ml) were drawn into heparinized tubes and neostigmine bromide solution (500 μg in 0.05 ml water) was added and mixed

immediately. Samples were taken 15, 30, 45, 60, 75, 90, 120, 150 and 180 min after the dose. Urine was collected at approximately 30-min intervals. The protocol was approved by the Tower Hamlets District Ethics Committee.

Red cell partitioning

Physostigmine was added to heparinized blood samples collected from four male volunteers (22—36 years) to give a final concentration of 1 ng ml^{-1}. Blood samples were divided and centrifuged to separate the plasma. Plasma (2 ml) was transferred to an extraction tube containing 100 μg neostigmine bromide. The contents of the other centrifuge tube were shaken to reconstitute blood and a sample (2 ml) was transferred as described for plasma. The blood and plasma samples were extracted and assayed along with calibration standards as described above.

RESULTS AND DISCUSSION

The influence of pH on the polarographic properties of physostigmine is shown in Fig. 1. The curves at pH 9 and 7 were almost superimposed but at lower pH values the responses were lower. The use of alkaline eluent was compatible with this finding. Using acidic eluents with modified silicas [1, 2, 6] was considered inappropriate and there appeared to be no advantage in using acid eluents and changing the pH value post-column before electrochemical detection. The areas of chromatographic peaks obtained from repeat injections of physostigmine (10 ng) increased as the flow-rate was reduced and the detector temperature was raised. The maximum area obtained suggested that the oxidation processes involved a three-electron transfer. The chromatographic conditions were optimised to give maximum sensitivity in terms of peak height.

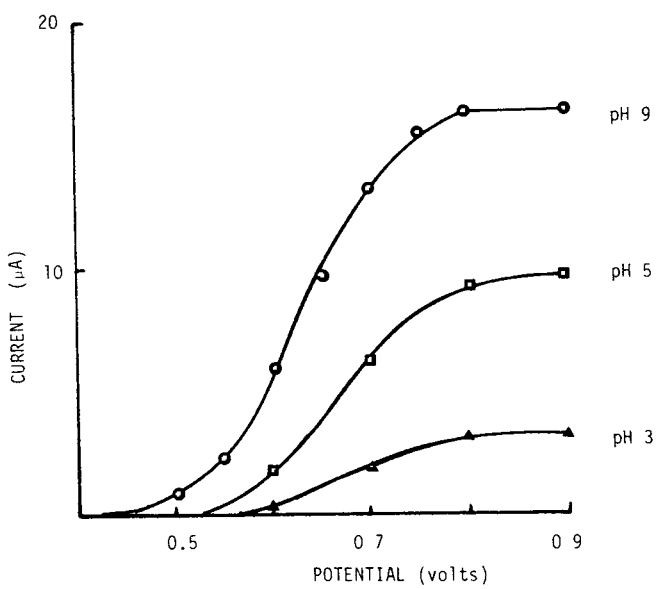

Fig. 1. Effect of buffer pH on current—voltage of physostigmine.

Under these conditions the efficiency of the oxidation process was approximately 66%.

The N,N-dimethylcarbamate analogue of physostigmine was chosen as the internal standard. This compound was suitable because of its extraction characteristics and its chromatographic properties, Figs. 2 and 3.

The N-ethyl homologue, eluting between physostigmine (N-methyl) and the propyl homologue, was insufficiently resolved from physostigmine to be used as the internal standard (Fig. 2). The extraction characteristics of the N-propyl carbamate were the least like those of physostigmine whereas the extraction of the dimethyl compound was very similar (Fig. 3). All the compounds gave the expected sigmoidal pH—extraction curves up to about pH 10 beyond which the concentrations in the ether layers declined. This was assumed to be due to decomposition by alkaline hydrolysis [7]. Between pH 8.5 and 11 the ratio of the amount of physostigmine to the amount of internal standard extracted varied by less than 2%. The ratio was unaffected by the nature of the sample — plasma, blood or urine — being extracted.

A potential problem when analysing esters in blood or plasma is hydrolysis

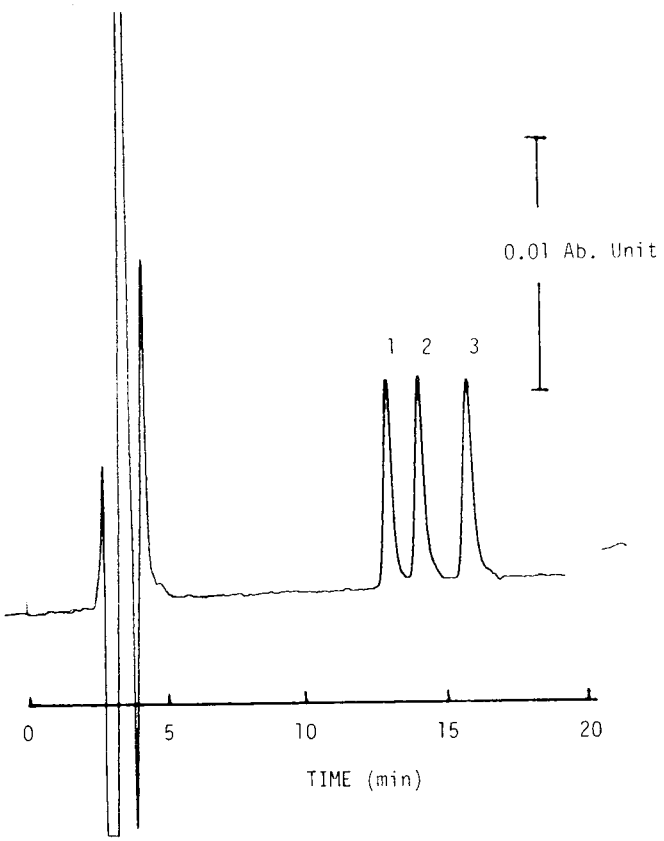

Fig. 2. Chromatogram of physostigmine (2) and its N-propyl (1) and N,N-dimethyl (3) analogues. The N-ethyl homologue, chromatographing between peaks 1 and 2, has been omitted for clarity.

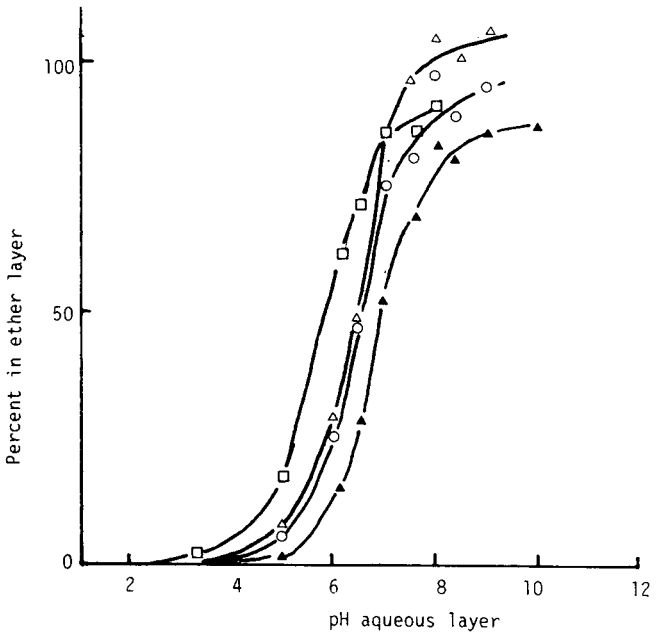

Fig. 3. Extractions of physostigmine and its analogues from various pH buffers into diethyl ether. (▲) N-Methyl carbamate (physostigmine); (○) N,N-dimethyl carbamate; (△) N-ethyl carbamate; (□) N-propyl carbamate.

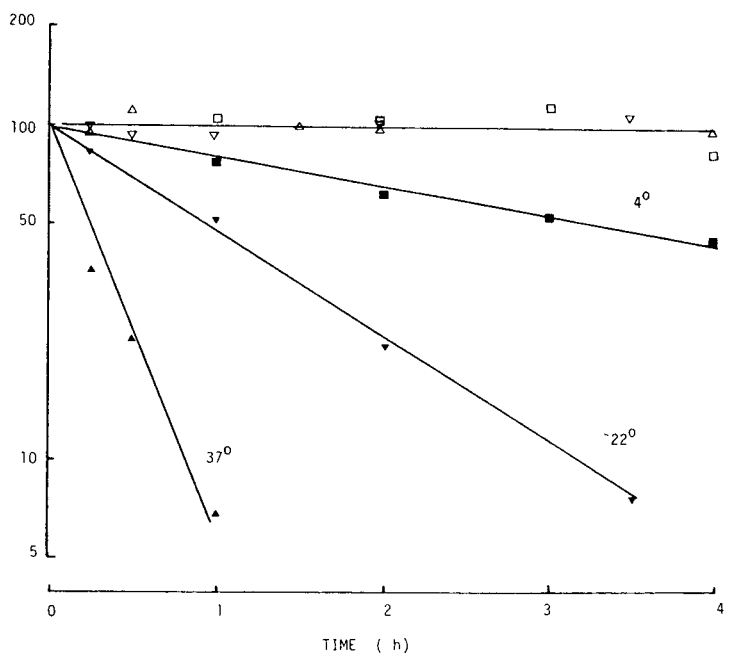

Fig. 4. Percentage physostigmine remaining in plasma as a function of time and temperature. Closed symbols: without added neostigmine; open symbols: with neostigmine (50 μg ml^{-1}).

of the compound by plasma esterases after blood samples have been taken. An indication of the magnitude of the problem with physostigmine is shown by Fig. 4. Physostigmine concentrations declined from an initial value of 5 ng ml^{-1}, with apparent first-order kinetics. At 37°C the apparent first-order half-time was approximately 15 min. At room temperature (22°C) 52% physostigmine was lost in 1 h. Even at 4°C the loss was not negligible. Physostigmine hydrolysis was enzymic. No loss occurred in the presence of excess neostigmine (Fig. 4, open symbols). Loss due to adsorption was discounted as adding neostigmine to tubes at the end of the incubation period failed to restore the physostigmine concentrations. When physostigmine was incubated in urine at 37°C for up to 4 h there was no measurable decrease in concentration. Neostigmine was chosen for its similarity to physostigmine, but being a quaternary ammonium compound its extraction into diethyl ether or benzene is negligible. Calibration standards were prepared containing neostigmine bromide (50 μg ml^{-1}) or venous blood was drawn into tubes containing sufficient neostigmine to give a final concentration of 50 μg ml^{-1}. To date, there has been no indication that neostigmine interferes with the assay of physostigmine.

Precision and sensitivity

The intra-assay precision was tested by replicate analyses of spiked samples. The intra-assay coefficients of variation (C.V.) obtained for assaying replicate 2-ml samples of plasma containing 10, 1 or 0.1 mg ml^{-1} physostigmine are shown in Table I. The results were calculated using data from the first electrode (oxidation, +0.7 V) and the second electrode (reduction, −0.2 V). The linearity of the method can be seen from the mean ratio (physostigmine response/internal standard response) as a function of concentration over two orders of magnitude (Table I). Intra-assay C.V. values (n = 6) for urine at 10 and 1 ng ml^{-1} were 3.8% and 3.9%, respectively. The mean concentrations and inter-assay C.V. values for the six stored plasma and blood samples were 2.86 ng ml^{-1} and 13.6%, and 2.96 ng ml^{-1} and 16.6%, respectively.

Sensitivity is not a fixed quantity but varies from sample to sample, run, laboratory, etc. It is also a function of the selectivity. Using 2 ml of plasma or blood, the limit of detection was in the order of 100 pg ml^{-1}. The sensitivity could be increased by taking a 4-ml sample for assay and using the sum of the oxidation and reduction signals. (The detector has a third output for this purpose, Fig. 5.) In this way, 50 pg ml^{-1} could be quantified with a C.V. of 14.5% (n = 5) and 25 pg ml^{-1} with a C.V. of 19.6% (n = 5).

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION USING OXIDATION OR REDUCTION SIGNALS (n = 6)

Concentration (ng ml^{-1})	Oxidation		Reduction	
	Mean ratio	C.V. (%)	Mean ratio	C.V. (%)
10	2.19	1.55	4.48	3.29
1.0	0.22	3.77	0.46	3.80
0.1	0.022	10.22	0.048	14.30

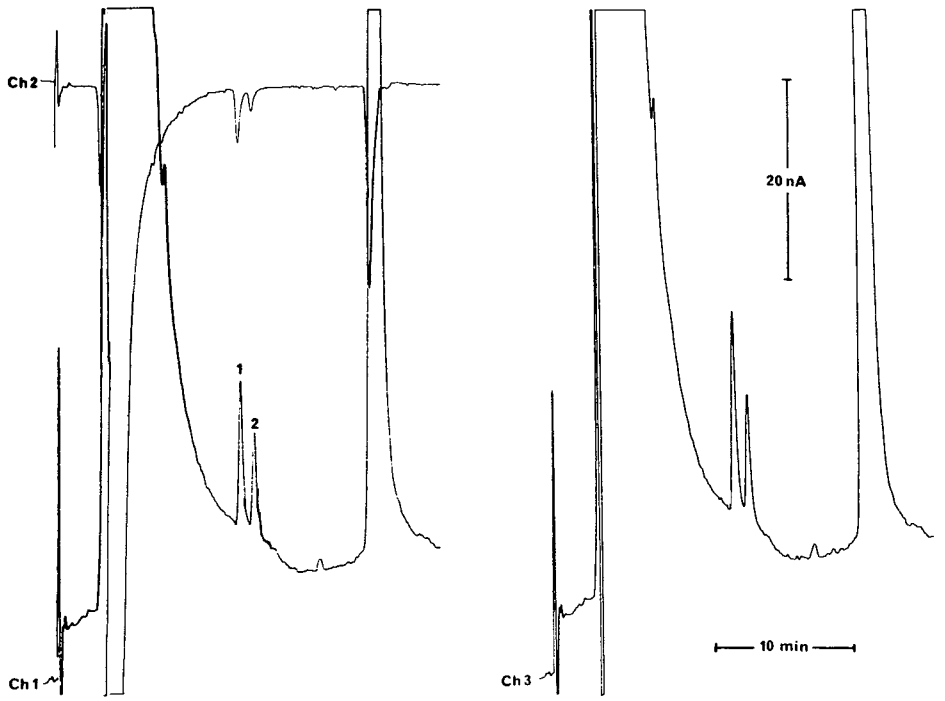

Fig. 5. Chromatogram of a plasma extract containing physostigmine (1) and internal standard (2) showing the three outputs of the ESA detector. Channel (Ch) 1, oxidation at + 0.7 V; channel 2, reduction at − 0.2 V and channel 3, the sum of the signals from channels 1 and 2. The plasma contained 0.5 ng ml^{-1} physostigmine and a 4-ml sample was extracted.

The eluent buffer pH was important in achieving the required selectivity and sensitivity. Although pH 8.6 buffer gave better resolution of physostigmine and internal standard, biological samples were assayed using buffer at pH 8.9. At the lower pH, a compound found in some biological extracts co-eluted with physostigmine. At pH 8.9 the chromatogram was free of interfering peaks in the region of physostigmine and internal standard.

Diethyl ether extraction of urine was unsuitable because it resulted in too many interfering peaks. When benzene was used the number and size of interfering peaks were considerably reduced, but the recovery of physostigmine was not affected. Benzene can be substituted for diethyl ether for extraction of blood and plasma. Benzene is more convenient as analytical grade reagent can be used without prior pretreatment. Diethyl ether has to be freshly distilled to remove electroactive anti-oxidants.

Red cell partitioning

The results of the erythrocyte-binding experiments illustrate the problem of determining plasma concentrations. When the partitioning was carried out in the presence of neostigmine bromide (50 $\mu g\ ml^{-1}$) the mean (n = 4) blood physostigmine concentration was 1.04 ng ml^{-1} and the range of red cell/plasma ratios 0.95—1.30. When plasma was separated before neostigmine was added, the mean blood concentration was 0.28 ng ml^{-1} and the red cell/plasma ratios

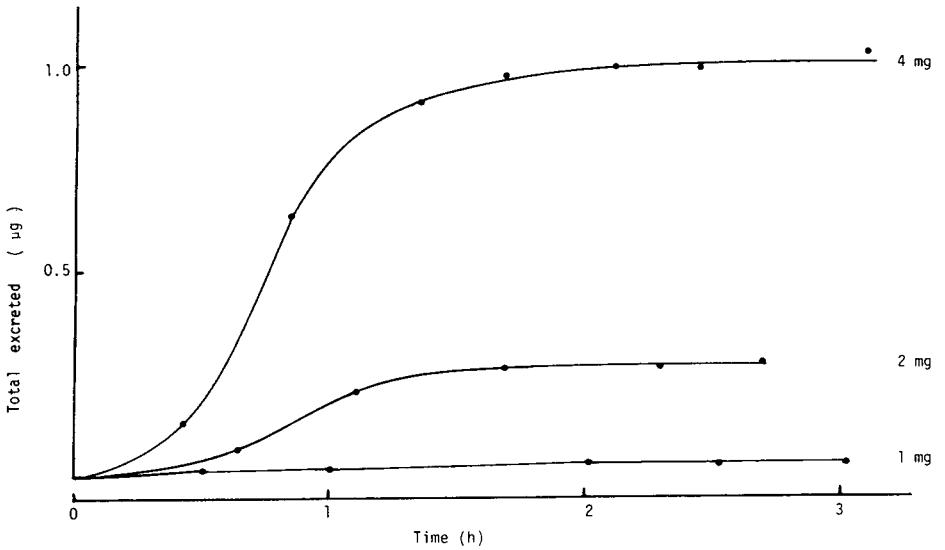

Fig. 6. Cumulative physostigmine urinary excretion curves in a volunteer who received 1, 2 and 4 mg physostigmine salicylate on separate occasions.

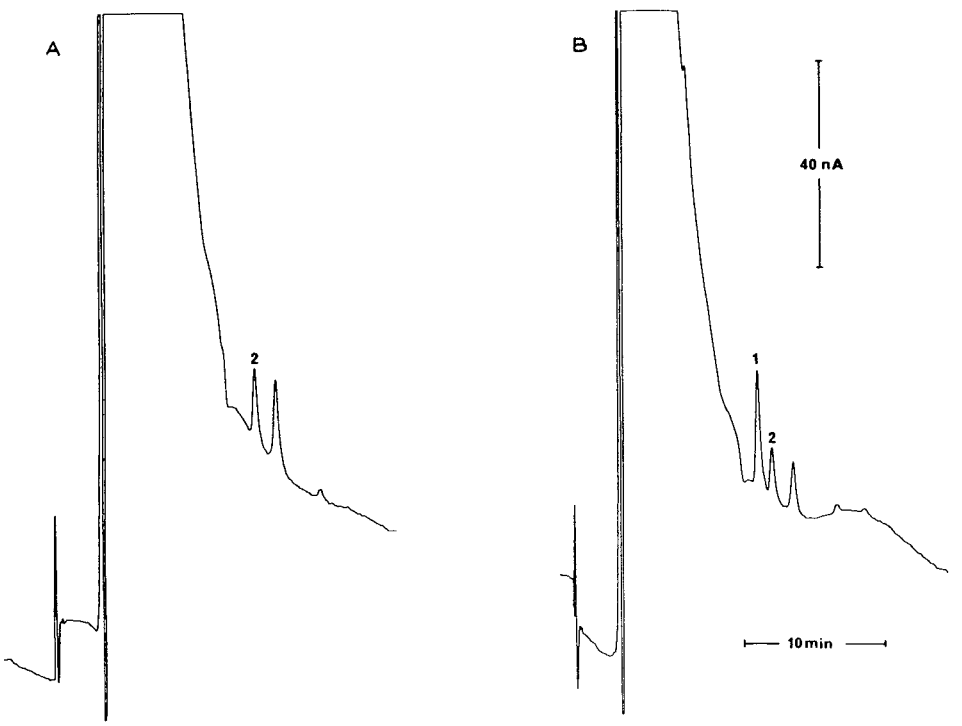

Fig. 7. Chromatograms, recorded from channel 3, of extracts from (A) pre-dose blood (4 ml) and (B) blood sampled 45 min after physostigmine salicylate (4 mg, orally). Peaks: 1 = physostigmine, 2 = internal standard.

were in the range 1.2—1.8. Neostigmine bromide must be added immediately after blood is sampled to prevent enzyme hydrolysis of physostigmine. However, as this perturbs the red cell binding, plasma assays will not give the true plasma concentration of the sample when the blood was taken. For this reason it is suggested that blood, rather than plasma, be used for analysis.

Physostigmine concentrations after oral dosing

After oral doses of 1 mg and 2 mg physostigmine salicylate, equivalent to 0.67 and 1.34 mg of physostigmine base, no drug could be detected in blood samples collected for up to 3 h after the dose. Although the concentrations were very low, physostigmine was detectable in urine. The maximum excretion rates (Fig. 6) occurred during the first hour indicating that the blood concentrations had reached maximum values, but that these were below the detection limit of the assay, < 50 pg ml^{-1}. The total excreted in the urine, in 3 h amounted to 0.005% and 0.02% of the dose after 1 mg and 2 mg, respectively. After 4 mg, orally, the total excreted into the urine accounted for approximately 0.04% of the dose.

Blood collected 45 min after the 4-mg dose contained the highest physostigmine concentration, 0.78 ng ml^{-1} (Fig. 7). Concentrations after the peak declined rapidly, suggesting a half-time of approximately 25—30 min (Table II). Physostigmine was undetectable in the 3-h blood sample.

TABLE II

BLOOD CONCENTRATIONS OF PHYSOSTIGMINE AFTER 4 mg PHYSOSTIGMINE SALICYLATE, ORALLY

Time (min)	Concentration (ng ml^{-1})
0	N.D.*
15.5	0.13
31	0.30
45	0.78
60	0.48
75.5	0.30
90	0.16
120	0.08
150	0.04
180	N.D.

*N.D. = Not detected.

CONCLUSION

LC with electrochemical detection is suitable for assaying physostigmine in biological fluids at concentrations likely to be encountered in pharmacokinetic studies. Blood and urine concentrations after oral doses are reported here. The assay is currently being applied to samples collected after intravenous infusions (0.6 mg physostigmine salicylate over 10 min) and the data will be reported when the study is complete.

REFERENCES

1 D.J. de Wildt, A.J. Porsius and H.H. van Rooy, J. Chromatogr., 225 (1981) 381.
2 J.Y.-K. Hsieh, R-K. Yang and K.L. Davis, J. Liquid Chromatogr., 5 (1982) 1691.
3 R. Whelpton, J. Chromatogr., 272 (1983) 216.
4 S. Ellis, J. Pharmacol. Exp. Ther., 79 (1943) 364.
5 M. Polonovski and C. Nitzberg, Bull. Soc. Chim Fr., 19 (1916) 33.
6 M. Kneczke, J. Chromatogr., 198 (1980) 529.
7 I. Christenson, Acta Pharm. Suec., 6 (1969) 287.

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CHROMBIO. 2578

DETERMINATION OF METABOLITES OF CYTOCHROME P-450 MODEL SYSTEMS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatographic techniques were developed for the simultaneous detection of metabolites in a cytochrome P-450 model system composed of NADH, haemoglobin and methylene blue. Monohydroxylated metabolites were determined following aniline, acetanilide and phenol hydroxylations. 4-Aminoantipyrine, 7-hydroxycoumarin and *p*-nitrophenol were determined after dealkylation of 4-N,N-dimethylaminoantipyrine, 7-ethoxycoumarin and *p*-nitroanisole. These substrates are commonly used for measuring cytochrome P-450 activities. Treatment of the samples was minimal, consisting of a simple deproteinization, and did not involve any organic extraction. Separations were carried out on reversed-phase columns and the products were detected by UV adsorption. Separations were completed in less than 15 min and the detection limits were between 0.5 and 4 μM.

INTRODUCTION

Hydroxylation and dealkylation reactions are commonly tested on microsomal cytochrome P-450. Most of the methods traditionally used for the calculation of activities are based on the determination of one major product of the reaction or of one side-product. These methods suffer from two kinds of drawbacks: as they are often spectrophotometric, there may possibly be interference from other compounds in complex mixtures, and with titration of only one major product, other products are assumed to be minor. This might not be a general case and study of the products of reaction could help in differentiating the different forms of cytochrome P-450 by their regioselectivities.

In an attempt to study reactions catalysed by cytochrome P-450 model systems, an approach using high-performance liquid chromatography (HPLC)

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was devised. This allowed both a qualitative and a quantitative method of separation of the reaction products. Both gas and liquid chromatography fitted this requirement, but gas chromatography, although generally more sensitive, was rejected because of the necessary derivatization of aqueous samples. In contrast, HPLC needed minimal preparation of aqueous samples and gave reproducible results within a reasonable time with good sensitivity.

Three hydroxylation reactions on aromatic rings (aniline, phenol and acetanilide) were studied by HPLC. Three oxidative dealkylations were also examined by HPLC: N-demethylation of 4-N,N-dimethylaminoantipyrine, O-de-ethylation of 7-ethoxycoumarin and O-demethylation of *p*-nitroanisole. These chromatographic techniques were applied to the study of activities and regioselectivities of model systems of cytochrome P-450 [1] (an example of which will be given).

The cytochrome P-450 system of liver microsomes is composed of NADPH (electron donor), NADPH cytochrome P-450 reductase (electron carrier) and cytochrome P-450. The model system given here as an example of application is NADH—methylene blue and haemoglobin. The aim was to compare hydroxylations and dealkylations by haemoglobin and cytochrome P-450 and to examine the use of methylene blue as an electron carrier.

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma and Aldrich. Bovine haemoglobin was prepared from freshly drawn blood as described by Heidelberg and Lansteiner [2], with minor modifications. Solutions were concentrated with an Amicon Hollow Fibers system and stored in liquid nitrogen.

Conditions

Experiments were conducted in the dark at 37 °C in 0.1 *M* phosphate buffer (pH 7.4). The model system was composed of 1 m*M* NADH, 10 μM methylene blue, 0.1 *M* haemoglobin and substrate. The concentrations of the different substrates were aniline 30 m*M*, phenol 20 m*M*, acetanilide 10 m*M*, 4-N,N-dimethylaminoantipyrine 20 m*M*, 7-ethoxycoumarin 0.5 m*M* and *p*-nitroanisole 5 m*M*. 7-Ethoxycoumarin and *p*-nitroanisole were previously dissolved in 5% ethanol and 9% polyethylene glycol 400, respectively. Reactions were started by addition of concentrated haemoglobin. Haemoglobin concentrations were measured according to Drabkin [3].

Preparation of samples for HPLC and determination of the products

Samples of 0.5 ml were deproteinized by addition of 40 μl of trichloroacetic acid (TCA) (30%, w/v) or 0.6 ml of methanol (at 4 °C). This was necessary in order to avoid irreversible adsorption of the proteins on the phase of the HPLC column.

Precipitation by TCA to form insoluble salts in an acidic medium was found to be the most efficient method, but oxidative damage of some products occurred. As TCA is rapidly eluted from the column, there is no interference with peaks of products. The use of perchloric acid instead of TCA with subse-

quent precipitation of perchlorate by potassium carbonate did not give any UV absorption but oxidized all the products considerably. Formation of insoluble salts in a neutral medium with heavy metals was inefficient. Dehydration with solvents miscible with water was a mild precipitation method that was used when the use of TCA was prohibited. Methanol was found to be the best precipitating agent after acetone (which absorbs considerably at 280 nm).

Samples were then centrifuged for 5 min at 800*g* and 10—25 μl of the supernatant injected into a Waters Assoc. 6000A HPLC system equipped with an octadecylsilane column. Two types of reversed-phase C_{18} columns were used at 25°C: a Rad-Pak C_{18}, 10-μm (Waters Assoc.) and a Chromatem RP-18, 5-μm (Touzart et Matignon). UV detection was performed at 254, 280 or 340 nm with an M440 detector (Waters Assoc.).

Product identity was confirmed by checking the co-elution of an internal standard. Calibration graphs were established with standards under the same experimental conditions.

RESULTS AND DISCUSSION

Determination of metabolites of aniline hydroxylation

The classical method [4] permits only the determination of 4-aminophenol, which gives indophenol by reaction with phenol and is detected by spectro-

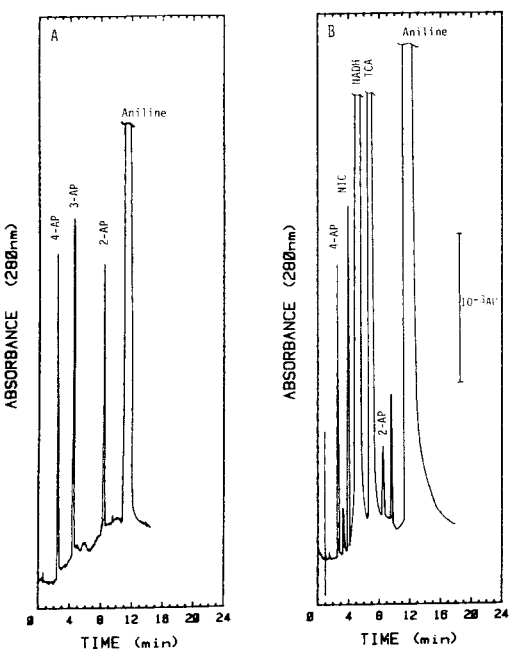

Fig. 1. (A) Chromatogram of standards of aniline and 2-, 3- and 4-aminophenols (2-AP, 3-AP and 4-AP). Deproteinization was accomplished with TCA: 15 μl of the supernatant were injected onto a Chromatem column. Mobile phase 0.2 *M* Tris—acetate buffer (pH 8) plus 0.75% (v/v) triethylamine; flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 1 h. TCA = trichloroacetic acid; NIC = nicotinamide. During the reaction the NADH is partially denatured to nicotinamide. Conditions as stated in the text.

photometry. This very sensitive reaction does not allow the detection of 2- and 3-aminophenols, which were followed by our method during aniline hydroxylation.

A separation of the three isomers on a Dupont Zipax SCX reversed-phase column with 0.1 *M* phosphoric acid (pH 2.9) as mobile phase and a flow-rate of 0.8 ml/min was described by Sakurai and Ogawa [5]. At this pH, substantial tailing of the aminophenols was observed. When the pH was increased to 7.5—8, 4- and 3-aminophenols no longer tailed but 2-aminophenol still did. This might be due to an interaction between the amino group of the aminophenol and the ungrafted silanol remnants in the column, as addition of 0.75% of triethylamine efficiently decreased the tailing. It should be noted that the pH of the mobile phase has to be checked after the addition of this very basic compound.

Fig. 1A shows the chromatogram of aniline plus 2-, 3- and 4-aminophenol standards on a Chromatem column. Fig. 1B shows the chromatogram of an incubation medium after 1 h: 4- and 2-aminophenols are produced. 3-Aminophenol is not detected (detection limits as in Table I).

TABLE I

LINEAR REGRESSION ANALYSIS FOR METABOLITES IN THE HPLC METHODS

x = molarity of the solution injected (μM), y = optical density recorded (O.D. $\times 10^3$).

Compound	Range		Slope	y-Intercept	Correlation coefficient
	μM*	pmol			
4-Aminophenol	2.0—50	30—750	0.052	0.052	0.999
3-Aminophenol	1.0—50	15—750	0.079	0.038	0.999
2-Aminophenol	3.0—50	45—750	0.074	— 0.006	0.999
Hydroquinone	1.0—50	12—565	0.080	0.003	0.999
Resorcinol	2.5—50	29—565	0.035	0.027	0.999
Catechol	3.0—50	34—565	0.030	0.006	0.999
4-Acetaminophenol	0.5—25	5—250	0.252	0.035	0.997
3-Acetaminophenol	1.0—25	10—250	0.123	0.052	0.999
2-Acetaminophenol	4.0—25	40—250	0.043	0.039	0.999
4-Aminoantipyrine	1.2—3.5	6—161	0.088	0.016	0.999
7-Hydroxycoumarin	1.0—50	5—230	0.134	— 0.037	0.997
p-Nitrophenol	1.0—50	5—230	0.101	— 0.026	0.999

*The lower end of the range is the detection limit: optical density = 10^{-4}, equivalent to a signal-to-noise ratio of 2:1. Statistical parameters based on the responses of five standards.

Determination of metabolites of phenol hydroxylation

The possible products examined were the 2-, 3- and 4-monohydroxyphenols, i.e., catechol, resorcinol and hydroquinone. Separation with gradient programming [6] was considered to be too complicated.

Precipitation of the protein was effected with methanol and not TCA, as hydroquinone and catechol are especially sensitive to oxidation in the presence of TCA.

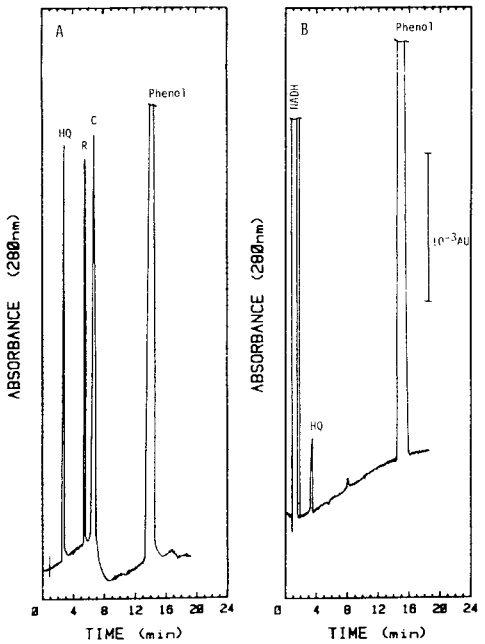

Fig. 2. (A) Chromatogram of standards of phenol, catechol (C), resorcinol (R) and hydroquinone (HQ). Deproteinization was accomplished with methanol: 25 μl of the supernatant were injected onto a Chromatem column. Mobile phase, tetrahydrofuran—0.1 *M* ammonium acetate (pH 5) (2:98, v/v); flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 20 min. Conditions as stated in the text.

Catechol was no longer detectable when the pH of the mobile phase was higher than 5, either for stability reasons or owing to intramolecular bonding between the adjacent hydroxy groups, which increased its affinity for the column. A 0.1 *M* ammonium acetate—acetic acid buffer (pH 5) was therefore chosen.

Fig. 2A shows the separation of standards of monohydroxyphenols and phenol on a Chromatem column. Fig. 2B shows the chromatogram of an incubation medium after 20 min: hydroquinone was found but not catechol or resorcinol (see Table I for detection limits).

Determination of metabolites of acetanilide hydroxylation

Acetanilide and 2-, 3- and 4-acetaminophenols, which are not sensitive to TCA, were separated on a Chromatem column at basic pH. The chromatograms in Fig. 3A and B show separations of standards and metabolites (4-, 3- and 2-acetaminophenols), respectively, in an incubation medium after 31 min (see Table I for detection limits)

Determination of metabolites of 4-N,N-dimethylaminoantipyrine N-demethylation

4-N,N-Dimethylaminoantipyrine is oxidized by cytochrome P-450 into formol and 4-aminoantipyrine. This reaction is usually followed by titration of formol with Nash reagent and detection at 412 nm according to procedures derived from the method of Nash [7].

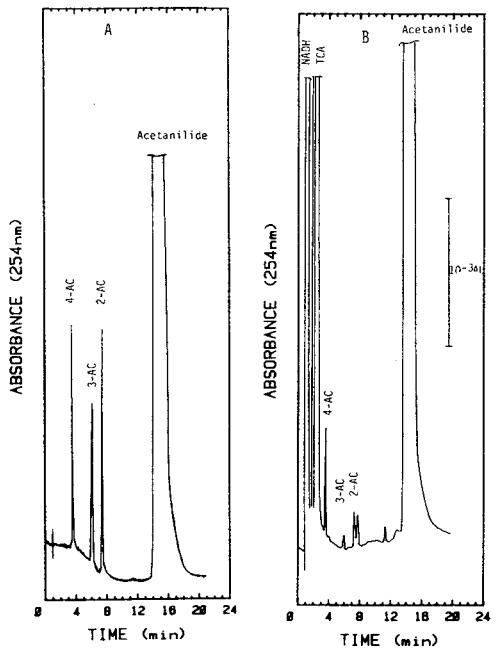

Fig. 3. (A) Chromatogram of standards of acetanilide and 2-, 3- and 4-acetaminophenols (2-AC, 3-AC and 4-AC). Deproteinization was accomplished with TCA: 10 μl of the supernatant were injected onto a Chromatem column. See Table I for detection levels. (B) Chromatogram of an incubation medium after 31 min. Conditions as stated in the text.

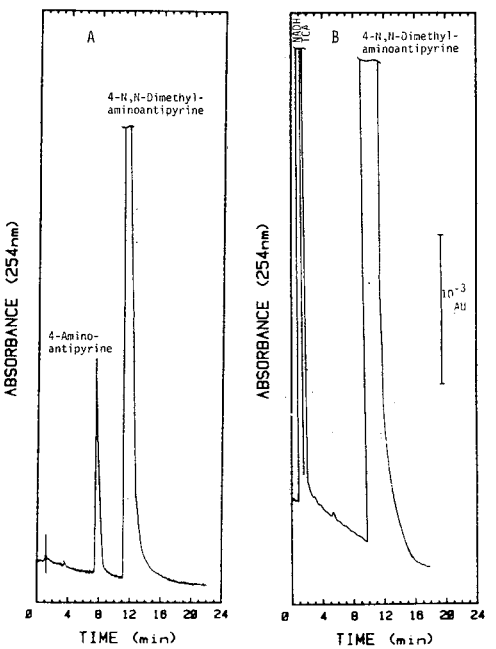

Fig. 4. (A) Chromatogram of standards of 4-N, N-dimethylaminoantipyrine and 4-aminoantipyrine. Deproteinization was accomplished with methanol: 10 μl of the supernatant were injected onto a Rad-Pak column. Mobile phase, methanol—0.1 *M* Tris—acetate buffer (pH 8) (35:65, v/v); flow-rate, 3 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 1 h 25 min. 4-Aminoantipyrine would have been easily detected if it had appeared. Conditions as stated in the text.

Interference was found with derivatives of haemoglobin at this wavelength, so determination of 4-aminoantipyrine by HPLC was preferred. The separation was effected at pH 8 on a Rad-Pak column. Separations of standards and of compounds in an incubation medium (after 1 h 25 min) are shown in Fig. 4A and B, respectively. The expected product, 4-aminoantipyrine, was not found in this model system at our detection limit (1.2 μM or 6 pmol) (Table I). By comparison, activities of different forms of cytochrome P-450 vary from 0.8 to 2.2 μM/min, which is at the level of our detection limit in 1 min [8, 9].

Determination of metabolites of 7-ethoxycoumarin O-de-ethylation

7-Ethoxycourmarin is transformed by cytochrome P-450 into 7-hydroxycoumarin and acetaldehyde. 7-Hydroxycoumarin is commonly determined by spectrofluorimetry (λ_{exc} = 366 nm, λ_{em} = 454 nm) [10]. It was very easily separated from 7-ethoxycoumarin by HPLC at an acidic pH on a Rad-Pak column and detected at 340 nm. A fluorescence detector would decrease the detection limit, which was 1 μM under these conditions. A typical separation of standards is given in Fig. 5A and the chromatogram of the reaction medium after 2 h 30 min in Fig. 5B. 7-Hydroxycoumarin, which was not detected, would have given an easily detected peak (Table I). With different forms of cytochrome P-450 the concentrations of 7-hydroxycoumarin range from 0.2 to 1.3 μM/min [7, 8], which means that in the worst case detection will occur after 5 min.

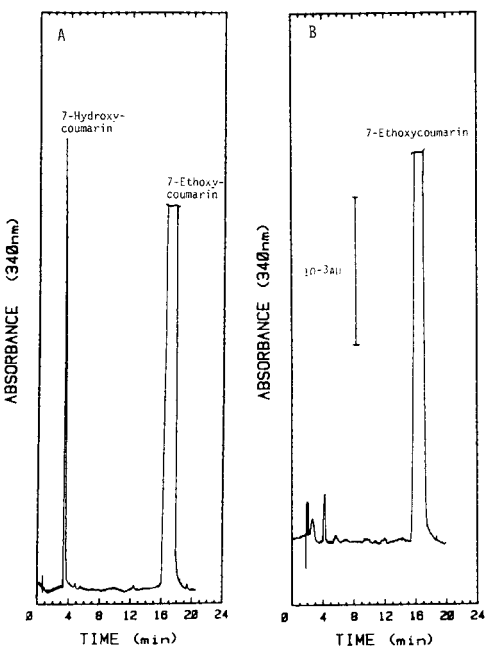

Fig. 5. (A) Chromatogram of standards of 7-ethoxycoumarin and 7-hydroxycoumarin. Deproteinization was accomplished with methanol: 10 μl of the supernatant were injected onto a Rad-Pak column. Mobile phase, methanol—0.1% acetic acid (50:50, v/v); flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 2 h 35 min. 7-Hydroxycoumarin was not found at a detectable level, which would have been very easy. Conditions as stated in the text.

Determination of metabolites of p-nitroanisole O-demethylation

p-Nitroanisole is converted by cytochrome P-450 into *p*-nitrophenol and formol. If *p*-nitrophenol is evaluated by altering the pH to 7.5 to give the yellow phenate form [11], colorimetric interference occurs in our model system. Standards were therefore separated on a Rad-Pak column at acidic pH to give mostly the phenol form and a single peak instead of unresolved peaks (phenol and phenate) (Fig. 6A). Again, the haemoglobin-containing system does not show any detectable *p*-nitrophenol-forming activity (Fig. 6B). *p*-Nitrophenol at concentrations from 0.2 to 0.6 μM/min is obtained from several forms of cytochrome P-450 [7, 8] and it would then be detected in at least 5 min (Table I).

These methods were developed to check the comparison between a cytochrome P-450 model system and a cytochrome P-450 system. Several workers [12—14] have tried to replace cytochrome P-450 with another haemoprotein such as haemoglobin, but until now experiments have been only conducted with aniline as substrate.

Our aim was to determine whether there was similar behaviour of an NADH—methylene blue—haemoglobin system and of a liver microsomal system towards typical substrates of cytochrome P-450, by comparing substrate specificities and reaction regioselectivities [1]. It was found that hydroxylation reactions but not dealkylation reactions occurred in this haemoglobin system

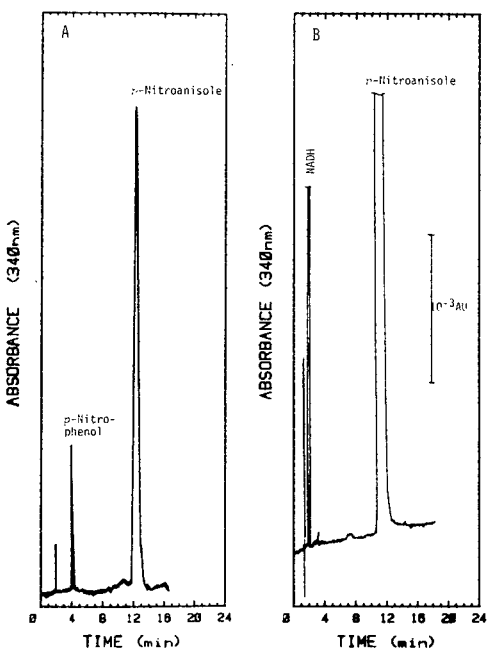

Fig. 6. (A) Chromatogram of standards of *p*-nitrophenol and *p*-nitroanisole. Deproteinization was accomplished with methanol: 10 μl of the supernatant were injected onto a Rad-Pak column. Mobile phase, methanol—0.1% acetic acid (45:55, v/v); flow-rate, 2 ml min^{-1}. See Table I for detection levels. (B) Chromatogram of an incubation medium after 2 h 30 min. No detectable activity was found (the peak of *p*-nitrophenol would have been well resolved). Conditions as stated in the text.

TABLE II

REGIOSELECTIVITES IN HYDROXYLATIONS OF ANILINE, ACETANILIDE AND PHENOL

Percentages of isomers formed from aniline, acetanilide and phenol in a model system (30 m*M* aniline, 10 m*M* acetanilide or 20 m*M* phenol, 0.1 m*M* methylene blue, 1 m*M* NADH and 0.1 *M* oxyhaemoglobin) and in a liver microsomal system [15, 16].

Compound	Metabolite	Model system	Cytochrome P-450 system
Aniline	4-Aminophenol	80	85
	3-Aminophenol	< 4	0
	2-Aminophenol	16	15
Acetanilide	4-Acetaminophenol	29	94
	3-Acetaminophenol	11	1
	2-Acetaminophenol	60	5
Phenol	Hydroquinone	100	100
	Resorcinol	< 10	0
	Catechol	< 12	0

(within our detection limits). Phenol was hydroxylated in the *para* positions. Aniline was hydroxylated in the *para* and *ortho* positions with a regioselectivity similar to that known for cytochrome P-450 (Table II). There was substrate specificity towards aniline and phenol. Although acetanilide was hydroxylated by our model system in the *ortho*, *meta* and *para* positions similarly to cytochrome P-450, the regioselectivities were totally different (Table II). The use of these methods allowed us to show that the haemoglobin—cytochrome P-450 comparison was valid only in the particular case with aniline as substrate when hydroxylations were concerned, and was not valid for these dealkylations.

CONCLUSION

Analysis by HPLC prevented interferences from other compounds and allowed the determination of the regioselectivity of a haemoglobin system. These methods might be adjusted for cytochrome P-450 and help to differentiate between different forms according to the different regioselectivities obtained towards the same substrate. They could be of great help in the comparison of model and microsomal systems when studying metabolites of drugs.

With HPLC, pre-treatment of the sample is minimal, no loss of product occurs, in contrast to extraction for gas chromatographic analysis, and there is no need to calculate an extraction yield for each compound. If other products are to be detected, gradients of mobile phase are possible, as was well demonstrated for benzo[*a*]pyrene metabolites [17].

The pH of the sample is very important with regard to the retention of polar compounds on the columns. Detection limits ranged from 1 to 4 μM or from 3 to 40 pmol, depending on the metabolites to be analysed and on the method used (Table II). They may be improved by use of another detection system such as fluorimetry or by decreasing the amount of precipitating agents. With phenol, derivatization with 4-aminoantipyrine could increase the sensitivity ten-fold but after a 40-min reaction [18]. The reproducibility was within 5% and all analyses were completed within 15 min.

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REFERENCES

1 L. Esclade, D. Guillochon and D. Thomas, submitted for publication.
2 M. Heidelberg and K. Landsteiner, J. Exp. Med., 38 (1923) 561—564.
3 D. Drabkin, Amer. J. Sci., 217 (1949) 710—711.
4 B.B. Brodie and J.J. Axelrod, J. Pharmacol. Exp. Ther., 94 (1948) 22—38.
5 H. Sakurai and S. Ogawa, J. Chromatogr. Sci., 14 (1976) 499—500.
6 G. Kung-Jou Chao and J.C. Suatoni, J. Chromatogr. Sci., 20 (1982) 436—440.
7 Y. Nash, Biochem. J., 55 (1953) 416—421.
8 C. Bonfils, C. Dalet, I. Dalet-Beluche and P. Maurel, J. Biol. Chem., 258 (1983) 5358—5362.
9 R. Sato, T. Ayoama and Y. Imai, in N. Mitsuhiro (Editor), Oxygenases Oxygen Metabolism, Academic Press, New York, 1982, pp. 321—332.
10 V. Ullrich and P. Weber, Hoppe Seyler's Z. Physiol. Chem., 353 (1972) 1171—1177.
11 K.J. Wetter and B. Seidel, J. Pharmacol. Exp. Ther., 146 (1964) 61—66.
12 J.J. Mieyal, R.S. Ackerman, J.L. Blumer and L.S. Freeman, J. Biol. Chem., 251 (1976) 3436—3441.
13 A.A. Akhrem, S.Y. German and D.I. Metelitsa, React. Kinet. Catal. Lett., 8 (1978) 217—221.
14 D. Guillochon, B. Cambou, L. Esclade and D. Thomas, Enzym. Microb. Techol., 6 (1984) 161—164.
15 V. Ullrich and H.J. Staudinger, Handb. Exp. Pharmacol., 28 (1971) 251—263.
16 R. Snyder, L.S. Longacre, C.M. Witmer and J.K. Kocsis, Rev. Biochem. Toxicol., 3 (1981) 123—153.
17 E.A. Elnenay and W.P. Schoor, Anal. Biochem., 111 (1981) 393—400.
18 G. Blo, F. Dondi and C. Bighi, J. Chromatogr., 295 (1984) 231—235.

Journal of Chromatography, 341 (1985) 383—390
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LIQUID CHROMATOGRAPHIC ANALYSIS OF CLONAZEPAM IN HUMAN SERUM WITH SOLID-PHASE (BOND-ELUT®) EXTRACTION

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SUMMARY

A simple, sensitive, selective and precise liquid-column chromatographic assay for clonazepam is described, in which 1 ml of serum containing 50 μg/l methylclonazepam as an internal standard is extracted by elution from a Bond-Elut® column with 400 μl of methanol. An aliquot of the eluate is injected on to a reversed-phase column and eluted with a mobile phase of acetonitrile—phosphate buffer (30:70) at a flow-rate of 2 ml/min at a column temperature of 50°C. Detection is at 254 nm. Chromatography is complete in 12 min. A sensitivity of 2 ng/ml is attained when 1 ml of serum is extracted. Analytical recovery of the clonazepam added to serum ranged from 91% to 99% with a coefficient of variation of 6.0%. This assay for clonazepam has good precision, with coefficients of variation of 11% at 15 ng/ml and 2.6% at 50 ng/ml. There was no interference from any of the commonly used antiepileptics.

INTRODUCTION

Clonazepam or 5-(2-chlorophenyl)-3-dihydro-7-nitro-1,4-benzodiazepine-2-one is a close structural and pharmacological analogue of nitrazepam. It has been effectively used in the treatment of petit mal epilepsy and minor motor seizures of childhood, and in refractory grand mal epilepsy, focal motor seizures, temporal lobe epilepsy, myoclonic epilepsy, and by intravenous route, in status epilepticus. It is usually used in patients who are already resistant to other antiepileptic drugs [1].

A thin-layer chromatographic (TLC) method for the assay of clonazepam was reported by Wad and Hanifl [2]. Since then, modifications of this method have been described [3]. However, the sensitivity of this method limits its usefulness to heavy overdose situation. Radioimmunoassay methods for clonazepam suffers [4—7] from cross-reactivity of clonazepam antibody with its 7-amino

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and 7-acetylamino metabolites. Currently gas—liquid chromatographic (GLC) procedures using electron-capture detection [8—12] of either the unchanged drug [8, 12—15] or derivatives [16—20] are widely used for the assay of clonazepam. The unchanged drug produces poor response with electron-capture detection, hence the need for acid hydrolysis and/or derivatization of the drug. The laborious extraction procedures often required for the isolation of the drug from plasma, chemical manipulation and long retention times make GLC methods very time-consuming. There are relatively few liquid chromatographic (LC) methods published for the analysis of clonazepam [21—23]. A normal-phase separation for clonazepam was reported by Perchalsky and Wilder [21] using dihydrodiazepam as internal standard. However, clonazepam and carbamazepine are not well resolved under normal-phase conditions. Revel and Sanjuan [23] used reversed-phase LC to separate clonazepam from carbamazepine but could not resolve chlordiazepoxide from clonazepam. Jambor [24] reported good recovery of clonazepam with chloroform extraction. However, the internal standard, dihydrodiazepam, was found to be unstable.

A liquid-column chromatographic method which obviates these drawbacks and offers considerable improvement in speed, accuracy, and selectivity in the monitoring of clonazepam is described here. The solid-phase extraction procedure used in this method is very simple, does not utilize large amounts of organic solvents, and ten samples can be processed in about 15 min. Both chlordiazepoxide and carbamazepine are well resolved from clonazepam in this assay.

EXPERIMENTAL

Chromatography

The analysis was carried out on a Series 3B (Perkin-Elmer, Norwalk, CT, U.S.A.) liquid chromatograph equipped with a Model 7125 (Rheodyne, Cotati, CA, U.S.A.) injector, an LC-15 fixed-wavelength ultra-violet detector (Perkin-Elmer), a 10-mV recorder Model BD 40 (E and K Scientific Products, Saratoga, CA, U.S.A.) and a 15 cm × 4.6 mm I.D. reversed-phase column (Altex, subsidiary of Beckman Instruments, Berkeley, CA, U.S.A.) packed with Ultrasphere ODS C_{18}, 5 μm particle size. The column was mounted in an LC 100 (Perkin-Elmer) oven maintained at 50°C. The column was eluted with acetonitrile—0.02 mol/l phosphate buffer, pH 3.8 (30:70) at a flow-rate of 2.0 ml/min, and the column effluent was monitored at 254 nm.

Extraction apparatus

C_{18} Bond-Elut® columns and a Vac-Elut® apparatus were obtained from Analytichem (Harbor City, CA, U.S.A.).

Reagents and standards

All reagents were of reagent-grade purity. All inorganic reagents were made up in distilled water. Clonazepam and the internal standard, methylclonazepam, were obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Clonazepam (10 mg) and 10 mg of methylclonazepam (internal standard) were

dissolved in 100 ml of methanol. These standards were stable at 4°C for at least six months. The working solution was prepared by diluting the stock solution 100-fold with water to obtain a solution containing 1 μg/ml of each drug. The serum standards containing 10, 25, 50, 100 ng/ml clonazepam were prepared by adding working solution to pooled drug-free human serum. Serum and plasma were used interchangeably. The serum standards are stable at 4°C for at least two weeks. The working internal standard was made by combining equal volumes of internal standard solution and glycine buffer to obtain a solution having a concentration of 50 ng/ml. The working internal standard is stable for one week at 4°C.

Buffers. Phosphate buffer (0.02 *M*) was prepared by dissolving 2.7 g of anhydrous potassium phosphate monobasic crystals in 1 l of distilled water. The pH of this solution was adjusted to 3.8 with orthophosphoric acid.

Glycine buffer (1 *M*) was made by dissolving 75.1 g of glycine (amino acetic acid, Sigma, St. Louis, MO, U.S.A.) in 1 l of water, and adjusting the pH to 10.5 with sodium hydroxide.

Mobile phase

The acetonitrile—phosphate buffer (30:70) mobile phase was prepared by adding 300 ml of acetonitrile to 700 ml of 0.02 mol/l phosphate buffer (pH 3.8). This solution was filtered before use.

Procedure

Place Bond-Elut columns on the top of the Vac Elut vacuum manifold. Pass two column volumes of methanol and distilled water through each column. Disconnect the vacuum as soon as the water has run through the columns to prevent them from drying out. Place 100 μl of internal standard (methyl-clonazepam) in 1 *M* glycine buffer onto each column, then pipette 1 ml of standard, control or patient sample onto each column. Connect the vacuum and wash each column with two column volumes of distilled water followed by 50 μl of methanol. Disconnect the vacuum, and place a rack containing appropriately labeled 75 × 10 mm glass tubes to collect eluent. Add 200 μl methanol to each column, and connect the vacuum and collect eluent. Add another 200 μl of methanol, collect, and combine the eluents.

Evaporate the methanol to dryness under a gentle stream of air in a water bath at 45°C. Reconstitute with 40 μl of methanol and inject all of the sample into the high-performance liquid chromatograph.

RESULTS

Recovery

Analytical recovery was calculated on drug-free pooled human serum spiked with known amounts of clonazepam to achieve the concentrations shown in Table I. A constant amount of internal standard was added to each sample. The samples were then processed as described and the recoveries shown in Table I were obtained. The analytical recovery ranged from 91% to 99% over the entire range. Absolute recovery of the drug averaged about 50%.

TABLE I

RECOVERY OF CLONAZEPAM FROM PLASMA SAMPLES ($n = 20$)

Drug added (ng/ml)	Drug recovered (ng/ml)	Standard deviation (ng/ml)	Coefficient of variation (%)	Percentage recovery
10	9.30	0.37	4	93
25	22.60	1.40	6	91
50	47.00	1.25	3	94
100	99.90	0.92	1	99

Linearity

Clonazepam was added to plasma in amounts equivalent to 15—100 μg/l, and a constant amount of internal standard was added to each sample. Concentration and peak height ratios were linear over this range.

Detection and sensitivity

The drugs and internal standard were detected at 254 nm, 1 ng of clonazepam standard could be detected by monitoring at 0.008 a.u.f.s. The minimum detectable concentration was 2 ng when 1 ml of serum sample was extracted. The sensitivity of this method allows for easy quantitation of 5 ng/ml clonazepam in 0.5 ml of serum.

Precision

Precision data were obtained by analyzing plasma samples containing two different concentrations of the drug as shown in Table II. For within-run determination the coefficients of variation (C.V.) ranged from 1.6% to 5%; for day-to-day precision, the C.V. ranged from 2.6% to 11%.

TABLE II

PRECISION OF CLONAZAPAM ASSAY

Concentration (ng/ml)	S.D. (ng/ml)	Coefficient of variation (%)
Within-run ($n = 14$)		
13.60	0.70	5.0
45.40	0.70	1.6
Day-to-day ($n = 10$)		
13.70	1.50	11.0
45.20	1.20	2.6

Interference

Possible interference from commonly used drugs was assessed by injecting concentrations of drugs that might be expected in overdose situations and measuring their retention times (Table III). Any drug found to elute sufficiently close to clonazepam or the internal standard was further evaluated by adding known amounts of the interfering drug to drug-free pooled plasma so as to quantitate the effect on clonazepam or the internal standard.

Carbamazepine, which normally interferes in the normal-phase assay of clonazepam, posed no problem. Furthermore, chlordiazepoxide could be resolved from clonazepam with the 25-cm column, a separation that was not possible in the other methods. Thus, none of the drugs tested were found to interfere.

Accuracy

Fig. 1 illustrates typical chromatograms obtained by this procedure: seventeen samples were assayed for clonazepam using the LC method. The results were compared with those obtained by GLC [9]. The coefficient of correlation was 0.973, the slope 0.744 and the intercept 6.2.

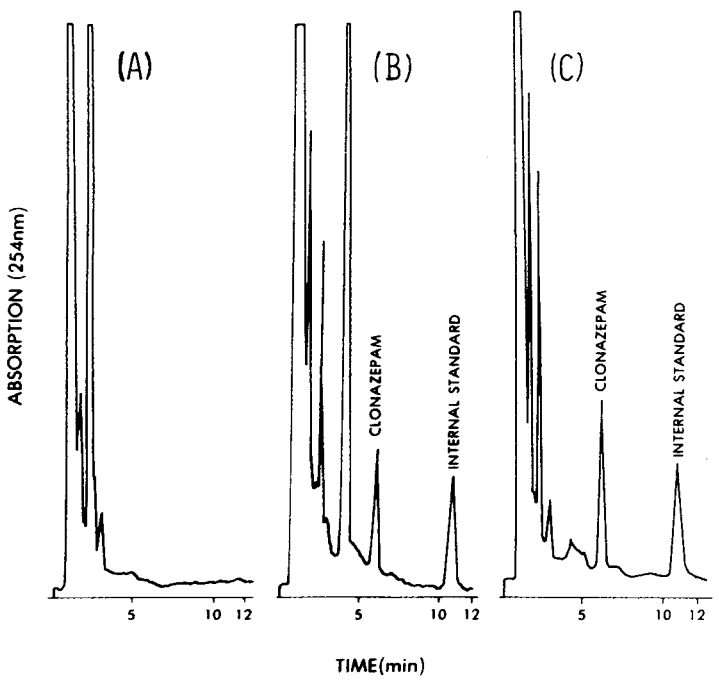

Fig. 1. Chromatogram of (A) clonazepam-free serum; (B) a patient's serum with 42 μg/l clonazepam; (C) a patient's serum with 57 μg/l clonazepam.

DISCUSSION

Clonazepam is an established anticonvulsant agent widely used in the treatment of epilepsy [25, 26]. The narrow therapeutic index and risk of increased seizure frequency in cases of overdose make routine monitoring of clonazepam plasma concentrations during treatment with the drug not only useful but necessary [26]. The high-performance liquid chromatographic procedure described would be adequate for such a purpose.

In the development of our present assay method, various chromatographic conditions were evaluated by injecting a solution of the drug in methanol to assess the effect of different parameters such as the pH of the mobile phase, the composition of the mobile phase, and the detection wavelength. All these factors were adjusted until optimal assay conditions were achieved.

TABLE III

RETENTION TIMES OF SOME OTHER DRUGS WITH THE MOBILE PHASE

Drug	Retention time (min)	Drug	Retention time (min)
Allyl cyclopentenyl barbituric acid	1.9	Mephobarbital	3.8
Alphenol	1.9	Meprobamate	N.D.
Amitriptyline	27.0	Mesoridazine besylate	11.2
Amobarbital	3.4	Methadone	N.D.
Anafranil	N.D.*	Methaqualone	6.4
Aprobarbital	1.2	Methyl clonazepam	11.0
Barbituric acid	0.4	Methyl nitrazepam	8.6
Butabital	1.5	Methyprylon	1.4
Butalbital	2.2	N-Acetyl procainamide	N.D.
Carbamazepine	3.8	Pentathal	7.6
Chlordiazepoxide	7.0	Nirvanol	1.0
Chlorpromazine	42.0	Nortriptyline	21.0
Clonazepam	6.0	Oxazepam	17.0
Demoxepam	3.0	Pentobarbital	3.2
Desipramine	16.0	Perphenazine	40.2
Diallyl barbituric acid	1.0	Phenobarbital	1.4
Diazepam	16.8	Phenytoin	3.2
Ethinamate	N.D.	Procainamide	N.D.
Ethosuximide	0.6	Promazine	21.0
Flurazepam	10.0	Propoxyphene	N.D.
Gentamicin	N.D.	Propranolol	5.0
Glutethimide	4.4	Protriptyline	16.0
Heptobarbital	3.2	Quinidine	2.3
Hexobarbital	3.4	Secobarbital	4.4
Imipramine	22.0	Thioamyl	1.1
Lidocaine	1.6	Thioridazine hydrochloride	N.D.
Mebutamate	N.D.	Trifluoperazine hydrochloride	N.D.
Medazepam	25.0	Trifluprom azine hydrochloride	N.D.
Meperidine	2.8	Tybamate	N.D.
		Vinbarbital	1.9

*N.D. = Not detected.

At 306 nm it was possible to monitor the concentration of clonazepam, but the peak height of clonazepam was 20% greater at 254 nm.

In several published methods phosphate buffer or borate butter was employed for the extraction of clonazepam, but we found that the glycine buffer (1 mol/l) used by Wad and Hanifl [2] for the TLC determination of diazepam and its metabolites to yield better recovery of clonazepam from serum than phosphate buffer.

Different compositions of the mobile phase (phosphate buffer—acetonitrile) were investigated, for example 60:40, 65:35, 72:28 and 70:30. The 70:30 composition was found to be the best. With the other combinations one of two things usually happened. Either the drug eluted too fast and was interfered with by serum constituents, or the internal standard peak was not sharp. Furthermore, with a flow-rate of 2 ml/min and 70:30 composition of mobile phase the assay could be performed in just 12 min.

Methylclonazepam was chosen as an internal standard because it is chemically similar to clonazepam. The usefulness of this internal standard can be appreciated by considering the fact that of over seventy drugs tested for interference none was found to interfere with clonazepam and the internal standard.

The time factor should also be considered. As the chromatography is complete in 12 min, a skilled technician can analyze thirty samples in about 6 h. This is possible by the solid-phase extraction method which can process ten samples in 15 min.

REFERENCES

1 G.F. Rossi, C. DiRocco Maisa and M. Meglio, in S. Garatlini, E. Mussini and L.O. Randall (Editors), The Benzodiazepines, Raven Press, New York, 1973, p. 461.
2 N.T. Wad and E.J. Hanifl, J. Chromatogr., 143 (1977) 214.
3 N. Wad, H. Rosenmund and E. Hanifl, J. Chromatogr., 128 (1976) 231.
4 W.R. Dixon, R.L. Young, A. Holazo, M.L. Jack, R.E. Weinfeld, K. Alexander, A. Liebman and S.A. Kaplan, J. Pharm. Sci., 65 (1976) 701.
5 W.R. Dixon, J. Earley and E. Postma, J. Pharm. Sci., 64 (1975) 937.
6 B.F. Erlanger, F. Borek, S.M. Beiser and S. Lieberman, J. Biol. Chem., 234 (1959) 1090.
7 W.R. Dixon, R.L. Young, R. Ning and A. Liebman, J. Pharm. Sci., 66 (1977) 235.
8 J. Naestoft and N.E. Larsen, J. Chromatogr., 93 (1974) 113.
9 J.A.F. de Silva, C.V. Puglisi and N. Munro, J. Pharm. Sci., 63 (1974) 520.
10 J.P. Cano, J. Guintrand, C. Aubert and A. Viola, Arzneim.-Forsch., 27 (1977) 338.
11 G.J.G. Parry and D.G. Ferry, J. Chromatogr., 128 (1976) 166.
12 A.G. de Boer, J. Röst-Kaiser, H. Bracht and D.D. Breimer, J. Chromatogr., 145 (1978) 105.
13 R.H. Min and W.A. Garland, J. Chromatogr., 139 (1977) 121.
14 R.H. Min, W.A. Garland, K.C. Khoo and G.S. Torres, Biomed. Mass Spectrom., 12 (1978) 692.
15 E.B. Solow and C.P. Kenfield, J. Anal. Toxicol., 1 (1977) 155.
16 J.M. Wilson, P.N. Friel, A.J. Wilensky and V.A. Raisys, Ther. Drug Monitor., 1 (1979) 387.
17 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
18 D. Shancott and R. Lemiux, Clin. Biochem., 9 (1975) 283.
19 A.K. Dhar and H. Kutt, Clin. Chem., 25 (1979) 137.
20 A.K. Dhar and H. Kutt, J. Chromatogr., 222 (1981) 203.
21 R.J. Perchalsky and B.J. Wilder, Anal. Chem., 50 (1978) 554.

22 D.R.A. Uges and P.A. Bouma, Pharm. Weekbl., 111 (1976) 877.
23 V. Revel and M. Sanjuan, Ther. Drug Monitor., 2 (1980) 283.
24 L. Jambor, Abstracts of the California Association of Toxicologists Meeting, April, 1982, p. 455.
25 T.R. Brone and J.K. Penry, Epilepsia, 14 (1973) 277.
26 P.L. Morselli, in P. Deniker, C. Radonco-Thoimas and A. Villeneuve (Editors), Neuropsychopharmacology, Pergamon Press, Oxford, 1978, p. 877.

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CHROMBIO. 2585

DOSAGE DU SULPIRIDE ET DU SULTOPRIDE PAR CHROMATOGRAPHIE LIQUIDE À HAUTE PERFORMANCE EN VUE DE LEUR ÉTUDE PHARMACOCINETIQUE

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SUMMARY

Quantitative analysis of sulpiride and sultopride by high-performance liquid chromatography for pharmacokinetic studies

A high-performance liquid chromatographic method with UV detection (226 nm) for the analysis of sulpiride and sultopride in body fluids has been developed. Plasma, red blood cell (RBC) and urine samples were extracted by chloroform at pH 10. Internal standards were a new substituted benzamide {N-[(ethyl-1-pyrrolidinyl-2)methyl] methoxy-2-ethylsulphonyl-5-benzamide, DAN} for the sulpiride assay and sulpiride for the sultopride assay. The detection limit in plasma and RBC was 10 ng/ml for sulpiride and 15 ng/ml for sultopride. The proposed techniques were selective, reliable and sensitive enough to be used for pharmacokinetic studies and drug monitoring. Some plasma and RBC data from pharmacokinetic studies in healthy volunteers (sulpiride) or patients (sultopride) are presented. Half-lives determined from either plasma or RBC concentrations were similar (7 h for sulpiride and 5 h for sultopride).

INTRODUCTION

Contrairement aux neuroleptiques des autres classes, les benzamides substituées dont le sulpiride et le sultopride montrent un très haut degré d' affinité pour les récepteurs dopaminergiques centraux (sites D_2). C'est cette sélectivité d'action qui pourrait expliquer leurs capacités relativement faible à induire des troubles extrapyramidaux, à la différence des neuroleptiques typiques comme l'halopéridol [1—3].

De nombreuses méthodes de dosage du sulpiride en milieux biologiques sont décrites dans la littérature; les dosages étant effectués soit par colorimétrie [4], soit par spectrophotométrie [4], soit par spectrofluorimétrie [5]. Cependant

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ces méthodes manquent de spécificités et prennent le risque de doser avec le sulpiride des métabolites; de plus la méthode spectrofluorimétrique donne des blancs élevés (constituants endogènes) [5].

Au cours de travaux antérieurs une méthode de dosage du sulpiride par spectrophotométrie in situ des chromatogrammes sur couche mince et détection dans l'ultraviolet (UV) avait été décrite [6, 7]. Cette méthode, plus sensible (2 μg/ml de plasma) que celle de Segura et al. [8] par chromatographie sur couche mince quantitative (TLC) et inhibition de fluorescence, avait permis de réaliser l'étude pharmacocinétique du sulpiride après son administration en clinique à la dose de 400 mg [9]. Le sulpiride est prescrit couramment aux doses de 50—100 mg [2, 10], aussi la limite de détection de la méthode précédente ne permettait pas son utilisation pour la réalisation d'études pharmacocinétiques à ces posologies.

Frigerio et Pantarotto [11] décrivent une méthode par chromatographie en phase gazeuse (GC) après dérivatisation dont la sensibilité de l'ordre du μg/ml parait insuffisante. Lorsque la GC est couplée à la spectrométrie de masse, le seuil de détection est abaissé considérablement mais l'appareillage est particulièrement coûteux et complexe. Mizuchi et al. [12] proposent une méthode de dosage par radioimmunoassay d'une très grande sensibilité.

Peu de méthodes de dosage du sulpiride par chromatographie liquide à haute performance (HPLC), suffisamment sensibles pour permettre le suivi des concentrations plasmatiques pendant quatre à cinq demi-vies, sont publiées [13, 14]. Seuls Alfredsson et al. [13] rapportent une méthode permettant de doser 10 ng de sulpiride par ml de plasma; la détection est faite en fluorescence. La méthode de dosage du sulpiride par HPLC et détection dans l'UV proposée est d'une sensibilité équivalente.

Les méthodes de dosage du sultopride rapportées dans la littérature sont variées. Les dosages sont effectués soit par spectrofluorimétrie [5, 15, 16], soit par HPLC [17, 18].

Lors de la réalisation de l'étude pharmacocinétique du sultopride en psychiatrie, la TLC avait été utilisée comme méthode de dosage [19]. La sensibilité était de 1 μg/ml de plasma, les cinétiques plasmatiques et urinaires étant suivies pendant 10 et 24 h, respectivement. Cette sensibilité s'est révélée être insuffisante pour un suivi cinétique sur 48 h; une méthode de dosage du sultopride plus sensible par HPLC a été développée.

Ce travail montre que les méthodes employées pour le dosage du sulpiride et du sultopride peuvent être appliquées à des études pharmacocinétiques par leurs sélectivités, leurs reproductibilités et leurs sensibilités.

MATÉRIEL ET MÉTHODE

Appareillage

L'appareil utilisé est un chromatographe liquide à haute performance SP 8100 Spectra-Physics (Les Ulis, France). Ce système est équipé d'une vanne à boucle Valco de 50 μl, d'un four et d'un passeur automatique d'échantillon SP 8110. Cet appareil est relié à un détecteur à longueur d'onde variable (Schoeffel SF 770) et à un miniordinateur intégrateur (SP 4100). La colonne est une LiChrosorb RP8 Merck (10 μm; 250 × 4.6 mm I.D.).

Produits de référence

Le sulpiride ou N-[(éthyl-1-pyrrolidinyl-2)méthyl] méthoxy-2-sulfamoyl-5-benzamide, le sultopride ou N-[(éthyl-1-pyrrolidinyl-2)méthyl] méthoxy-2-éthylsulfonyl-5-benzamide et le produit DAN ou N-[(éthyl-1-pyrrolidinyl-2-)-méthyl] méthoxy-2-amino-4-éthylsulfonyl-5-benzamide, nous ont été gracieusement fournis par les Laboratoires Delagrange (Paris, France).

Réactifs

Sulpiride, solution à 0.1 g/l dans l'eau distillée, à diluer au 1:10 et au 1:100 extemporanément. Sulpiride, étalon interne pour le dosage du sultopride, solution à 0.1 g/l dans le méthanol. Sultopride, solution à 0.1 g/l dans l'eau distillée. Produit DAN, étalon interne pour le dosage du sulpiride, solution à 0.1 g/l dans le méthanol, à diluer au 1:10 extemporanément. Phase mobile: 0.1 *M* acétate d'ammonium—méthanol (10:90). Tampon pH 10: 0.1 *M* glycocolle (dans 0.1 *M* chlorure de sodium)—0.1 *M* hydroxyde de sodium (51:49). Acide trichloracétique à 20%; 1 *M* acide sulfurique, 2 *M* et 0.5 *M* hydroxyde de sodium.

L'eau ainsi que les solvants utilisés ont été bidistillés par nos soins et filtrés sur Millipore (0.45 μm). Tous les produits chimiques utilisés sont des produits purs pour analyse.

Conditions chromatographiques

Débit de la phase mobile: 1 ml/min. Température du four: 50 °C. Sensibilité du détecteur: 0.02 ou 0.04. Atténuation: 2, 4, 8 ou 16. Vitesse de déroulement du papier de l'enregistreur: 1 cm/min. Pression: 43 bars.

Dosage du sulpiride dans le plasma, les érythrocytes et les urines

Dans des tubes à extraction introduire: plasma (4 ml), érythrocytes (2, 3 ou 4 g), urine (0.5, 1, 2 ou 4 ml), de l'eau distillée q.s.p. 4 ml. Ajouter pour les érythrocytes uniquement 0.5 ml de 1 *M* acide sulfurique, laisser en contact 5 min puis ajouter 0.5 ml de 2 *M* hydroxyde de sodium. Pour le plasma et les urines ajouter 0.2 ml de 0.5 *M* hydroxyde de sodium. Ajouter ensuite 1 ml de tampon pH 10. Vérifier le pH. L'ajuster à 10 avec 0.5 *M* hydroxyde de sodium si nécessaire. Extraire par deux fois 20 ml de chloroforme. Centrifuger. Congeler. Prélever deux fois 15 ml de phase organique et les introduire dans une capsule contenant l'étalon interne (0.2 ml pour le plasma et les érythrocytes, 0.8 ml pour les urines). Laisser le solvant s'évaporer à température ambiante (2 h environ). Reprendre le résidu par trois fois 1 ml de chloroforme et transvaser dans de petits tubes à hémolyse en verre. Laisser à nouveau le solvant s'évaporer. Reprendre le résidu par la phase mobile (0.4 ml pour le plasma et les érythrocytes; 1 ml pour les urines). Injecter 50 μl de cette solution préalablement filtrée sur millipore 0.2 μm.

Pour l'établissement des gammes d'étalonnage, ajouter: à six échantillons de 4 ml de plasma ou de 4 g d'érythrocytes, prélevés avant administration du médicament, 0.2 et 0.5 ml d'une solution de sulpiride à 0.001 g/l, puis 0.3, 0.5, 1 et 2 ml d'une solution à 0.01 g/l; à quatre échantillons d'urine (4 ml) prélevée avant administration du médicament: 0.5, 0.75, 1 et 2 ml d'une solution de sulpiride à 0.1 g/l. Suivre ensuite le mode opératoire adopté pour les échantillons à doser.

Dosage du sultopride dans le plasma, les érythrocytes et les urines

Dans des tubes à extraction, introduire: plasma (2, 3 ou 4 ml), érythrocytes (2, 3 ou 4 g), urines (0.5, 1, 2, 4 ou 10 ml), de l'eau distillée q.s.p. 5 ml (plasma, érythrocytes) ou 10 ml (urines). Ajouter pour le plasma et les érythrocytes 1.5 ml d'acide trichloracétique; agiter, laisser en contact 5 min, puis ajouter 1.5 ml de 2 *M* hydroxyde de sodium et laisser en contact 2 min. Pour les urines, ajouter 0.2 ml de 0.5 *M* hydroxyde de sodium. Dans les trois cas, vérifier le pH, ce dernier devant être de 10. Ajuster si nécessaire. Ajouter 20 ml de chloroforme. Agiter durant 20 min. Centrifuger. Congeler. Prélever 15 ml de phase chloroformique et les introduire dans une capsule contenant l'étalon interne (0.3 ml pour le plasma et les érythrocytes, 0.8 ml pour les urines). Abandonner à la température ambiante jusqu'à évaporation (1 h environ). Reprendre le résidu par trois fois 1 ml de chloroforme et transvaser dans des tubes à hémolyse en verre. Laisser le solvant s'évaporer. Reprendre le résidu par la phase mobile (0.4 ml pour le plasma et les érythrocytes, 1 ml pour les urines). Injecter 50 µl de cette solution préalablement filtrée sur Millipore 0.2 µm.

Pour l'établissement des gammes d'étalonnage, ajouter: à cinq échantillons de 4 ml de plasma et de 4 g d'érythrocytes prélevés avant administration du médicament: 0.02, 0.05, 0.1, 0.2 et 0.4 ml d'une solution de sultopride à 0.1 g/l; à quatre échantillons de 4 ml d'urine prélevée avant administration du médicament: 0.25, 0.5, 1 et 2 ml d'une solution de sultopride à 0.1 g/l. Suivre ensuite le mode opératoire adopté pour les échantillons à doser.

Mode de calcul

Les pics sont intégrés et les rapports des surfaces des pics, produit à doser sur l'étalon interne, sont exprimés en fonction de la concentration théorique.

Calcul de la concentration des échantillons à analyser

Les rapports des surfaces permettent d'obtenir par l'équation de la droite d'étalonnage la concentration en principe actif de l'échantillon.

RESULTATS

Dosage du sulpiride

Temps de rétention. La Fig. 1 représente les tracés obtenus avec des érythrocytes surchargés de sulpiride (0.125 µg/g et 0.75 µg/g) traités selon le protocole proposé. Le temps de rétention du sulpiride est de 4.4 min, celui de l'étalon interne de 6.3 min. Aucune interférence de substances endogènes du plasma, des érythrocytes ou de l'urine n'est observée.

Linéarité. Les coefficients de corrélation pour différentes gammes d'étalonnage dans le plasma, les érythrocytes et les urines sont de: 0.9980 ± 0.00047 ($n = 10$), 0.99986 ± 0.00015 ($n = 5$) et 0.9970 ± 0.002 ($n = 14$), respectivement, et les pentes correspondantes de: 0.821 ± 0.012, 0.502 ± 0.006 et 0.209 ± 0.003. L'ordonnée à l'origine est dans tous les cas très proche de zéro: 0.0242 ± 0.010 pour le plasma, 0.0154 ± 0.0147 pour le érythrocytes et 0.0492 ± 0.0373 pour les urines.

Répétabilité. La répétabilité de l'injection est déterminée sur quatre solutions titrant 0.050, 1.25, 2.5 et 5 µg/ml de plasma; chaque solution étant injectée dix

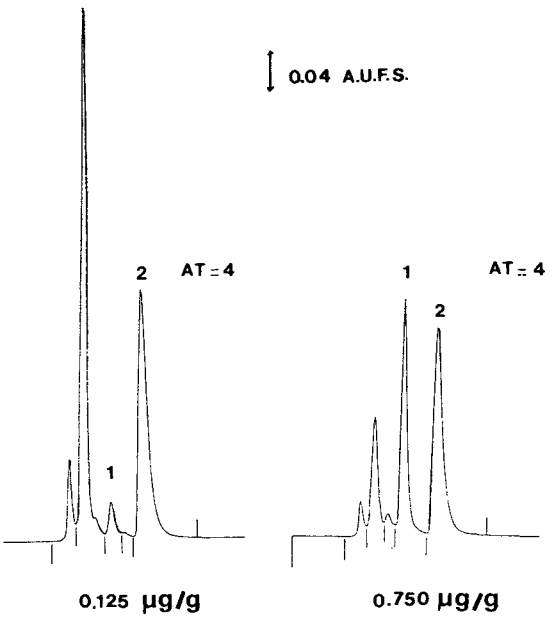

Fig. 1. Enregistrements dans l'ultraviolet de deux points d'une gamme d'étalonnage de sulpiride dans les érythrocytes. Pics: 1 = sulpiride (4.4 min); 2 = produit DAN (étalon interne) (6.3 min).

fois. Les coefficients de variation sont de 2.1, 1.3, 1.0 et 0.90% respectivement. La répétabilité entre gammes d'étalonnage préparées dans du plasma, des érythrocytes et de l'urine de différents sujets est évaluée pour chaque concentration. Les résultats sont rapportés Tableau I.

Coefficient d'extraction. Le coefficient d'extraction est de 98% [2].

Sensibilité. La limite de détection de la méthode est de 10 ng par ml de plasma.

Dosage du sultopride

Temps de rétention. La Fig. 2 représente les tracés obtenus avec des plasmas surchargés de sultopride (0.5 µg/ml et 2.5 µg/ml). Le temps de rétention du sultopride est de 5.40 min, celui de l'étalon interne de 4.40 min. Aucune interférence de substances endogènes du plasma, des érythrocytes ou de l'urine n'est observée.

Linéarité. Le rapport, R, surface intégrée du pic correspondant au sultopride/surface intégrée du pic correspondant à l'étalon interne varie linéairement avec la concentration comme le montrent les coefficients de corrélation obtenus par ajustement linéaire des données (méthode des moindres carrés). Ces derniers sont de: 0.998294 ± 0.001278 ($n = 10$) pour le plasma, 0.998016 ± 0.001064 ($n = 10$) pour les érythrocytes et 0.994496 ± 0.002451 ($n = 14$) pour les urines et les pentes de: 0.154 ± 0.002, 0.133 ± 0.001 et 0.057 ± 0.001, respectivement. L'ordonnée à l'origine est dans tous les cas très proche de zéro: -0.0025 ± 0.0032 pour le plasma, -0.0058 ± 0.00922 pour les érythrocytes et -0.00986 ± 0.0198 pour les urines.

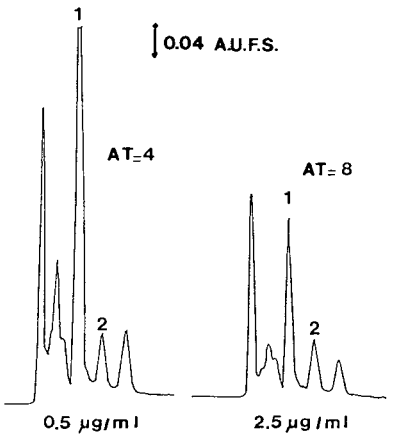

Fig. 2. Enregistrements dans l'ultraviolet de deux points d'une gamme d'étalonnage de sultopride dans le plasma. Pics: 1 = sulpiride (étalon interne) (4.4 min); 2 = sultopride (5.4 min).

Répétabilité. La répétabilité de l'injection est déterminée sur deux solutions titrant 6.25 et 25 µg/ml d'urine; chaque solution étant injectée dix fois. Les coefficients de variation sont de 0.23% et 0.785%, respectivement. La répétabilité entre gammes d'étalonnage préparées dans du plasma, des érythrocytes et de l'urine de différents sujets est calculée pour chaque concentration. Ces résultats sont rapportés Tableau I.

Coefficient d'extraction. Le coefficient d'extraction est de 98%.

Sensibilité. La limite de détection de la méthode est de 15 ng par ml de plasma.

TABLEAU I

COEFFICIENTS DE VARIATION POUR DIFFÉRENTES GAMMES D'ÉTALONNAGE EXTRAITES SELON LE PROTOCOLE ADOPTÉ

Concentration ($mg\ l^{-1}$)	Coefficients de variation (%)					
	Sulpiride			Sultopride		
	Plasma ($n = 10$)	Erythrocytes ($n = 5$)	Urine ($n = 14$)	Plasma ($n = 10$)	Erythrocytes ($n = 10$)	Urine ($n = 14$)
0.050	4.14	3.02				
0.125	3.52	7.68				
0.500				3.18	2.33	
0.750		0.592				
1.25	2.52			1.55	4.50	
2.50	2.02	1.06		0.56	1.08	
5.00	1.50	1.24		0.50	2.39	
6.25						1.98
10.00				1.31	0.57	
12.50			1.60			0.71
18.75			1.21			
25.00			1.53			2.28
50.00			1.43			1.01

DISCUSSION ET CONCLUSION

Lors de l'étude pharmacocinétique du sulpiride après administration intramusculaire de trois doses (50, 100 et 200 mg), les concentrations en sulpiride dans le plasma et l'urine étaient déterminées par HPLC avec détection dans l'UV à 197 nm. L'étalon interne utilisé était la nicotinamide et la durée de l'analyse de 14 min [2].

Les conditions chromatographiques sont modifiées de façon à réduire le temps d'analyse. L'étalon interne retenu est de la même série chimique que le sulpiride. La méthode proposée est donc mieux adaptée à la réalisation d'études pharmacocinétiques ou à des dosages de routine permettant d'assurer une bonne surveillance thérapeutique.

La sensibilité de notre méthode est identique à celle décrite par Alfredsson et al. [13] qui utilisent un système de détection en fluorescence. Par contre le temps d'analyse est considérablement raccourcit 8 min au lieu de 20 min.

Cette méthode de dosage est plus sensible que celle décrite par Frigerio et Pantarotto [11] par GC et détection par ionisation de flamme; le sulpiride étant dosé après méthylation. La limite de détection est de 3.5 ng injectés au lieu de 20 ng dans la méthode de Frigerio et Pantarotto [11].

Des échantillons de plasma, d'érythrocytes et d'urine prélevés après administration de sulpiride à l'homme par voies intraveineuse et orale ont été dosés par cette méthode. Un exemple de cinétiques plasmatique et érythrocytaire est donné Fig. 3. Le coefficient de distribution du sulpiride entre érythrocytes et plasma est voisin de 1. Les cinétiques d'absorption et d'élimination du sulpiride

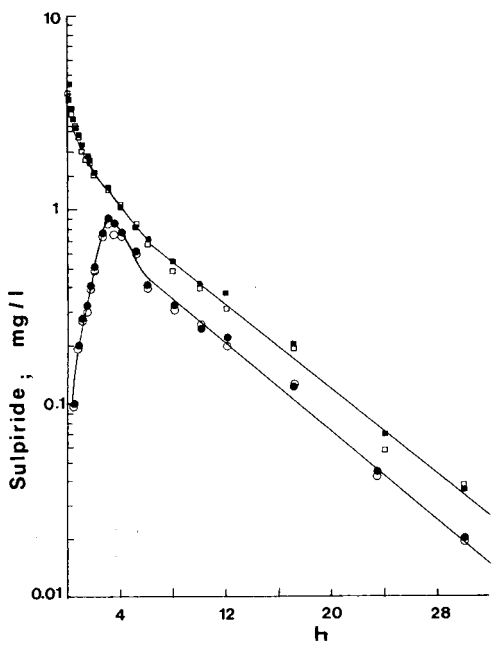

Fig. 3. Variations du logarithme de la concentration en sulpiride dans le plasma (■ et ●) et dans les érythrocytes (□ et ○), en fonction du temps, après administration de 100 mg par voie intraveineuse (■ et □) et de 200 mg par voie orale (gélules) (● et ○).

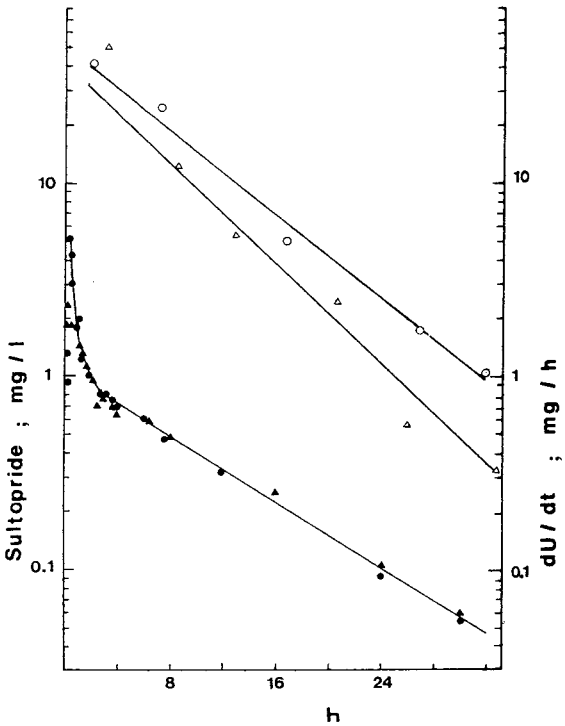

Fig. 4. Variations du logarithme de la concentration en sultopride dans le plasma (• et ▲) et de la vitesse d'élimination urinaire, dU/dt, (○ et △), en fonction du temps, après administration de 445.74 mg par voie intramusculaire (• et ○) et de 375.96 mg par voie orale (gouttes buvables) (▲ et △).

ainsi que la biodisponibilité des formes orales peuvent être évaluées aussi bien à partir des données érythrocytaires qu'à partir de l'évaluation des concentrations dans le plasma. Elles peuvent également être évaluées à partir des données urinaires.

Pour le dosage du sultopride, nous avons développé une méthode plus sensible que celles décrites dans la littérature [5, 15—19]. Cette méthode de dosage nous a permis de réaliser l'étude pharmacocinétique du sultopride chez des patients hospitalisés pour désordres psychiatriques après son administration par voies intramusculaire et orale (gouttes buvables) à la dose de 400 mg [1]. Un exemple des cinétiques obtenues est donné Fig. 4.

RESUMÉ

le dosage du sulpiride et du sultopride est effectué par chromatographie liquide à haute performance avec détection dans l'ultraviolet (226 nm). Les échantillons de plasma, d'érythrocytes et d'urine sont extraits par le chloroforme à pH 10. L'étalon interne utilisé est une nouvelle benzamide substituée, le produit N-[(éthyl-1-pyrrolidinyl-2)méthyl] méthoxy-2-amino-4-éthylsulfonyl-5-benzamide (DAN) pour le dosage du sulpiride et le sulpiride pour le dosage du sultopride. La limite de détection dans le plasma et les érythrocytes est de

10 ng/ml pour le sulpiride et 15 ng/ml pour le sultopride. Les méthodes proposées, sélectives, reproductibles et sensibles peuvent être utilisées pour la réalisation d'études pharmacocinétiques et pour la surveillance thérapeutique. Des exemples de cinétiques plasmatiques et érythrocytaires chez des sujets sains (sulpiride) ou des patients (sultopride) sont donnés. Les temps de demi-vie déterminés à partir des données plasmatiques et érythrocytaires sont identiques (7 h pour le sulpiride et 5 h pour le sultopride).

BIBLIOGRAPHIE

1 J. Bres et F. Bressolle, Thérapie, (1985) sous presse.
2 F. Bressolle, J. Bres, M.D. Blanchin et R. Gomeni, J. Pharm. Sci., 73 (1984) 1128.
3 M. Lecuyer, Sem. Hop. Paris, 26 (1984) 1872.
4 G. Pitel et T.H. Luce, Ann. Pharm. Fr., 28 (1970) 409.
5 T. Kleimola, O. Leppänen, J. Kanto, R. Mäntylä et E. Syvälahti, Ann. Clin. Res., 8 (1976) 104.
6 F. Bressolle, J. Bres et S. Brun, J. Chromatogr., 174 (1979) 421.
7 F. Bresolle, J. Bres, E. Fuseau et A.M. Siouffi, J. Pharm. Clin., 2 (1982) 141.
8 J. Segura, L. Borga et O.M. Bakke, Arch. Int. Pharmacodyn., 223 (1976) 88.
9 J. Bres, F. Bressolle, M.D. Blanchin, E. Rechencq et L. Monnier, Congrès Hispano Français de Biopharmacie et de Pharmacocinétique, Barcelone, 2—6 Avril 1979, Vol. III, Coop. COIMOFF, Madrid, 1979, pp. 25—38.
10 F. Bressole, A. Faure et J. Bres, dans J.M. Aiache et J. Hirtz (Rédacteurs), 2ème Congrès Européen de Biopharmacie et Pharmacocinétique, Salamanque, 24—27 Avril 1984, Vol. III, 1984, pp. 287—296.
11 A. Frigerio et C. Pantarotto, J. Chromatogr., 130 (1977) 361.
12 A. Mizuchi, S. Saruta, N. Kitagawa et Y. Miyachi, Arch. Int. Pharmacodyn. Ther., 254 (1981) 317.
13 G. Alfredsson, G. Sedvall et F.-A. Wiesel, J. Chromatogr., 164 (1979) 187.
14 N. Verbiese-Genard, M. Hanocq et L. Molle, J. Pharm. Belg., 35 (1980) 24.
15 O. Denisoff et L. Molle, Arzneim.-Forsch., 28 (1978) 2156.
16 M. Murasaki, S. Yamazumi, K. Okamoto, A. Takahashi et S. Miura, Clin. Eval. (Jpn.), 9 (1981) 577.
17 K. Nishihara, Y. Konda et Z. Tamura, Chem. Pharm. Bull., 31 (1983) 4144.
18 N. Verbiese-Genard, M. Hanocq et L. Molle, dans A. Frigerio et L. Renoz (Rédacteurs), Recent Developments in Chromatography and Electrophoresis, Elsevier, Amsterdam, 1979, pp. 79—86.
19 J. Bres, A.F. Gaillot, D. Monsoncles, J.C. Penochet et G. Vidal, Progress in Clinical Pharmacy, Vol. III, Elsevier/North-Holland Biomedical Press, Amsterdam, 1981.

Journal of Chromatography, 341 (1985) 401—409
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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METHOD FOR THE DETERMINATION OF 4′-DEOXYDOXORUBICIN, 4′-DEOXYDOXORUBICINOL AND THEIR 7-DEOXYAGLYCONES IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

4′-Deoxydoxorubicin (4′-DOX) is a new and structurally similar analogue of the anticancer drug adriamycin (ADR). Based on known pathways of metabolism of ADR a high-performance liquid chromatographic method for the separation and identification of 4′-DOX and five possible metabolites was developed. Sensitivity for serum is 10 ng/ml for 4′-DOX and its alcoholic product 4′-deoxydoxorubicinol (4′-DOL) and 2 ng/ml for four of its aglycone products with coefficients of variation in k' of less than 5% throughout the day. An extraction step with better than 80% recovery of 4′-DOX and five reference metabolites from serum is described. Analysis of patient sera identified two metabolite peaks. These co-eluted with the reference metabolites of 4′-DOL and the 7-deoxyaglycone of 4′-DOX. Pharmacokinetics of the parent drug followed a two-compartment model. Both the metabolites were produced quickly and disappeared quickly.

INTRODUCTION

An irreversible toxicity to the heart with a high risk of mortality limits the clinical administration of the therapeutically active anthracycline anticancer drug adriamycin (ADR) to a cumulative dose of 550 mg/m^2 [1]. Many analogues have been synthesised in the hope that one will be equally or more cytotoxic than the parent drug but without its cardiotoxicity [2, 3]. 4′-Deoxydoxorubicin (4′-DOX) is one of the most promising newly synthesised anthracyclines. Structurally it is identical to ADR differing only by the absence of an oxygen atom at position 4 on the daunosamine sugar group (Fig. 1). Like ADR it has a broad spectrum of anti-tumour activity but is thought to be more effective [4, 5]. Of all the new anthracycline analogues tested 4′-DOX appeared to be the least cardiotoxic [6]. However, its systemic toxicity is greater than

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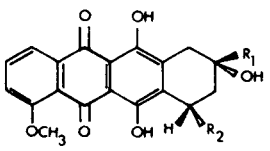


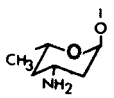
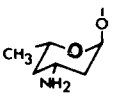
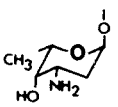
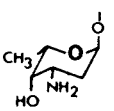
COMPOUND	R_1	R_2
4'- Deoxydoxorubicin (4'- DOX)	$-C(=O)-CH_2OH$	
4'- Deoxydoxorubicinol (4'- DOL)	$-CH(OH)-CH_2OH$	
Adriamycin (ADR)	$-C(=O)-CH_2OH$	
Adriamycinol (AOL)	$-CH(OH)-CH_2OH$	
4'- Deoxydoxorubicin aglycone (4'-DOX- one), also Adriamycin aglycone (ADR-one)	$-C(=O)-CH_2OH$	OH
4'- Deoxydoxorubicinol aglycone (4'- DOL-one), also Adriamycinol aglycone (AOL-one)	$-CH(OH)-CH_2OH$	OH
4'- Deoxydoxorubicin 7 - deoxyaglycone (4'-DOX-done), also Adriamycin 7 - deoxyaglycone (ADR-done)	$-C(=O)-CH_2OH$	H
4'- Deoxydoxorubicinol 7-deoxyaglycone (4'- DOL-done), also Adriamycinol 7 - deoxyaglycone (AOL-done)	$-CH(OH)-CH_2OH$	H

Fig. 1. The structure of 4′-deoxydoxorubicin and five of its possible metabolites plus the structure of adriamycin and its metabolites.

ADR and its administration is limited to approximately half the therapeutic dose of ADR [5, 7, 8]. Preliminary reports indicate that 4′-DOX is also non-cardiotoxic in man [9, 10]. Although, in one study a patient developed clinical signs of congestive heart failure after only three courses of 30 mg/m^2 4′-DOX [10].

Two major pathways of metabolism have been described for ADR [11—14]: reduction of the carbonyl group on the alkyl side-chain by a cytoplasmic aldo—keto reductase enzyme to yield the alcohol adriamycinol (AOL, Fig. 1) and microsomal reductive glycosidic cleavage of ADR and AOL to 7-deoxyaglycones

(Fig. 1). It is not unreasonable to expect 4′-DOX also to be metabolised in a similar fashion because of its close structural similarity to ADR.

In this paper a high-performance liquid chromatographic (HPLC) method is presented for determining 4′-DOX, 4′-deoxydoxorubicinol (4′-DOL) and their 7-deoxyaglycones in the serum of cancer patients.

MATERIALS AND METHODS

Apparatus

HPLC was performed throughout using an Altex Model 110A pump and an Altex Model 210 injection port with a 20-μl injection loop (Beckman-RIIC, High Wycombe, U.K.); a Gilson Spectro-Glo filter fluorimeter with narrow-band interference filters at 480 nm (excitation) and 560 nm (emission) and a 10-μl quartz micro flow cell (Gilson, Villiers-le-Bel, France); a Shimadzu CR-1B computing integrator (supplied by Scotlab Instrument Sales, Bellshill, U.K.) and 250 mm × 4.6 mm I.D. stainless-steel columns packed with μBondapak C_{18} (10 μm particle size, Waters, Northwich, U.K.) using a Shandon HPLC column packer (Shandon, Runcorn, U.K.). Columns were packed according to the following method: 4 g of μBondapak in a propan-2-ol slurry was packed into a column under a pressure of 480 bars in an upward direction with 100 ml propan-2-ol, the column was inverted and packing was continued in a downward direction with 50 ml propan-2-ol and finally conditioned in a downward direction with 50 ml methanol and 50 ml of the mobile phase described in this paper.

Reagents and reference compounds

All methanol, propan-2-ol and chloroform were HPLC-reagent grade (Fisons Scientific Apparatus, Loughborough, U.K.). Orthophosphoric acid and all other solvents and chemicals were of analytical reagent grade (AnalaR, BDH, Poole, U.K.). Water was deionised and double-distilled in a quartz glass still. Pure adriamycin·HCl and adriamycinol·HCl were a gift from Dr. S. Penco (Farmitalia, Milan, Italy). 4′-Deoxydoxorubicin containing 5 mg lactose per mg of drug was from Farmitalia. Daunorubicin·HCl (DNR), the internal standard, was from May and Baker (Dagenham, U.K.). 4′-DOL was synthesised from 4′-DOX by reduction with sodium borohydride in a reaction analogous to the reduction of ADR to AOL [15]. Quinone groups reduced during the reaction were regenerated by bubbling air through the reaction mixture for 2 h. Purity of the alcohol was assessed by thin-layer chromatography (TLC) using 20 × 10 cm glass plates coated with a 250-μm layer of silica gel G (Analtech uniplates, Scotlab Instrument Sales) and three different ascending solvent systems (S). S1 was chloroform—methanol—water (80:20:3); S2 chloroform—methanol—acetic acid—water (80:20:14:6) and S3 ethyl acetate—ethanol—acetic acid—water (80:10:5:5) [15]. Spots were visualised under UV light at 254 nm. The reaction mixture still contained a significant quantity of the reduced quinone compound (a yellow non-fluorescent substance) but was otherwise pure. 4′-DOL, a red substance, was separated from the reduced quinone compound and purified by preparative TLC using 20 × 20 cm glass plates coated with a 1-mm layer of silica gel G (Analtech uniplates) after running the plate in S3 for 10 cm, drying and then running in S2 for 18 cm. The alcohol was eluted from

the silica gel using propan-2-ol and spectro-photometric analysis confirmed the red compound had a UV—visible spectrum identical to 4′-DOX. Four aglycones were used as reference compounds. The aglycone group of ADR and 4′-DOX is identical (Fig. 1), consequently their aglycone metabolites would be identical as well (see Fig. 1). The aglycones of 4′-DOX (4′-DOX-one) and 4′-DOL (4′-DOL-one) and the 7-deoxyaglycones of 4′-DOX (4′-DOX-done) and 4′-DOL (4′-DOL-done) were synthesised and characterised as previously described [16].

Calibration

Integrator calibration was performed by an external standard method. Five stock solutions containing mixtures of 100 μg/ml ADR, AOL, 4′-DOX, 4′-DOL, DNR, 4′-DOX-one, 4′-DOL-one and 10 μg/ml 4′-DOX-done and 4′-DOL-done were prepared in methanol and diluted in methanol. For the calibration 20 ng of the two 7-deoxyaglycones and 100 ng of all the other reference compounds were applied on to the column. Standard solutions were stored in PTFE-lined capped bottles at −20°C and were made up fresh every two months. Detector response was linear over the range 2—2000 ng injected on column for all nine reference compounds ($r = 0.996$). The limit of detection was set at the 3:1 signal-to-noise ratio.

Chromatographic conditions

The mobile phase consisted of 5 m*M* (final concentration) orthophosphoric acid—propan-2-ol, pH 3.2 (74:26). Elution was isocratic at a flow-rate of 1.2 ml/min. Mobile phase was filtered (0.22 μm filter pore size, Waters) and sonicated at 12 μm for 15 min before use (MSE Instruments, Crawley, U.K.). The column was maintained at ambient room temperature.

Rapid extraction from serum

Blood samples were collected from patients receiving 40 mg/m^2 ADR and 30 mg/m^2 4′-DOX and were allowed to clot in plain glass tubes. Sera were then separated by centrifugation at 1000 *g* for 10 min and stored at −20°C in plain PTFE-lined screw capped tubes. The technique used to extract 4′-DOX and its metabolites was identical to that used to extract ADR and its metabolites and has already been described in detail [16]. Essentially, the method involved direct extraction from 1 or 2 ml serum with 5 vols. chloroform—propan-2-ol (2:1) for 30 min with vortexing (100 ng DNR was added as an internal standard). After centrifugation at 2000 *g* for 15 min at 4°C, the upper aqueous phase was discarded and the lower organic phase transferred to a clean tube and evaporated to dryness at 40°C. The extract was redissolved in either 40, 50 or 100 μl of methanol and 20 μl was applied on to the HPLC column.

RESULTS

High-performance liquid chromatography

In Fig. 2 is an example of the separation of 4′-DOX and its reference metabolites (Fig. 2A) and ADR and its metabolites (Fig. 2B). Retention times (t_R) and capacity factors (k') are shown in Table I. In control experiments over an 8-hr period at 27 ± 0.5°C the coefficient of variation (C.V.) in k' was less than

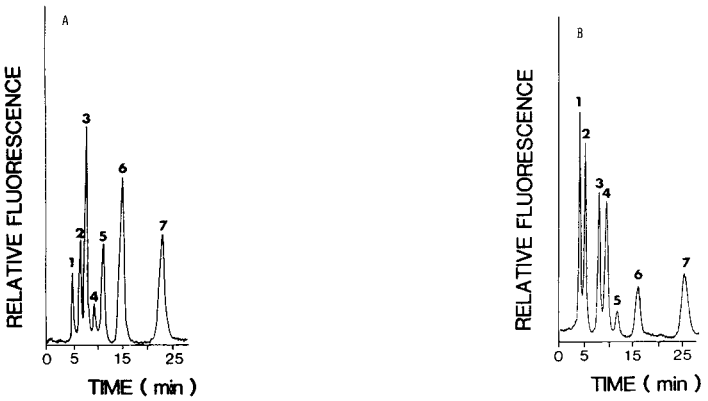


Fig. 2. Chromatographic separation of reference compound mixtures. (A) 4′-DOX and five of its possible metabolites. Peaks are identified as: 1 = 4′-DOL; 2 = 4′-DOX; 3 = 4′-DOL-one; 4 = DNR; 5 = 4′-DOX-one; 6 = 4′-DOL-done; 7 = 4′-DOX-done. (B) ADR and metabolites. Peaks are identified as: 1 = AOL; 2 = ADR; 3 = AOL-one; 4 = DNR; 5 = ADR-one; 6 = AOL-done; 7 = ADR-done. Chromatographic conditions: mobile phase was 5 m*M* ortho-phosphoric acid—propan-2-ol, pH 3.2 (74:26) and the stationary phase was μBondapak C_{18} (250 mm × 4.6 mm I.D.). Elution was isocratic at a flow-rate of 1.2 ml/min and detection was by fluorescence at 480 nm (excitation) and 560 nm (emission). Chemical structures of all reference compounds are to be found in Fig. 1.

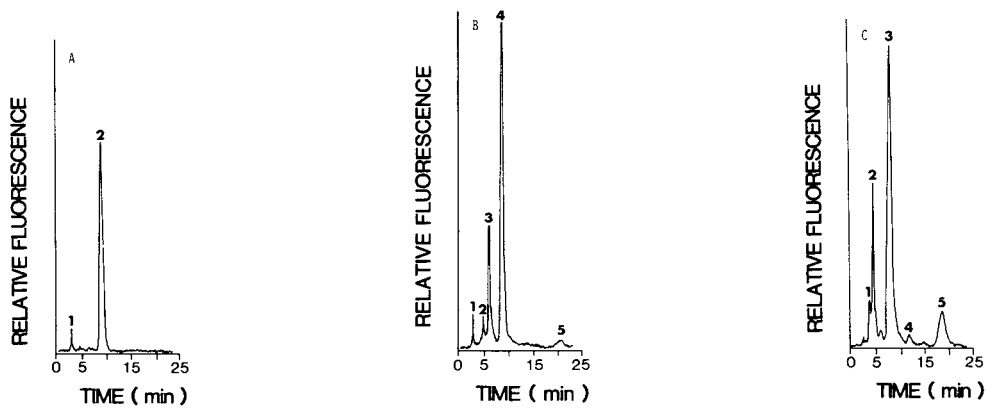


Fig. 3. Chromatographic analysis of serum from cancer patients. (A) Serum extract of blood taken prior to drug administration. Peaks are identified as: 1 = serum peaks; 2 = DNR (50 ng/ml). (B) Serum extract of blood taken 20 min after administration of 30 mg/m^2 4′-DOX as an i.v. bolus injection. Peaks are identified as: 1 = serum peaks; 2 = 4′-DOL (6.7 ng/ml); 3 = 4′-DOX (173 ng/ml); 4 = DNR; 5 = 4′-DOX-done (3.4 ng/ml). (C) Serum extract of blood taken 20 min after administration of 40 mg/m^2 ADR as an i.v. bolus injection. Peaks are identified as 1 = AOL (31 ng/ml); 2 = ADR (138 ng/ml); 3 = DNR; 4 = AOL-done (8 ng/ml); 5 = ADR-done (49 ng/ml). Chromatographic conditions as in Fig. 2 except for C where the flow-rate was 1.3 ml/min.

5% for all of the nine reference compounds (for example, C.V. for 4′-DOX was 2.0%, for 4′-DOL 2.1% and 4′-DOX-done 2.7%). The limit of detection as an amount injected on to the column was 5 ng for 4′-DOX and 4′-DOL and 1 ng for the four 4′-DOX aglycones. Limit of detection in serum after extraction of 1 ml was 10 ng/ml for 4′-DOX and 4′-DOL and 2 ng/ml for the four 4′-DOX

TABLE I

SEPARATION OF 4′-DEOXYDOXORUBICIN AND ITS REFERENCE METABOLITES BY ISOCRATIC REVERSED-PHASE HPLC USING μBONDAPAK C_{18}

Compound*	t_R(min)	k'	Compound*	t_R(min)	k'
4′-DOX	5.9	1.4	ADR	5.4	1.2
4′-DOL	4.8	0.9	AOL	3.9	0.6
4′-DOL-one	7.4	2.0	DNR	8.7	2.5
4′-DOX-one	10.6	3.2			
4′-DOL-done	13.8	4.5			
4′-DOX-done	21.1	7.4			

*Chemical structures to be found in Fig. 1.

aglycones. Extraction from serum did not introduce peaks which interfered with the identification of 4′-DOX and its reference metabolites (Fig. 3A).

Serum extraction

Extraction efficiency from blood bank serum of 100 ng 4′-DOX was 81.4 ± 2.6% S.D. (n = 10) and 100 ng of 4′-DOL was 80.6 ± 4.3% S.D. (n = 10). The rapid extraction technique has already been shown to recover the four 4′-DOX aglycones from serum with efficiency greater than 80% [16]. Over a concentration range of 20 ng/ml to 2 μg/ml (10—1000 ng injected on to the column) extraction efficiency of 4′-DOX was linear with r = 0.941 [actual concentration (y) = 1.3 × extracted concentration (x) − 4.7].

Analysis of patient serum

In Fig. 3B is a chromatogram of a serum extract of blood taken from a patient 20 min after receiving 30 mg/m^2 4′-DOX as an intravenous (i.v.) bolus injection. Peaks were identified which corresponded to 4′-DOX (173 ng/ml),

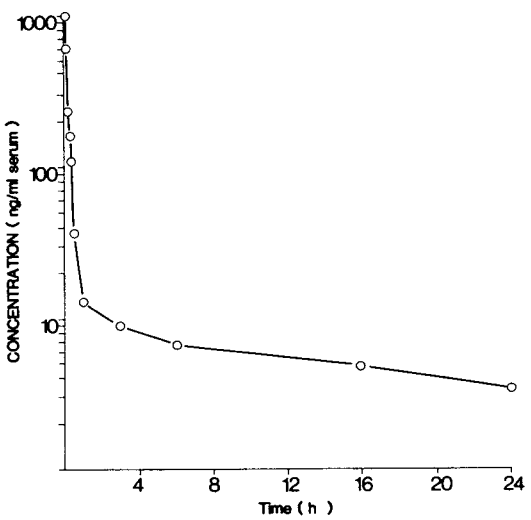


Fig. 4. Serum profile of 4′-deoxydoxorubicin in a patient after administration of 30 mg/m^2 as an i.v. bolus injection.

4′-DOL (6.7 ng/ml) and 4′-DOX-done (3.4 ng/ml). A chromatogram of a serum extract of blood taken from a patient 20 min after receiving 40 mg/m^2 ADR as an i.v. bolus injection is shown in Fig. 3C. Here, peaks corresponded to ADR (138 ng/ml), AOL (31 ng/ml), AOL-done (8 ng/ml) and ADR-done (49 ng/ml).

A patient serum profile of 4′-DOX is shown in Fig. 4. Pharmacokinetic parameters were calculated from an extended least-squares computer fit to the experimental data points contained in Fig. 4. The rate of disappearance of 4′-DOX from the serum fitted a bi-exponential decay indicative of a two-compartment open pharmacokinetic model where: serum concentration $c = 1120e^{-6.9t} + 8.4e^{-0.04t}$. Serum concentrations of 4′-DOX fell quickly immediately after its administration ($t_{1/2\alpha}$ 6 min) to a level of 12 ng/ml by 1 h. From 4 to 16 h it was necessary to extract from 2 ml serum and at 24 h 2 ml twice and combine extracts because the concentration of 4′-DOX in 1 ml of serum was less than the limit of detection of the HPLC assay (10 ng/ml). The $t_{1/2\beta}$ was calculated to be 18.8 h, $AUC_{0-\infty}$ (area under the curve) 387 ng/ml h and clearance 77.5 l/h/m^2. The serum profiles of 4′-DOL and 4′-DOX-done are to be found in Fig. 5. Both metabolites were present in serum at their peak concentrations after only 3 min and were no longer detectable by 30 min. The AUC of 4′-DOL was 12 ng/ml h and it accounted for 2.9% of the total concentration of serum 4′-DOX. The AUC of 4′-DOX-done was 2.3 ng/ml h and it accounted for only 0.6% of the total concentration of serum 4′-DOX.

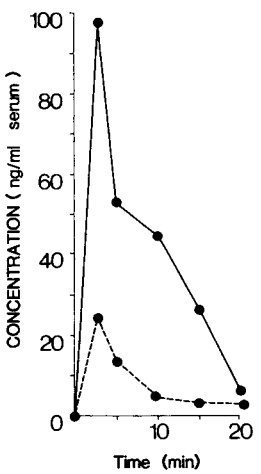


Fig. 5. Serum profiles of 4′-DOL (——) and 4′-DOX-done (— — —) in a patient after administration of 30 mg/m^2 4′-DOX as an i.v. bolus injection.

DISCUSSION

Preliminary reports of human metabolism of 4′-DOX suggest that it is converted to either 4′-DOL alone [17] or to 4′-DOL and the aglycone 4′-DOX-one [18]. The HPLC method described in this paper has been designed to clearly separate and identify the aglycone metabolites of 4′-DOX as well as the parent drug and 4′-DOL. In the patient studied the 7-deoxyaglycone of 4′-DOX (4′-DOX-done) was detected along with 4′-DOL. The behaviour of these two

metabolites was rather unusual: both were at their maximum serum concentration after only 3 min. To determine whether or not these metabolites could be chemical artefacts, purity and stability studies of 4′-DOX were carried out. These showed that 4′-DOX was 99.9% spectrophotometrically pure and that it did not degrade into aglycone or 4′-DOL artefacts but into non-fluorescent components. Presently, the metabolism of 4′-DOX is being investigated in our laboratory in a larger group of patients.

In vitro studies have shown that the 7-deoxyaglycone metabolites of ADR and AOL are by-products of semi-quinone free radicals [19, 20] whose generation by the heart has been implicated in causing ADR-induced cardiotoxicity [21, 22]. 7-Deoxyaglycone metabolites of ADR have been detected in cancer patients [15, 16] and their appearance has been cited as evidence that ADR free radical formation occurs in vivo in man [19]. As early as 1971 it was suggested that high aglycone metabolite levels were associated with ADR cardiotoxicity in animals [23, 24]. 5-Iminodaunorubicin, an analogue of ADR that does not cause appreciable cardiotoxicity in animals, is not metabolised to a 7-deoxyaglycone in vivo and is also not converted into free radicals in vitro [25]. A new, simple HPLC method which can detect 4′-DOX, 4′-DOL and low levels of the potentially important 7-deoxyaglycone metabolites will aid in the clinical evaluation of this useful new anthracycline.

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REFERENCES

1 D.M. Løesch, Cancer Topics, 4 (1982) 18.
2 A. Di Marco, A.M. Casazza, T. Dasdia, A. Necco, G. Pratesi, P. Rivolta, A. Velcich, A. Zaccara and F. Zunino, Chem. Biol. Interact., 19 (1977) 291.
3 A. Bargiotti, A.M. Casazza, G. Cassinelli, A. Di Marco, S. Penco, G. Pratesi, R. Supino, A. Zaccara, F. Zunino and F. Arcamone, Cancer Chemother. Pharmacol., 10 (1983) 84.
4 S.E. Salmon, R.M. Liu and A.M. Casazza, Cancer Chemother. Pharmacol., 6 (1981) 103.
5 A.M. Casazza, G. Savi, G. Pratesi and A. Di Marco, Eur. J. Cancer Clin. Oncol., 19 (1983) 411.
6 F. Formelli and A.M. Casazza, Drugs Exp. Clin. Res., 10 (1984) 75.
7 F. Formelli, C. Pollini, A.M. Casazza, A. Di Marco and A. Marniani, Cancer Chemother. Pharmacol., 5 (1981) 139.
8 F. Formelli and A.M. Casazza, Curr. Chemother. Immunother., 2 (1982) 1451.
9 H.S. Garewal, A. Robertone and S.E. Salmon, Proc. Amer. Soc. Clin. Oncol., 3 (1984) 41.
10 E.E. Holdener, W. ten Bokkel Huinink, H.H. Hansen, U. Bruntsch, M. von Glabbeke, H.J. Senn, H.M. Pinedo and M. Rozencweig, Proc. Amer. Soc. Clin. Oncol., 3 (1984) 139.
11 M.A. Asbell, E. Schwartzbach, F.J. Bullock and D.W. Yesair, J. Pharmacol. Exp. Ther., 182 (1972) 63.
12 N.R. Bachur, R.C. Hildenbrand and R.S. Jaenke, J. Pharmacol. Exp. Ther., 191 (1974) 331.
13 W. Bolanowska and T. Gessner, Xenobiotica, 12 (1982) 125.
14 H. Loveless, E. Arena, R.L. Felstead and N.R. Bachur, Cancer Res., 38 (1978) 593.

15 S. Takanashi and N.R. Bachur, Drug Metab. Dispos., 4 (1976) 79.
16 J. Cummings, J.F.B. Stuart and K.C. Calman, J. Chromatogr., 311 (1984) 125.
17 P. Dodion, T.A. Davis, M. Rozencweig, M. Watthieu, N. Crespeigne, M. Beer, and N.R. Bachur, Proc. Amer. Soc. Clin. Oncol., 3 (1984) 26.
18 J.G. McVie, F.J. Varossieau, H. Weenan, M.M. de Planque and W. ten Bokkel Huinink, Proc. Amer. Soc. Clin. Oncol., 3 (1984) 26.
19 N.R. Bachur, S.L. Gordon, M.V. Gee and H. Kon, Proc. Nat. Acad. Sci. U.S., 76 (1979) 954.
20 P.L. Gutierrez, M.V. Gee and N.R. Bachur, Arch. Biochem. Biophys., 223 (1983) 68.
21 I.B. Afanas'ev, N.I. Polozova and G.I. Samokhvalov, Bioorg. Chem., 9 (1980) 434.
22 M.E. Scheulen, H. Kappus, A. Nienhaus and C.G. Schmidt, J. Cancer Res. Clin. Oncol., 103 (1982) 39.
23 E. Herman, R.M. Mhatre, I.P. Lee, J. Vick and V.S. Waravdeker, Pharmacology, 6 (1971) 230.
24 R.M. Mhatre, E.H. Herman, V.S. Waravdekar and I.P. Lee, Biochem. Med., 6 (1972) 445.
25 J.H. Peters, G.R. Gordon, D. Kashiwase and E.M. Acton, Cancer Res., 44 (1984) 1453.

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SEMI-AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CYCLOSPORINE A IN WHOLE BLOOD USING ONE-STEP SAMPLE PURIFICATION AND COLUMN-SWITCHING

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SUMMARY

A highly sensitive and semi-automated high-performance liquid chromatographic method, utilizing acetonitrile protein precipitation and column-switching, is described for the determination of cyclosporine A in whole blood. Following a rapid manual acetonitrile treatment of the blood samples, the supernatant is loaded automatically onto a 5-μm high-speed protein separation column without any further clean-up operations. The fraction containing cyclosporine A is switched to a 3-μm C_{18} reversed-phase high-speed column by a microprocessor-controlled column-switching unit for final separation and detection by absorption at 214 nm.

Minimal sample handling and efficient separation resulted in a high recovery (75 ± 3%) of cyclosporine A from blood and a detection limit as low as 2 μg/l with a highly reproducible and linear response up to 2500 μg/l using 0.5 ml of sample. A separation cycle including regeneration of the first column is finished in 15 min, and this system was used continuously for ca. 1000 blood samples from heart, liver, kidney, pancreas and bone marrow recipients without change in separation parameters or material replacement.

The method described allows accurate and very fast daily routine monitoring of cyclosporine A in large numbers of blood samples from transplant recipients.

INTRODUCTION

Cyclosporine A (CyA, OL 27-400, Sandimmune®) is a hydrophobic cyclic undecapeptide of fungal origin [1], which is being used increasingly for immunosuppression in patients undergoing organ transplantation [2]. This compound is effective in preventing rejection of heart—lung, liver, kidney,

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pancreas and bone marrow grafts by interfering selectively with T-lymphocyte activation and proliferation [3].

Concentrations of CyA in blood that exceed therapeutic levels are associated with adverse effects, such as nephrotoxicity, hepatotoxicity, hirsutism and tremor [4, 5]. Since patients show large individual variations in CyA metabolism and absorption rates after oral application, in addition to drug interactions, methods should be available for the frequent and rapid estimation of the drug to provide adequate immunosuppression without a significant incidence of toxicity [6].

Since cyclosporine A is taken up by erythrocytes, and its distribution in blood depends on temperature, drug concentration, haematocrit and lipoprotein content, the drug should be measured in whole blood [7]. This may be done by a radioimmunoassay (RIA) method [8], but the one that was developed for CyA shows a cross-reactivity to certain metabolites of CyA [9]. The proportions of the unchanged drug and its metabolites seem to be affected by graft function in liver graft recipients and may also be altered by drug interactions.

Several high-performance liquid chromatographic (HPLC) methods have therefore been developed to measure native CyA in blood or plasma samples. A semi-automated method that excludes labour-intensive sample extraction and purification procedures has been published recently, but needs still an additional sample hexane wash system [10] for sample purification.

The aim of the present study was the development of a highly sensitive and reproducible HPLC analysis system for CyA measurement in blood samples which would involve minimal technical equipment with one-step sample purification omitting any further clean-up of the protein precipitation supernatant.

MATERIALS AND METHODS

Reagents

Cyclosporines A, D (Val-2-cyclosporine) and dihydro-C (Thr-2-cyclosporine) [11] were a gift from Biochemie (Vienna, Austria).

Water was purified with a Milli-Q system (Millipore), and fractions with a specific resistance higher than 18 MΩ/cm were used for HPLC analysis and sample preparation.

Acetonitrile (Art. 30, chromatography grade), methanol (Art. 6007) and trifluoroacetic acid (Art. 8262) were obtained from Merck (Darmstadt, F.R.G.).

Heparinized or EDTA blood for preparation of standards was drawn from healthy volunteers. For stock solutions, the cyclosporines were dissolved in methanol (2.5—250 μg/ml) and stored at 4°C.

Mobile phases

A and B were acetonitrile and water (purified as described above), respectively, and C was acetonitrile—water (71:29). Trifluoroacetic acid was added to mobile phases B (160 μl/l) and C (50 μl/l), and a continuous stream of helium was allowed to pass through the mobile phases.

Sample preparation

A 500-μl volume of whole blood (plasma or bile may also be used) was pipetted into a 15-ml glass test-tube, and cell lysis/protein precipitation was initiated by the addition of 890 μl of acetonitrile (≡ 68% acetonitrile). The stoppered tube was vortexed for 30 sec (Vortex Genie® mixer), incubated for 10 min at room temperature and then centrifuged at 1200 *g* for 10 min. The 1000 μl of the supernatant were transferred to a fresh tube and mixed with 615 μl of water (42% aqueous acetonitrile). This purified sample was filled into 2-ml glass automatic sampler vials (Model 125, Spark Holland), which were each capped with a septum. CyA blood standards were prepared the same way, except that the blood was incubated for 30 min at 37°C with different amounts of CyA stock solutions (less than 5% methanol) prior to treatment with acetonitrile.

Chromatography

A diagram of the HPLC system is shown in Fig. 1A and B. Sample volumes of 1000 μl are loaded automatically (Model 125, Spark Holland automatic sampler) onto an Ultrapore® RPSC protein separation column (5 μm mean particle diameter, 75 x 4.6 mm I.D.; Altex Berkeley, CA, U.S.A.) kept at 333°K by a thermostatted waterjacket (Braun, Thermomix). The autosampler

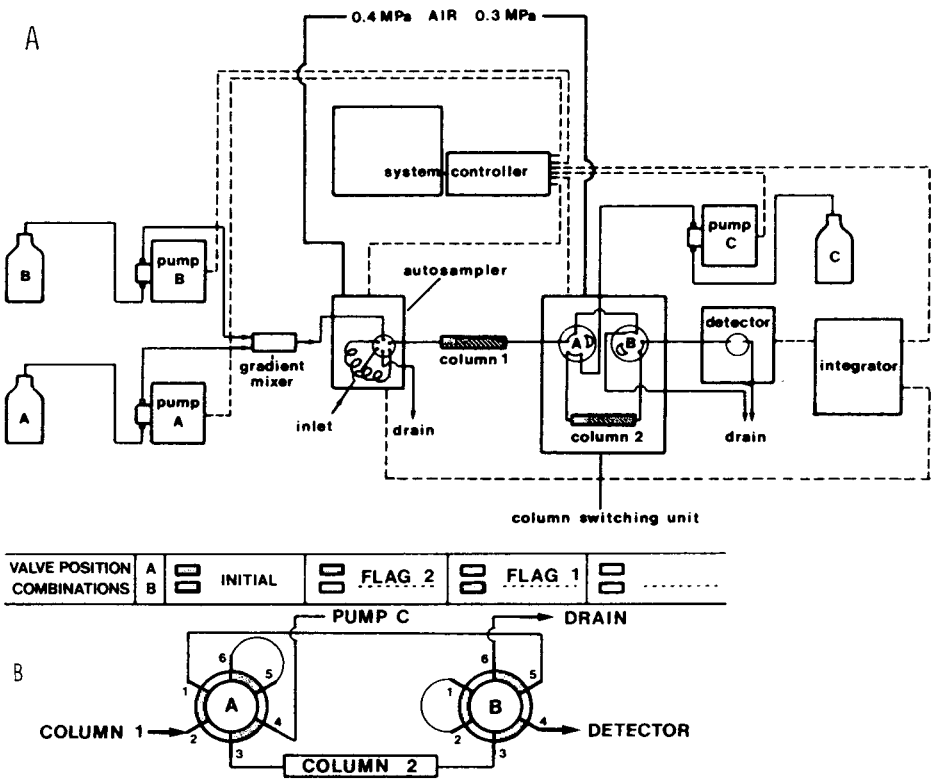

Fig. 1. (A) Scheme of the chromatography system; dashed lines represent microprocessor or autosampler control connections. (B) Scheme of the column switching device.

is started by a Beckman Model 421 CRT HPLC system controller, and itself activates data processing by a Chromatopac CR2AX Integrator (Shimadzu, Kyoto, Japan). The mobile phase consists of 42.4% B and 57.6% A, delivered at a flow-rate of 1 ml/min by a Model 110A and a Model 112 pump (Beckman), respectively (back-pressure ca. 4 MPa), and mixed in a gradient system (Beckman). At time 5.90 min after injection initiation ($t = 4.0$ min) flag 1 (column-switching unit, ventil 1) is activated for 1.80 min, and the fraction containing CyA is diverted automatically onto an Ultrasphere® ODS column (3 μm mean particle diameter, 75 x 4.6 mm I.D.; Altex), which was also kept at 333°K. The column-switching unit was Model 9210 (Spark Holland); its connections and valve positions are shown in Fig. 1A. CyA is eluted from this column with mobile phase C at a flow-rate of 1 ml/min using pump C (Model 112, Beckman). Cyclosporine is detected by its UV absorption at 214 nm using a fixed-wavelength detector (Model 160 absorbance detector, Beckman). Peak height, peak area calculation and baseline drawing are performed by the Chromatopac CR2AX. Concentrations of CyA in blood are calculated by relating peak area to standard curve area measurements run intermittent to unknown samples. In parallel with CyA detection on column II, the Ultrapore RPSC column is regenerated by washing with 90% mobile phase A. (After 11.0 min, the percentage of B is changed to 5% in 1 min, and after 13.0 min it is brought to the initial percentage of 42.4% in 1 min.) Under these conditions, column I can be used for at least 1000 sample determinations without significant increase in back-pressure and without changes in separation parameters, provided each sample is carefully freed from precipitated material as described above. Since a minimum of contaminating material is loaded onto column II in addition to CyA, no increase in back-pressure could be detected for ca. 1000 samples. The cycle finishes at time 3.30 min with activation of flag 4 (integrator stop) and starts again at 4.00 min with automatic injection of the next sample.

RESULTS

Sample treatment

Whole blood was drawn into heparinized or EDTA-coated tubes and prepared for CyA analysis. Coated tubes should be used since the method is sensitive enough to show sample dilution by addition of heparin solutions for inhibition of clot formation. Recovery of CyA from whole blood after acetonitrile treatment was 75 ± 3% for all CyA concentrations tested. Lysis of erythrocytes by addition of Zaponin® (Coulter Electronics, Luton, U.K.) prior to acetonitrile treatment showed no influence on cyclosporine recovery (data not shown). (The cyclosporine fraction that was not recovered seems to be occluded in the protein precipitate.)

Chromatograms

Separation of CyA from most of the contaminating sample components by protein separation column I could be monitored by activating flag 1 to divert the effluent directly to the UV detector. A typical result, using a whole blood standard containing 1000 ng/ml CyA is shown in Fig. 2A. Such determinations

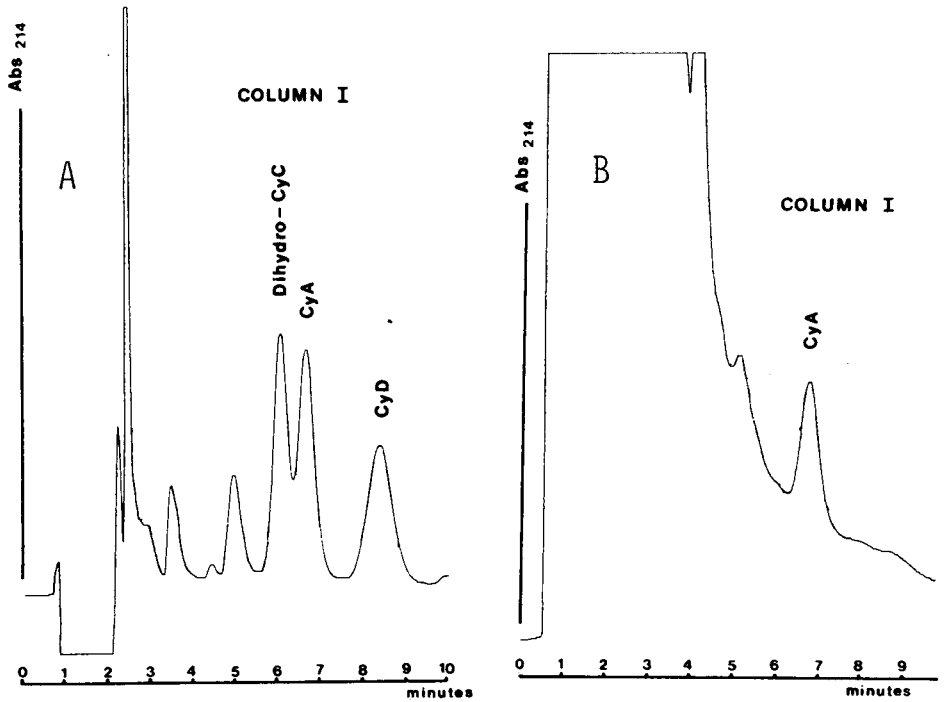

Fig. 2. (A) Separation of a mixture of three cyclosporines (ca. 1000 μg/l of each cyclosporine in methanolic solution) with column I. (B) Separation of a blood standard sample containing 1000 μg/l CyA with column I.

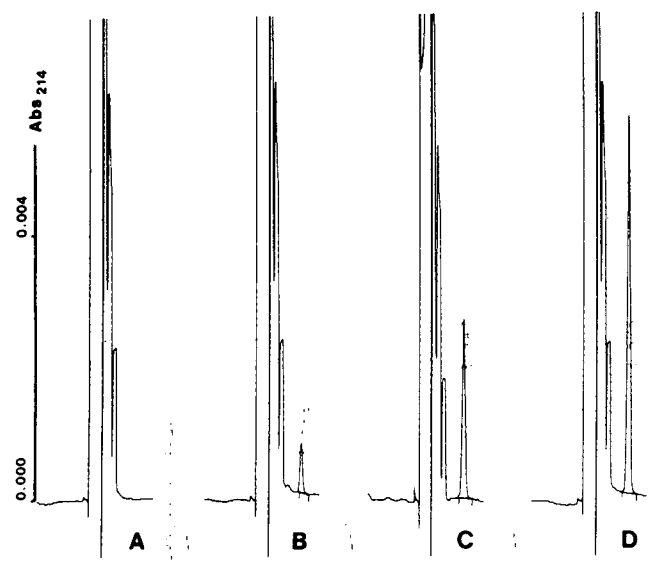

Fig. 3. Representative chromatograms obtained in the analysis of 0.5 ml of (A) blank blood, (B) a blood standard containing 50 μg/l CyA, (C) a blood sample from a kidney graft recipient containing 257 μg/l CyA and (D) a blood standard containing 500 μg/l CyA. Baseline, starting and end points of the peaks are written during data processing by the integrator.

were used to check the correct setting of the switching parameters (start of switching and duration). Reversal of acetonitrile concentrations in mobile phase (57.6%) and sample solute (42%), and the high separation capability of the RPSC 5 μm column I (Fig. 2B), result in a small CyA-containing fraction. Addition of trifluoroacetic acid to mobile phase B and washing with 95% acetonitrile while eluting CyA from column II allow continuous use of column I without further maintenance.

Typical chromatograms of the switched CyA fractions eluted from column II are shown in Figs. 3A—D. The large offset prior to CyA elution is caused by the injection of mobile phases A and B during automatic column switching. No interfering peaks were detected in the blood samples from healthy volunteers, in a number of patients not receiving CyA (pancreatitis, burns) and in graft recipients who showed CyA metabolites as assessed by RIA determination but had no unmetabolized native drug. No internal standard has been used. CyA concentrations in samples were calculated by comparing peak areas of samples with those of standards run daily.

Linearity

Cyclosporine blood standards were run daily, and these determinations gave a linear concentration—response relationship. Cyclosporine concentrations of 0, 10, 20, 100, 250, 500, 1000 and 1500 μg/l in blood were used for calibration, and from the data a mean slope of 0.2851 mV sec l μg^{-1} and a mean y-intercept value of 0.36 μV sec were calculated for the linear calibration curve by regression analysis. Linearity is achieved in the range 10—1500 μg/l as shown by a mean correlation coefficient of 0.998 ($n = 22$; days 1, 8 and 15).

Precision and reproducibility

The precision of the method was evaluated by analysing blood standard samples ranging from 10 to 1500 μg/l CyA. A set of standards was used to establish a calibration curve and then standards containing 10—1500 μg/l CyA were analysed at days 1, 8 and 15. Within-day variability is very low, the coefficient of variation ranging from 0.5% to 1.75% ($n = 12$; triplicate measurements of CyA standards with 20, 250, 500 and 1000 μg/l CyA), and results shown in Table I demonstrate the high reproducibility (low day-to-day

TABLE I

ANALYSIS OF BLOOD SAMPLES SPIKED WITH CYCLOSPORINE A

Calculated from duplicate measurements of each CyA blood standard at days 1, 8 and 15.

Spiked concentration (μg/l)	Measured concentration (mean ± S.D., μg/l)	Relative error (%)
10	9.38 ± 0.14	6.2
20	18.9 ± 0.36	5.7
100	95.9 ± 1.93	4.8
250	251 ± 0.18	0.1
500	496 ± 6.35	0.8
1000	1019 ± 24.7	1.9
1500	1490 ± 6.41	0.67

variation) of the method (seven CyA blood standards measured at days 1, 8 and 15 in duplicate).

Sensitivity

The sensitivity of the method was investigated by analysing blood samples spiked with 10 or 20 μg/l cyclosporine. These concentrations could be measured with high precision and accuracy (Tables I and II). The relative error is less than 7% for blood samples to which cyclosporine has been added in a concentration of 10 μg/l, and the mean response factor is the same. The mean absolute response was 3140 ± 64 μV sec for 10 μg/l CyA and peak areas as low as 600 μV sec, which corresponds to 2 μg/l CyA, could be clearly dissolved and integrated. Pure and helium-degassed mobile phases were required to achieve a low detection limit.

TABLE II

SENSITIVITY OF DETECTION OF CYCLOSPORINE A IN BLOOD

CyA concentration (μg/l)	Response factor (× 10) ($\mu g\ sec^{-1}\ l^{-1}\ mV^{-1}$)	Coefficient of variation (%)
10	31.82	2.5
20	34.60	3.4
100	35.70	2.1
250	34.83	0.3
500	34.95	1.9
1000	35.06	0.4

Application of the method

The method has been used for analysis of more than 1000 blood samples from heart, liver, pancreas, bone marrow and kidney graft recipients. CyA trough levels were monitored daily and compared with concentrations measured by the RIA method. The ratio between HPLC and RIA concentrations may vary from 1.1 up to 10.1 for CyA in kidney and liver graft

TABLE III

CONCENTRATION OF CYCLOSPORINE A IN BLOOD FROM A PATIENT

The patient, who had a liver graft, received a single (160 mg CyA = 2.3 mg/kg) or multiple oral doses (4 × 50 mg CyA).

Time (h)	CyA concentration (μg/l)	
	Single dose	Multiple doses
1	570	280
4	1695	630
6	1140	555
8	928	1060
10	642	635
12	347	380

recipients [12]. Table III shows the results of CyA determinations after a single oral dose (time = 0 h) or reduced multiple oral doses (given at 0.5 and 10 h), which resulted in lower CyA peak values.

The HPLC method is extremely useful for rapid determination of CyA in critical clinical situations (signs of acute nephro- or hepatoxicity). A sample analysis can be performed within 1 h and is specific for the unchanged drug, which seems to be the main toxic component [13].

DISCUSSION

Several HPLC methods for the determination of CyA in blood have been published (for a review see ref. 10), but they need relatively expensive and complicated equipment and daily maintenance of the separation columns. The new method presented here provides fully automated sample analysis after only manual sample purification performed in a single step. A large number of blood samples from graft recipicients can be loaded onto the autosampler after manual precipitation of proteins. A sample cycle is finished in 15 min, and this CyA determination system could be run continuously overnight. In most cases, perioperative monitoring of CyA in our laboratory could be finished the same day for all patients, and dosages could be adjusted the same evening for those receiving the drug twice daily (ca. hundred kidney and ten liver transplantations per year).

Acetonitrile treatment of the blood is efficient in precipitating most of the contaminating material and in extracting CyA. For the new method described here, a hexane wash to remove lipophilic material [16], which is an essential step in any other HPLC method reported so far, is not necessary. Sample purification is reduced to one simple precipitation step. Therefore no other HPLC grade chemicals, as used in manual extraction methods (diethyl ether, methanol, hexane) [14, 15] or automated sample wash (hexane) [10], are necessary. High-speed micropore columns (5 μm and 3 μm, respectively) with small dimensions allow for low flow-rates and minimal consumption of reagents, and lead to high reproducibility and increased sensitivity. Minimal sample handling and acetonitrile extraction resulted in high and reproducible recovery of CyA from blood and made possible external standardization without loss of accuracy. Concentrations as low as 10 μg/l CyA in blood could be measured with confidence. This makes the method suitable for routine monitoring of graft recipients who receive maintenance doses of CyA and show high metabolization rates.

By changing the separation conditions of column I, the method may be adapted for determination of CyA metabolites.

In conclusion, the new method reported here employs (a) a simple and short one-step sample purification, (b) sample separation on micropore columns (5 μm and 3 μm, respectively) which could be easily monitored for both columns used, (c) column switching, (d) fast analysis and sensitive detection in 15 min providing rapid sample throughput, (e) minimal technical equipment and minimal routine care for the HPLC system. The instrumentation consists of a standard gradient HPLC system with one additional pump for regenerating column I, and a column-switching device. The method is suitable for meeting

the growing demand for rapid and sensitive monitoring of unchanged CyA in blood samples from graft recipients with a minimum of sample handling and of technical equipment.

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REFERENCES

1 A. Ruegger, M. Kuhn, H. Lichti, H.R. Loosli, R. Huegenin, C. Quiquerez and A. von Watburg, Helv. Chim. Acta, 59 (1976) 1075.
2 R.Y. Calne, Brit. J. Surg., 67 (1980) 765.
3 C. Weil, Med. Res. Rev., 4 (1984) 221.
4 R.Y. Calne, D.J.G. White, S. Thirn, D.B. Evans, P. McMaster, D.C. Dunn, G.N. Craddock, B.D. Pentlow and K. Rolles, Lancet, ii (1978) 1323.
5 A. Laupacis, Transplant. Proc., 15 (1983) 2748.
6 D.J.G. White, D. McNaughton and R.Y. Calne, Transplant. Proc., 15 (1983) 454.
7 W. Niederberger, M. Lemaire, G. Maurer, K. Nussbaumer and O. Wagner, Transplant. Proc., 15 (1983) 2419.
8 P. Donatsch, E. Abisch, M. Homburger, R. Traber, M. Trapp and R. Voges, J. Immunoassay, 2 (1981) 19.
9 S.G. Carruthers, D.J. Freeman, J.C. Koegler, W. Howson, P.A. Keown, A. Laupacis and C.R. Stiller, Clin. Chem., 29 (1983) 180.
10 H.T. Smith and W.T. Robinson, J. Chromatogr., 305 (1984) 353.
11 R. Wenger, Transplant. Proc., 15 (1983) 2230.
12 W. Woloszcuk, J. Schindler, G. Hamilton and E. Roth, Lancet, ii (1984) 638.
13 C. Cunningham, P.H. Whiting, M.D. Burke, D.N. Wheatley and J.G. Simpson, Transplant. Proc., 15 (1983) 2712.
14 M.C. Allwood and R. Lawrance, J. Clin. Hosp. Pharmacy, 6 (1981) 195.
15 R.E. Kates and R. Latini, J. Chromatogr., 309 (1984) 441.
16 R.J. Sawchuk and L.L. Cartier, Clin. Chem., 27 (1981) 1368.

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Note

Confidence limits for a gel-permeation system

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A semi-automatic gel-permeation system designed for the study of human glomerular filtration of dextrans has recently been described by Cullen et al. [1]. These authors used two columns of TSK chromatography media (Toyo Soda, Tokyo, Japan) in series under pressure for the separation of discrete dextran fractions. Calibration of the assembly was effected with the use of no less than seventeen narrow-cut dextran fractions, each of which is closely characterised in terms of weight-average molecular weight (MW). For each fraction, a mean elution volume (V_e) was found from generous (at least ten-fold) replications over a range of column loads. Thus an almost unique collection of data was available for the statistical study of probable errors. Dextrose and dextran of weight-average MW $> 2 \cdot 10^6$ daltons were used as end-markers and in the first instance, the mean elution volumes of these were taken as the total separation volume (V_t) and the void volume (V_0), respectively, in evaluating partition coefficients $K_{av} = (V_e - V_0)/(V_t - V_0)$.

We found the relationship between $-\ln K_{av}$ (ordinates) and m ($= MW^{2/3}$) was sigmoidal in nature, with slopes increasing sharply as m declined towards zero or increased finally at the upper end of its range. Over part of the elution range, however, there was an indication of linearity.

EXPERIMENTAL

Data used in the present study are shown in Table I and were calculated from the original observations of Cullen et al. [1]. In an attempt to realize a closer approach to the classical linear form (Porath [2]; Hjertén [3]) a series of triads was used to derive values for V_0 and for $V_t - V_0$ as described earlier by Baker [4]. A triad is here defined as any group of three points (Table I), each

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TABLE I

ELUTION DATA

Point No.	Substance	Mean MW	No. of replicates	V_e (ml, mean ± S.E.M.)
1	Dextrose	180	19	38.56 ± 0.10
2	Dextran fraction	1170	10	35.13 ± 0.16
3	Dextran fraction	2500	10	33.95 ± 0.12
4	Dextran fraction	4100	11	33.37 ± 0.14
5	Dextran fraction	4500	10	32.75 ± 0.16
6	Dextran fraction	5250	12	32.66 ± 0.11
7	Dextran fraction	7900	10	31.57 ± 0.10
8	Dextran fraction	8825	12	31.67 ± 0.10
9	Dextran fraction	11500	12	31.01 ± 0.12
10	Dextran fraction	14700	10	30.02 ± 0.13
11	Dextran fraction	21975	12	29.06 ± 0.14
12	Dextran fraction	31900	10	28.12 ± 0.17
13	Dextran fraction	39200	10	27.63 ± 0.15
14	Dextran fraction	42150	12	27.47 ± 0.10
15	Dextran fraction	73625	12	25.95 ± 0.09
16	Dextran fraction	104450	12	24.93 ± 0.09
17	Dextran fraction	144960	12	24.28 ± 0.14
18	Dextran fraction	239825	12	23.23 ± 0.11
19	Dextran fraction	$> 2 \cdot 10^6$	11	18.29 ± 0.05

characterised by the crude data, i.e. mean elution volume and molecular weight. We thus obtained means for the above parameters from which a significantly linear correlation of $-\ln K_{av}$ with m was obtained over about half of the range of elution volumes from V_0 to V_t as given by the end-markers. With the calibration equation now determined, variabilities could be found by standard statistical procedures. Next, taking the partition coefficients as variables not subject to significant error, it was possible to assess quantitatively the errors to be expected when mean elution volumes were used to estimate molecular weights.

Programs written for a small computer (Sinclair ZX81) enabled the solution of simultaneous exponential equations to be carried out very rapidly, and provided the print-out of trial plots of calibration graphs.

RESULTS

In a preliminary trial, seven well spaced triads from the range of points 2—18 (Table I) were used to derive seven values for V_0 and for $V_t - V_0$. Those for V_0 ranged from 22.3 to 26.6 ml with a mean of 24.3 ml, while for $V_t - V_0$, the mean was 11.2 and the range was 10.2—12.4 ml. Using these means to calculate $Y = -\ln K_{av}$, a linear relationship of high significance was found as $Y = 0.001441m + 0.004881$ ($r = 0.9972$; eight degrees of freedom) for the points 4—13 in Table I. The mean $\bar{Y}$ corresponds to an elution volume of 30.39 ml, with $\bar{m}$ equivalent to a MW of 13 623 daltons. The 90% confidence interval (CI) for the above regression coefficient was defined by the limiting values 0.001357 and 0.001526. Points 2 and 3 (Table I) when added to the graph fell well within these limits, while point 14, plotted similarly, fell within the 90% CI for the variation of Y about the regression.

With this much established, the series of triads was extended arbitrarily to a sample of size 20 covering the range of points 2—14 inclusive. Now using the better estimates of means for V_0 = 25.65 ml (standard error = 0.15 ml) and for $V_t - V_0$ = 11.00 ml (± 0.12 ml), values for $-\ln K_{av}$ were computed for the points 1—15 and plotted. Correlation for points 2—14 inclusive showed the relationship $Y = 0.001490m + 0.00200$ ($r = 0.9991$, eleven degrees of freedom). The mean $\bar{Y} = 0.82431$ corresponds to V_e = 30.47 ml, with $\bar{m}$ = 554.62 equivalent to a MW of 13 060 daltons. The 90% CI for the regression coefficient was defined by limiting values of 0.001456 and 0.001524, while the standard deviation (S.D.) for $\bar{Y}$ at 90% confidence was $\bar{Y}$ ± 0.0439. Graphical representation of these results (Fig. 1) shows that variation of Y for a given molecular weight depends largely on the latter factor, since that induced by the error of the slope is comparatively small.

Using the same methods as above, it was found, also for points 2—14 (Table I), that the regression of m to Y could be expressed as $m = 670.0Y + 2.330$. The limiting values of the regression coefficient for 90% confidence were 654.7 and 685.3, with the 90% CI $\bar{m}$ ± 29.44. Again, the error in m at any given value for Y consists mainly of the component of variance arising from the scatter about the regression. Graphical representation of the errors (90% CI) in the regression of m to $-\ln K_{av}$ took a form similar to that of Fig. 1.

In the original work of Cullen et al. [1] calibration was effected by use of

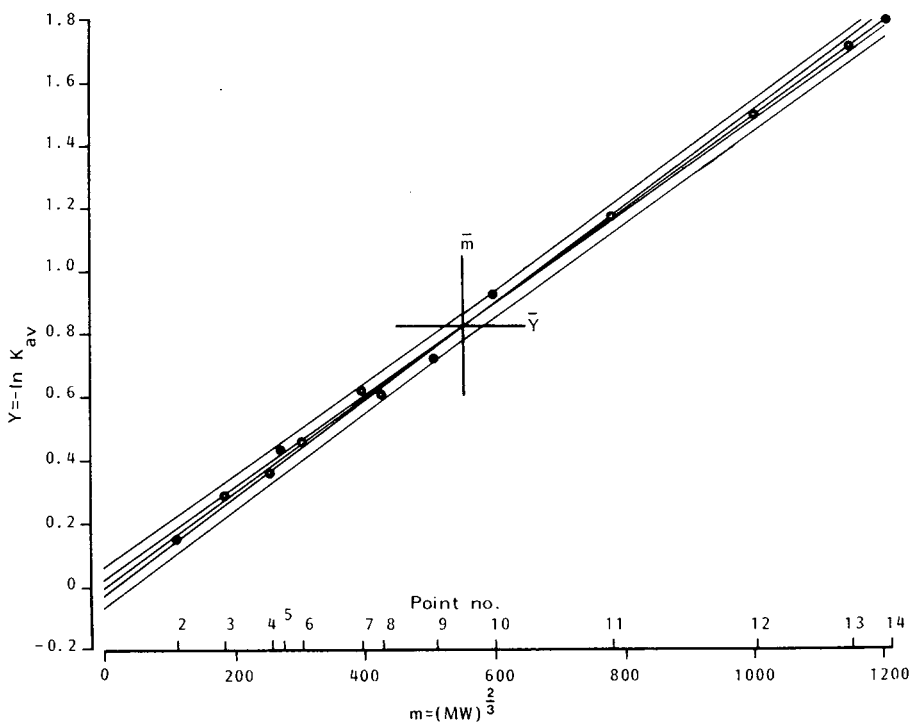

Fig. 1. Regression of $Y = \ln K_{av}$ to $m = MW^{2/3}$. Mean slope with 90% CI for slope and for variation of Y about the slopes as outer limits. $K_{av} = (V_e - 25.65)/11.00$; V_e values (ml) as means, points 2—14 (Table I) inclusive.

the significant linear relationship between K_{av} and ln MW, and this method (B) was compared with the above (A). With data from Table I, using points 1 and 19 for V_0 and V_t, respectively, the regressions (a) $K_{av} = 1.7050 - 0.1178 \ln MW$ and (b) $\ln MW = 14.35 - 8.4165\ K_{av}$ ($r = 0.9959$, fifteen degrees of freedom) were established. With the same methods as used above with method A, 90% confidence intervals were calculated and a corresponding pair of graphs was now constructed for method B. The four graphs served as charts from which the ambits, i.e. intervals between maximum and minimum, could be found as 90% confidence intervals both for the mean elution volumes with given molecular weights and for the molecular weights estimated when the true mean weights were known. Results of these computations of ambits are shown in Fig. 2, from which it is clear that method A gives valuably greater precision for elution volumes than does method B. As estimators of molecular weights, both methods gave the same limits when the true molecular weight was approximately 2000 daltons, but with increasing molecular weights method A showed progressive superiority: only in the range 1000—2000 daltons did method B give an indication of greater precision.

Errors in the estimations of molecular weights from elution volumes may be expressed also as ± percentages of the known mean weights since it was found that the limits were almost completely symmetrical about the means. In these terms, errors (90% CI) of method B are between ± 34% (2000 daltons) and ± 30% (40 000 daltons), contrasting with those of method A, which range from ± 34% (2000 daltons) to ± 5.9% (40 000 daltons) (Fig. 2). When method B was applied to points 2—14 (Table I) only, the status of the procedure in comparison with method A was found to have deteriorated, while application of method B with the derived values $V_0 = 25.65$ and $V_t - V_0 = 11.00$ ml gave no improvement.

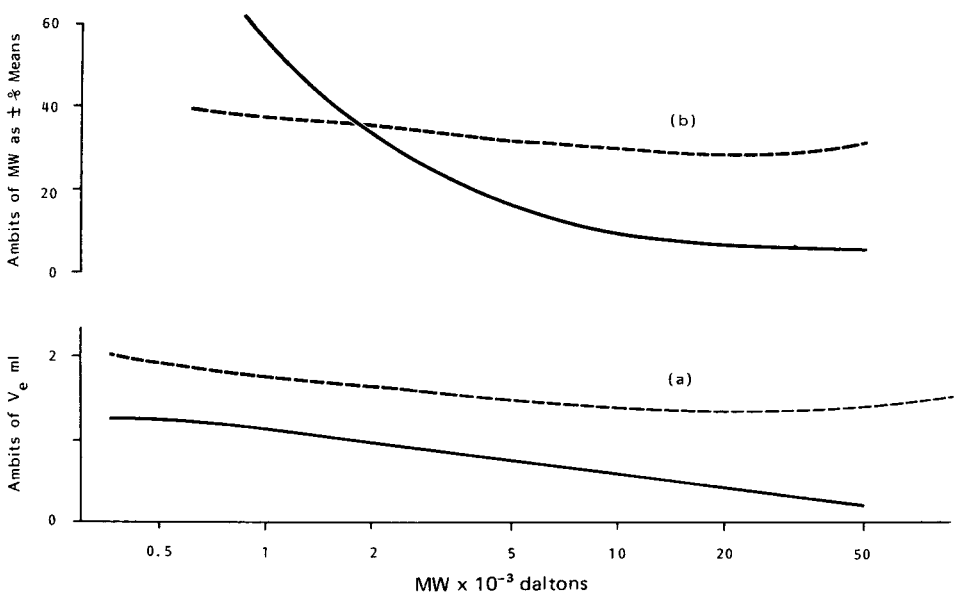

Fig. 2. Ambits (90% CI) for elution volumes as functions of molecular weights (a) and for molecular weights, as ± percentage MW, estimated from elution volumes (b). Solid lines show results for method A, broken lines for method B.

DISCUSSION

The results obtained show that the rectilinear calibration holds good for the system studied over a useful span of the interval between V_0 and V_t as indicated by dextrose and highly polymerised dextran. This range allows, with calibration method A, successful treatment of dextrans having molecular weights from 1500 to 50 000 daltons. Near the centre of the working range 90% of mean elution volumes will fall within an ambit of ± 0.20 ml. With lower molecular weights, e.g. 1000 daltons, 90% of these volumes should be spread over $\pm < 0.6$ ml, while at the other extreme (50 000 daltons) means for V_e should exceed an ambit of ± 0.10 ml from the central volume in only 10% of cases, one in twenty giving higher volumes and with the same proportion falling lower. These conclusions cannot be expected to apply to other than the isochemical series treated here.

Whereas the confidence limits for mean elution volumes may be of interest, as e.g. in deciding that volumes need be measured only to the nearest 0.1 ml, it is perhaps of greater importance to know how precisely molecular weights may be deduced once the mean for V_e has been estimated.

It is clear from Fig. 2 that calibration by method A is preferable, although the useful range is smaller than that with method B. It is also seen that only rough estimates are possible with the system studied if true molecular weights are much below 5000 daltons.

The formal use of confidence intervals in this study tends to give a disconcerting impression for two reasons. Firstly, in truth, fewer than 5% of cases will be values reaching limits for both sources of variation in the same sense. A proportion of the results that are high because of variation about the regression will be modified by a counter-balancing diminution due to the variability of the regression coefficient. Secondly, the central tendency will ensure that precision is influenced favourably in a preponderence of cases. Admittedly the statistical methods used, although robust, are only good approximations. In particular, it is known that although the standard molecular weights are truly invariate, the mean elution volumes are estimated means, with known small standard errors. None of the standard dextran fractions was homogenous; unfortunately, pure compounds are not available. It must be emphasized that mean elution volumes have been used throughout, so it is important that data be obtained by replication with rigid control of instrumental conditions, pumping rate and temperature being particularly pertinent.

To summarize the comparison of methods A and B treated here, it may be said that method A is preferable, but requires at least five to six standards to furnish an adequate sample of triads. Method B is quicker and more convenient to apply, but lacks precision, although the working range is wider. Its adequacy is equally dependent on the amount of data from standards. If method B is used in preliminary work, results of sufficient interest can then be re-examined by the use of the better but more laborious procedure.

It is noticeable that V_0 forms a far greater proportion of V_t than the 25—30% that would be expected. This may reflect that the gel may contain a proportion of highly permeable particles. Whatever the cause, it is tempting to speculate that the inductive use of triads may explain the practical superiority

of method A. Failure of the calibration at lower molecular weights as V_e tends towards V_t may be ascribed to the fact that the shorter molecules cannot assume a random coil configuration.

In the system studied, two TSK gels (3000 PW and 5000 PW) were used in series. The above considerations might well apply to systems in which other grades of gel of similar manufacture are to be employed for investigations with different ranges of molecular weights in an isochemical series.

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REFERENCES

1 M.P. Cullen, C. Turner and G.B. Haycock, J. Chromatogr., 337 (1985) 29.
2 J. Porath, Pure Appl. Chem., 6 (1963) 233.
3 S. Hjertén, J. Chromatogr., 50 (1970) 189.
4 R.W.R. Baker, J. Chromatogr., 154 (1978) 3.

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Note

3-Chloroformyl-7-methoxycoumarin as a fluorescent derivatization reagent for alcoholic compounds in liquid chromatography and its use for the assay of 17-oxosteroids in urine

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Several fluorogenic reagents have been reported for the derivatization of alcoholic compounds in high-performance liquid chromatography (HPLC); i.e. 4-dimethylamino-1-naphthoyl nitrile (DANN) [1], 2-methyl-1,1′-binaphthalene-2′-carbonyl nitrile (MBCN) [2], 1- and 9-anthroyl nitriles (1-AN and 9-AN) [3] and 4-diazomethyl-7-methoxycoumarin (DMC) [4]. Although it has been shown that 3-chloroformyl-7-methoxycoumarin (3CMC) gives fluorescent esters when refluxed with methanol and ethanol and it is readily synthesized [5], no further study of its application in liquid chromatography has been described.

We have found that MC3C also reacts sensitively with primary alcohols other than methanol and ethanol and secondary alcoholic compounds in acetone, benzene or their mixture at high temperatures to yield highly fluorescent esters, which can be separated by thin-layer chromatography (TLC) or HPLC. As an example in the practical use of the reagent, we have developed an HPLC method for the determination of 17-oxosteroids (androsterone, dehydroepiandrosterone and etiocholanolone), which have a secondary hydroxyl group in the molecule, in a small amount of human urine.

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EXPERIMENTAL

Materials and apparatus

All chemicals were of analytical-reagent grade unless otherwise noted. Deionized and distilled water was used. Organic solvents were distilled and dried in the usual manner. 3CMC was synthesized as described by Baker and Collis [5]. TLC plates were prepared by dispersing 30 g of silica gel (Wakogel-B5, Wako, Osaka, Japan) in 60 ml of water, applying the dispersion on glass plates (20 × 20 cm) at a thickness of 0.25 mm with a TLC spreader (Yazawa Kagaku, Tokyo, Japan) and heating the plates at 120°C for 1 h for the activation. Human urine samples were obtained from healthy volunteers of the Faculty of Pharmaceutical Sciences, Kyushu University.

Uncorrected fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorimeter using 1 × 1 cm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. A Jasco TWINCLE chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (20-μl loop) and a Hitachi 650-10S spectrofluorimeter fitted with a 18-μl flow-cell operating at 400 nm emission and 355 nm excitation; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. A stainless-steel column (150 × 4.0 mm I.D.) was packed with TSK gel ODS-120A (particle size 5 μm; Toyo Soda Kogyo, Tokyo, Japan) using a slurry technique with chloroform as solvent.

Procedure for TLC and HPLC of the reaction mixtures of alcoholic compounds

A mixture of 50 μl each of test solution in benzene or acetone and 10 m*M* 3CMC solution in benzene was placed in a screw-capped reaction vial (3.5 ml; Gasukuro Kogyo, Tokyo, Japan), which was heated at 100°C for 20 min. A 5-μl aliquot of the reaction mixture was applied on a TLC plate and then developed with benzene—ethyl acetate (8:2) at ca. 23°C. The fluorescent band of the ester was scraped off and the ester was extracted with 3.5 ml of chloroform. The reaction mixture (20 μl) was also injected into the chromatogram after the dilution with 0.9 ml of methanol and eluted with aqueous 70% methanol containing 2% acetic acid at a flow-rate of 0.5 ml/min for the esters of alcohols, with aqueous 80% methanol containing 2% acetic acid at a flow-rate of 1.0 ml/min for the esters of 17-oxosteroids and 17-α-hydroxypregnenolone, and with a mixture of tetrahydrofuran—methanol (1:12) containing 2% acetic acid at a flow-rate of 1.0 ml/min for cholesterol and cholestanol.

Assay procedure for 17-oxosteroids in urine

To 0.5 ml of urine were added 0.2 ml of 2*M* acetate buffer (pH 5.2) and 10 μl of a suspension of β-glucuronidase containing arylsulphatase (β-glucuronidase activity 95 400 U/ml, arylsulphatase activity 5110 U/ml; Sigma, St. Louis, U.S.A.) and the mixture was incubated at 37°C overnight to hydrolyze the conjugated forms of 17-oxosteroids [6]. To the resulting mixture, 5.0 ml of dichloromethane were added and mixed on a vortex-type mixer for 1 min. The organic layer (2.0 ml) was evaporated to dryness in vacuo below 30°C and the residue was dissolved in 0.5 ml of acetone by shaking on a

vortex-type mixer for 1 min. A 50-μl aliquot of the solution was treated as described in the procedure for HPLC. For the establishment of calibration curves, a series of 17-oxosteroid standard solutions (50—500 nmol/ml for each of the steroids, in acetone) was prepared, and the mixtures of water (0.5 ml, in place of urine sample) and standards (50 μl) were treated as described above without incubation. The peak heights in the chromatogram were used for the quantification of 17-oxosteroids.

RESULTS AND DISCUSSION

Reaction 3CMC with alcholic compounds and separation of their esters by TLC and HPLC

3CMC esters of alcoholic compounds examined (see Table I) have the fluorescence excitation and emission maxima around 355 and 400 nm, respectively, in methanol, acetone, ethyl acetate, chloroform, benzene or *n*-hexane, and the fluorescence increases in intensity with increasing polarity of the solvents.

The reaction of 3CMC with primary alcohols (see Table I) in benzene or acetone is complete within 60 min at 25°C or within 7 min at 100°C in a tightly closed reaction vial, whereas the reaction with secondary alcoholic compounds, including hydroxysteroids (see Table I), is complete within 20 min at 100°C.

Benzene and acetone as solvents for the reaction provide the most intense fluorescence for all the compounds examined; other solvents tested, acetonitrile, dioxane and tetrahydrofuran, give less intense fluorescence. Water interferes with the reaction. Acceleration of the reaction was not observed in the presence of trichloroacetic acid, acetic acid, pyridine, triethylamine, potassium carbonate or 15-crown-5 (a phase-transfer catalyst). Thus, the reaction in benzene, acetone or their mixture at 100°C for 20 min was employed in the procedure. Tertiary alcohols, such as 2-methyl-2-propanol and 2-methyl-2-butanol, did not react with the reagent.

TABLE I

R_F VALUES AND LIMITS OF DETECTION FOR 3CMC ESTERS OF ALCOHOLIC COMPOUNDS IN TLC

Compound	R_F	Detection limit (pmol)
Reaction blank	0.00	
	0.38	
Methanol	0.31	41
Ethanol	0.37	33
l-Pentanol	0.50	22
Benzyl alcohol	0.49	8
2-Propanol	0.44	82
2-Pentanol	0.52	82
Cyclohexanol	0.49	200
Etiocholanolone	0.48	2
Androsterone	0.50	2
Dehydroepiandrosterone	0.44	5
17-α-Hydroxypregnenolone	0.22	9
Cholesterol	0.61	15
Cholestanol	0.62	5

3CMC is stable in benzene for ten days or more at 25°C and in acetone for 8 h; a benzene solution of 3CMC was used in the recommended procedure. The reaction mixture of the hydroxyl compounds diluted ten times with methanol can be stored at room temperature (ca. 23°C) for at least three days in daylight.

3CMC esters of the compounds tested can be separated by TLC on silica gel plates and show single fluorescent bands (Table I). The limits of detection are at the picomole level, and in particular the sensitivity for hydroxysteroids is high (Table I). The 3CMC esters extracted from the bands on the TLC plate with chloroform are stable for ca. three days when dissolved in methanol, acetone or benzene after the removal of the extraction solvent. These observations indicate that 3CMC is usable as a fluorescent derivatization reagent for alcoholic compounds in liquid chromatography.

The esters of alcohols and hydroxysteroids, except those of cholesterol and cholestanol, are separated within 20 min by reversed-phase HPLC on a TSK gel ODS-120A column using aqueous 70% and 80% methanol containing a small amount of acetic acid as mobile phases, respectively (Fig. 1A and B). The peaks for benzyl alcohol and cyclohexanol cannot be separated under the HPLC conditions. The peaks of methanol, ethanol and 2-propanol overlap that of the reagent blank. The esters of cholesterol and cholestanol are eluted within 12 min with methanol containing small amounts of tetrahydrofuran and acetic acid (Fig. 1C) though they are retained on the column when aqueous methanol is used as mobile phase.

The calibration curves for the alcoholic compounds (see Fig. 1) are linear up to at least 2 nmol/ml. The limits of detection for alcohols and hydroxysteroids are 0.12—0.50 and 0.11—0.23 pmol in a 20-μl injection volume, respectively, at

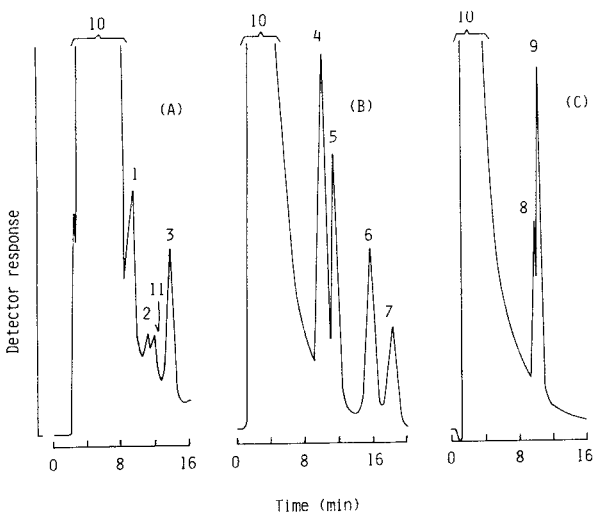

Fig. 1. Chromatograms of 3CMC esters of alcohols (A) and hydroxysteroids (B and C). Portions (50 μl) of a mixture of alcoholic compounds (alcohols, 50 μ*M* each; hydroxysteroids, 10 μ*M* each; in benzene) were treated according to the procedure for HPLC. Peaks: 1 = benzyl alcohol and cyclohexanol; 2 = 2-pentanol; 3 = 1-pentanol; 4 = etiocholanolone; 5 = androsterone; 6 = dehydroepiandrosterone; 7 = 17-α-hydroxypregnenolone; 8 = cholesterol; 9 = cholestanol; 10 = the components of the reagent blank; 11 = one of the components of the reagent blank.

a signal-to-noise ratio of 2. This sensitivity is almost the same as those in 1-AN and 9-AN and at least there times higher than those in DANN and MBCN.

3CMC also reacts with aliphatic primary and secondary amines such as 1-propylamine, 3-methyl-2-propylamine, benzylamine, 2-furfurylamine, di-*n*-butylamine, piperidine, spermine and histamine under the reaction conditions described. These aliphatic amines, however, do not interfere with the determination of alcoholic compounds by HPLC because their 3CMC derivatives can be eluted earlier than the 3CMC esters (within 4 min) under the HPLC conditions. 3CMC does not react with aromatic amines such as aniline and α-naphthylamine and catecholamines (dopamine, epinephrine and norepinephrine). No fluorescent derivative is provided from the reaction of 3CMC with phenolic compounds such as phenol and salicylic acid, but DANN, 1-AN and 9-AN give fluorescent products from phenolic compounds. DMC reacts with carboxylic compounds to form fluorescent derivatives. These observations indicate that 3CMC is more selective than the other reagents for alcoholic compounds.

Assay for 17-oxosteroids in human urine

We developed a sensitive HPLC method for the assay of totals of individual 17-oxosteroids in urine on the above-mentioned basis. The conjugated 17-oxosteroids are hydrolyzed by β-glucuronidase- and arylsulphatase-mediated reactions in the usual manner [6], and the resulting free 17-oxosteroids are extracted with dichloromethane. The steroids remaining after removal of the solvent from the extract are dissolved in acetone, and the acetone solution is subjected to derivatization with 3CMC followed by HPLC.

Fig. 2 shows a typical chromatogram obtained from the urine of a healthy man according to the assay procedure. Linear relationships were observed between the peak heights and the concentrations of androsterone, dehydroepiandrosterone and etiocholanolone up to at least 25 nmol/ml.

The recoveries of androsterone, dehydroepiandrosterone and etiocholanolone

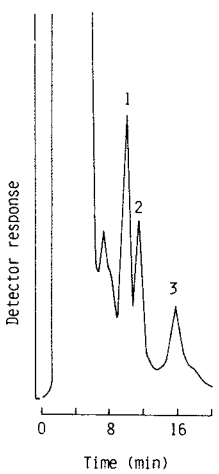

Fig. 2. Chromatogram from normal urine. Peaks (concentration in μg/ml): 1 = etiocholanolone (5.1); 2 = androsterone (2.8); 3 = dehydroepiandrosterone (3.0).

(10 nmol/ml of each) from urine were 95 ± 5.0, 92 ± 2.8 and 90 ± 6.2% (mean ± S.D., $n = 10$ for each), respectively.

The limits of detection for androsterone, dehydroepiandrosterone and etiocholanolone were 0.17, 0.28 and 0.15 pmol in a 20-μl injection volume (which correspond to 98, 162 and 87 ng/ml in urine), respectively, at a signal-to-noise ratio of 2. The sensitivity seems to be at least five times higher than that of the HPLC method using Dns hydrazine [7]. The precision was established by repeated assays ($n = 10$) using normal urine containing androsterone, dehydroepiandrosterone and etiocholanolone at 1.8, 1.8 and 2.2 μg/ml, respectively. The coefficients of variation were 2.8, 3.9 and 5.5%, respectively. The amounts (mg, mean ± S.D.) of androsterone, dehydroepiandrosterone and etiocholanolone in the 24-h urine of healthy persons assayed by this method were 2.6 ± 0.8, 2.2 ± 0.9 and 4.9 ± 0.7 in men (22—26 years, $n = 5$), and 1.2 ± 0.7, 0.9 ± 0.8 and 3.4 ± 0.8 in women (22—23 years, $n = 5$), respectively. The values of individual 17-oxosteroids are in good agreement with the published data [7].

This study shows that 3CMC should be useful as a fluorescent derivatization reagent in liquid chromatography of alcoholic compounds.

REFERENCES

1 J. Goto, S. Komatsu, N. Goto and T. Nambara, Chem. Pharm. Bull., 29 (1981) 899.
2 J. Goto, N. Goto and T. Nambara, Chem. Pharm. Bull., 30 (1982) 4597.
3 J. Goto, N. Goto, F. Shamsa, M. Saito, S. Komatsu, K. Suzaki and T. Nambara, Anal. Chim. Acta, 147 (1983) 397.
4 A. Takadate, T. Tahara, H. Fujino and S. Goya, Chem. Pharm. Bull., 30 (1982) 4120.
5 W. Baker and C.B. Collis, J. Chem. Soc., (1949) S12.
6 H.Ch. Curtius and M. Roth (Editors), Clinical Biochemistry — Principles and Methods, Walter de Gruyter, Berlin, New York, 1978, p. 700.
7 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 226 (1981) 1.

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Note

Simultaneous determination of cholestanol and cholesterol in human serum by high-performance liquid chromatography with fluorescence detection

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The concentration of cholestanol and the concentration ratio of cholestanol to cholesterol are increased in the sera of patients with cerebrotendinous xanthomatosis, obstructive jaundice and cholestatic hepatitis, whereas the cholesterol concentration in the serum of patients with cerebrotendinous xanthomatosis, compared with that for the other patients, remains within the normal range [1]. Therefore, the determination of cholesterol and cholestanol in serum gives important information, especially for the biological diagnosis of cerebrotendinous xanthomatosis [1].

However, the simultaneous determination of cholesterol and cholestanol in serum is difficult, because cholesterol and cholestanol are harldy separated by gas chromatography [2] or thin-layer chromatography [3] and the concentration of cholesterol in normal serum is about 500—1000 times higher than that of cholestanol. Only one method has been reported so far for the simultaneous determination of cholesterol and cholestanol: gas chromatography—mass fragmentography [4]. The method requires a large amount of serum (1.0 ml) and expensive equipment.

We have shown that 3-chloroformyl-7-methoxycoumarin (3CMC) [5] is a useful fluorescent derivatization reagent for alcoholic compounds in high-performance liquid chromatography (HPLC) [6]. We have recently found that epoxidized cholesterol (cholesterol-5α,6α-epoxide) and cholestanol can be completely separated by reversed-phase HPLC on a TSK gel ODS-120A column and thus developed a sensitive method for the simultaneous determination of cholesterol and cholestanol in a minute amount of human serum (50 μl) by

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HPLC with pre-column fluorescent derivatization with 3CMC after epoxidation of cholesterol.

EXPERIMENTAL

Materials and apparatus

All chemicals were of analytical-reagent grade unless indicated otherwise. Deionized, distilled water was used. Organic solvents were distilled and dried in the usual manner. Cholesterol was obtained from Nakarai Chemicals (Kyoto, Japan; standard reagent, purity 99%), Katayama Chemicals (Osaka, Japan; guaranteed reagent) and Sigma (St. Louis, MO, U.S.A.; standard reagent for chromatography, purity above 99%, and that from porcine liver, purity 99%). Cholestanol was purchased from Nakarai Chemicals and cholesterol-5α,6α-epoxide from Sigma. 3CMC was synthesized by the method of Baker and Collis [5]. Human sera were obtained from healthy volunteers in our laboratory and kept at −20°C until used.

Uncorrected fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorometer using 1 × 1 cm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. A Jasco TWINCLE chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (20-μl loop) and a Hitachi 650-10S spectrofluorometer fitted with an 18-μl flow cell as a fluorescence detector, operating at 400 nm emission and 355 nm excitation. A stainless-steel column (150 × 4.0 mm I.D.) was packed with TSK gel ODS-120A (particle size 5 μm) (Toyo Soda, Tokyo, Japan) using a slurry technique with chloroform as solvent.

Procedure

A mixture of 50 μl of serum and 1.0 ml of 0.2 *M* sodium hydroxide in 95% ethanol was placed in a PTFE screw-capped Pyrex culture tube (100 × 16 mm I.D.) (Iwaki Glass, Tokyo, Japan) and heated at 100°C for 10 min to hydrolyse esters of cholesterol and cholestanol. To the resulting mixture, 0.5 ml of water and 5.0 ml of *n*-hexane were added. The mixture was shaken on a vortex-type mixer for 10 min and centrifuged at 1000 *g* for 5 min. The organic layer (3.0 ml) was transferred into a glass-stoppered test-tube (10 ml) and the solvent was evaporated to dryness in vacuo at 30°C. The residue was dissolved in 1.0 ml of 0.3% (w/v) *m*-chloroperbenzoic acid solution in *n*-hexane by shaking on a vortex-type mixer for 1 min and incubated at 37°C for 30 min to epoxidize cholesterol. The mixture, after adding 4.0 ml of *n*-hexane, was washed with 2 ml of 0.2 *M* sodium hydroxide solution. The organic layer (3.0 ml) was transferred into another test-tube and the solvent was evaporated to dryness in vacuo at 30°C. The residue was dissolved in 0.2 ml of 20 m*M* 3CMC solution in acetone by shaking on a vortex-type mixer for 1 min. A 100-μl aliquot of the mixture was heated in a screw-capped reaction vial (3.5 ml) (Gasukuro Kogyo, Tokyo, Japan) at 100°C for 20 min. A 20-μl aliquot of the reaction mixture was injected into the chromatograph after the dilution with 0.9 ml of acetone and eluted with a mixture of methanol and tetrahydrofuran (13:1, v/v) containing 2% (v/v) of acetic acid at a flow-rate of 1.0 ml/min. The spectral

bandwidths in both the excitation and emission sides of the monochromator in the fluorescence detector were 2 nm for the first 8 min of the retention time and were then changed to 10 nm to obtain a high detector response. For the establishment of calibration graphs, series of cholestanol standard solutions in acetone (2—100 μg/ml) and cholesterol standard solutions in acetone (0.5—4.0 mg/ml) were prepared, and the standards (50 μl each) were carried through the procedure, alkali hydrolysis being omitted. The peak heights in the chromatograms were used for the quantification of cholestanol and cholesterol.

RESULTS AND DISCUSSION

The 3CMC esters of cholesterol and cholestanol were hardly separated by reversed-phase HPLC on a TSK gel ODS-120A column [6]. In the determination of cholestanol in serum by gas chromatography, a large amount of interfering cholesterol was removed by epoxidizing it with *m*-chloroperbenzoic acid in chloroform (product, cholesterol-5α,6α-epoxide) [7, 8]. The epoxidation can be carried out in *n*-hexane with warming for 30 min at 37°C. The esterification of cholesterol-5α,6α-epoxide and cholestanol with 3CMC in acetone is completed within 20 min at 100°C. The separation of 3CMC esters of cholesterol-5α,6α-epoxide and cholestanol can be achieved by HPLC with methanol containing small amounts of tetrahydrofuran and acetic acid as the mobile phase; tetrahydrofuran provides a rapid separation of peaks and acetic acid serves to sharpen the peaks.

Fig. 1 shows the chromatograms obtained from cholesterol and cholestanol standards according to the procedure without alkali hydrolysis. The detector response is adjusted by changing the spectral bandwidths in both the excitation and emission monochromators of the fluorescence detector as described in the procedure, because there is a very large difference in concentration between the 3CMC esters of cholesterol-5α,6α-epoxide and cholestanol. This could not be achieved by changing the sensitivity range of the fluorescence detector. The 3CMC ester of cholestanol is eluted at a retention time of 13.6 min (Fig. 1B). In the chromatogram from cholesterol, however, two peaks are observed at retention times of 4.8 and 13.6 min (Fig. 1A). The eluates from peaks 1 and 2 in Fig. 1A and B have fluorescence excitation maxima at 353 and 359 nm, respectively, and both emission maxima at 402 nm. The retention times and the fluorescence excitation and emission spectra of the eluates from peaks 1 and 2 in Fig. 1A are identical with those obtained with authentic samples of cholesterol-5α,6α-epoxide and cholestanol, respectively. These observations indicate that the commercial preparations of cholesterol contain a relatively large amount of cholestanol; the concentration ratio of cholestanol to cholesterol was in the range 0.0014—0.0023.

Esters of cholesterol and cholestanol in serum are hydrolysed at 100°C in aqueous ethanolic sodium hydroxide solution and the resulting free cholesterol and cholestanol are extracted with *n*-hexane. The extract is subjected to epoxidation and then to fluorescent derivatization, followed by HPLC.

Fig. 2 shows typical chromatograms obtained from the serum of a healthy man and the same serum fortified with cholestanol. The fluorescence excitation and emission spectra of peaks 1 and 2 in Fig. 2A and B are identical with those in Fig. 1A and B, respectively.

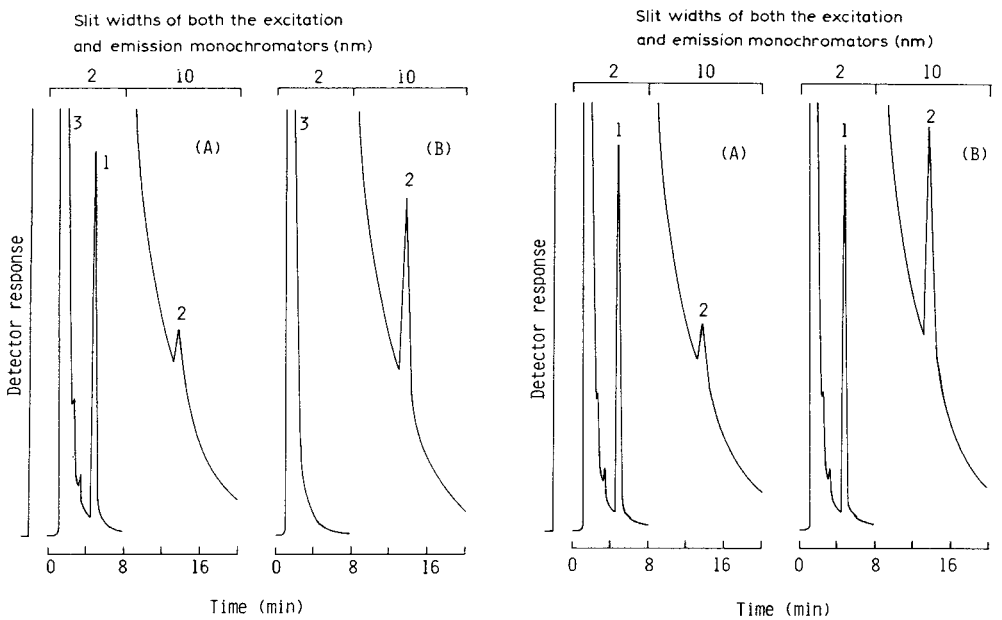

Fig. 1. Chromatograms of 3CMC esters of (A) cholesterol and (B) cholestanol. Portions (50 μl) of commercial preparations of cholesterol and cholestanol in acetone (2.0 mg/ml and 10 μg/ml, respectively) were treated according to the procedure without alkali hydrolysis. Peaks: 1 = cholesterol-5α,6α-epoxide; 2 = cholestanol (in A, cholestanol contained in the commercial preparation of cholesterol as impurity; in B, the commercial preparation of cholestanol); 3 = components of the reagent blank.

Fig. 2. Chromatograms from (A) normal serum and (B) the serum fortified with cholestanol (10 μg/ml). Peaks: 1 = cholesterol-5α,6α-epoxide; 2 = cholestanol.

The calibration graphs for cholesterol and cholestanol were linear up to at least 4.0 mg/ml and 100 μg/ml, respectively, and passed through the origin. The limits of detection for cholesterol and cholestanol were 2.72 and 0.15 pmol in a 20-μl injection volume, corresponding to 5.8 and 0.34 μg/ml in serum, respectively, at a signal-to-noise ratio of 2. The sensitivity may permit the assay of cholesterol and cholestanol in 0.1 and 5.7 μl of normal serum, respectively.

The recoveries of cholesterol and cholestanol (1.0 mg/ml and 5 μg/ml, respectively) from serum were 99.9 ± 5.8% and 98.8 ± 5.6% (mean ± S.D., n = 10), respectively. The precision was established by repeated assays (n = 10) using serum containing cholesterol and cholestanol at 1.76 mg/ml and 3.13 μg/ml, respectively. The coefficients of variation were 4.9% and 4.4%, respectively.

Comparison with a fluorimetric method for the assay of cholesterol in serum based on the enzymatic reaction (cholesterol ester hydrolase—cholesterol oxidase—peroxidase system) with fluorogenic substrate (tyramine) [9] showed a correlation coefficient of 0.955 (n = 15), and the regression equation for the present method (x) against the fluorimetric enzymatic method (y) was $y = 0.912\,x + 0.16$. The amounts of cholestanol and cholesterol in the serum of

TABLE I

CONCENTRATIONS OF CHOLESTANOL AND CHOLESTEROL AND THEIR RATIO IN SERA FROM NORMAL PERSONS

Age (years)	Sex*	Cholestanol (μg/ml)	Cholesterol (mg/ml)	Ratio of cholestanol to cholesterol × 100
22	M	2.44	1.94	0.13
24	M	2.83	1.93	0.15
24	M	4.13	1.89	0.22
25	M	1.10	1.35	0.08
25	M	1.70	1.33	0.13
26	M	2.63	1.40	0.19
26	M	1.80	1.21	0.15
26	M	2.19	1.94	0.11
26	M	1.20	0.90	0.13
30	M	2.84	2.11	0.13
33	M	3.13	1.92	0.16
38	M	3.03	1.50	0.20
54	M	3.95	1.90	0.21
22	F	2.95	1.94	0.15
33	F	4.44	2.20	0.20
Mean ± S.D.		1.70 ± 0.38	2.69 ± 1.00	0.16 ± 0.04

*M = Male; F = female.

fifteen healthy subjects (22—54 years) determined by this method and their concentration ratios are shown in Table I. The values are in good agreement with published data [1, 4, 8].

This study provides the first HPLC method for the determination of cholestanol along with cholesterol. The method is sensitive, rapid and simple and should be useful for biomedical investigations of cholesterol and cholestanol.

REFERENCES

1 S. Kamei, Y. Seyama, K. Ichikawa, T. Kasama, A. Okubo and T. Yamakawa, Jap. J. Clin. Chem., 7 (1978) 151.
2 T.T. Ishikawa, J.B. Brazier, L.E. Stewart, R.W. Fallat and C.J. Gluech, J. Lab. Clin. Med., 87 (1976) 345.
3 G. Salen, Ann. Intern. Med., 75 (1971) 843.
4 Y. Seyama, K. Ichikawa and T. Yamakawa, J. Biochem., 80 (1976) 223.
5 W. Baker and C.B. Collis, J. Chem. Soc., s12 (1949).
6 C. Hamada, M. Iwasaki, N. Kuroda and Y. Ohkura, J. Chromatogr., 341 (1985) 426.
7 L.F. Fieser and M. Fieser, Reagents for Organic Synthesis, Vol. 1, Wiley, New York, 1967, p. 135.
8 S. Serizawa, Y. Seyama, H. Otsuka, T. Kasama and T. Yamakawa, J. Biochem., 90 (1981) 17.
9 C. Hamada, M. Iwasaki, K. Zaitsu and Y. Ohkura, Chem. Pharm. Bull., 28 (1980) 3131.

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Note

Simple high-performance liquid chromatographic method for the determination of medroxyprogesterone acetate in human plasma

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Medroxyprogesterone acetate (MPA) is widely used in the treatment of breast and endometrial cancer [1]. Plasma concentrations of MPA in the literature have been determined mainly by radioimmunoassay (RIA) [2] and gas—liquid chromatography (GLC) [3]. RIA has the advantage of sensitivity (100 pg MPA) but its limited selectivity can lead to overestimation of MPA [4], whereas GLC methods are selective but require derivatisation. A simple high-performance liquid chromatographic (HPLC) method using Sep-Pak extraction cartridges has previously been reported [5].

We propose an alternative HPLC method which employs solvent extraction and a more conventional HPLC column. The method has been successfully automated and is therefore suitable for the rapid and inexpensive analysis of large batches of samples.

EXPERIMENTAL

Materials

All chemicals were of AnalaR grade and obtained from commercial sources. The solvents, methanol and hexane, were of HPLC grade. The steroids were

obtained from Sigma (Poole, U.K.). Pooled human plasma was collected from healthy subjects; EDTA was the anticoagulant.

Standards

Medroxyprogesterone acetate (6α-methyl-17α-hydroxyprogesterone acetate) was obtained from Sigma. Stock standards (1 mg/ml) and working standards (1 and 10 μg/ml) of MPA were prepared in methanol and stored at 4°C for up to one month.

The internal standard 16β-methylprogesterone, was obtained from Sigma and stock solutions (100 μg/ml) were prepared in methanol. The internal standard working solution (0.5 μg/ml) was prepared daily by dilution of the stock solution in 0.2 *M* phosphate buffer, pH 7.0.

Extraction procedure

Plasma (2.0 ml) was transferred to a disposable screw-capped glass tube and 1.0 ml of the internal standard working solution was added. After brief vortex-mixing (3 sec), 7.0 ml hexane were added and the tube was sealed with a PTFE-lined cap. After gentle mixing on a rolling mixer (Luckham, Burgess Hill, U.K.) for 30 min, the hexane layer was transferred to a clean glass tube and evaporated to dryness under nitrogen at 30°C. The residue was dissolved in 200 μl of the HPLC mobile phase and 150 μl were injected onto the HPLC column.

Chromatography

The stainless-steel HPLC column, 25 cm × 4.6 mm I.D., contained 5-μm reversed-phase particles of Spherisorb 5-ODS2 (Hichrom). The mobile phase comprised methanol—0.02 *M* acetate buffer, pH 4 (79:21), and was pumped at a flow-rate of 1.5 ml/min (Beckman pump, Model 110A). A variable-wavelength Pye Unicam LC-UV detector was used in conjunction with a pen recorder (J.J. Instruments) to monitor the eluent at 240 nm (the λ_{max} of MPA) at a sensitivity of 0.05 a.u.f.s. Injections were made using a Rheodyne 7125 manual loop injector or a Waters WISP automatic sampler with limited-volume inserts.

Calibration

Calibration standards were prepared by the addition of 10—50 μl of an MPA working standard to 2.0-ml aliquots of pooled blank plasma. At least six calibration standards containing between 5 and 250 ng/ml MPA were analysed with each batch of samples.

Recovery

Duplicate spiked plasma samples containing 100 ng/ml were analysed as described except that exactly 5.0 ml of the 7.0-ml hexane extract were evaporated under nitrogen. A 1 μg/ml solution of MPA in mobile phase was injected to give a peak height equivalent to 100% recovery. The recovery of MPA from the spiked plasma was calculated as follows:

$$\text{Recovery} = \frac{\text{mean sample peak height}}{1\ \mu\text{g/ml MPA peak height}} \times \frac{7}{5} \times 100\%$$

Reproducibility and stability

Pooled plasma was spiked with MPA at 25.8, 103 and 258 ng/ml, and aliquots were stored at −20°C.

Intra-assay variation was determined on two occasions by analysis of five samples at each concentration, spread randomly throughout the batch of samples. Inter-assay variation was assessed by analysis of one sample at each concentration on five separate occasions.

The stability of MPA at −20°C was assessed by comparing the assayed concentration with the original spiked concentration of MPA over a period of four months.

RESULTS

Selection of chromatographic conditions

Reversed-phase packing materials and a methanol—aqueous mobile phase were suitable for the chromatography of MPA. The retention of MPA by various types of column packing was examined by comparison of their phase capacity ratios (k') for MPA (see Table I). Since a 25-cm Spherisorb 5-ODS2 column gave the greatest retention of MPA, this was the column of choice. Although the retention of MPA was not affected by changes in pH or buffer molarity of the mobile phase, we preferred to control the pH and a 0.02 *M* acetate buffer at pH 4 was used. The methanol content of the mobile phase was optimised to achieve the best separation of low levels of MPA from endogenous peaks, in the shortest time. Under the conditions described, retention times of MPA and 16β-methylprogesterone, the internal standard, were 5.3 and 9.0 min respectively.

TABLE I

EFFECT OF COLUMN PACKING MATERIAL ON THE RETENTION OF MEDROXYPROGESTERONE ACETATE

HPLC conditions as described in text.

Column packing	Column length (cm)	Phase capacity ratio (k')* of MPA
Spherisorb 5-C_8	25	1.6
μBondapak C_{18}	30	1.8
Hypersil ODS	25	2.5
Spherisorb 5-ODS	25	2.8
Spherisorb 5-ODS2	25	3.5

*$k' = \frac{t_R - t_0}{t_0}$ where t_R = retention time of drug and t_0 = retention time of an unretained solute.

Chromatograms

Typical chromatograms of pooled plasma (A), a calibration standard (B), and a patient's sample (C) are shown in Fig. 1. The pooled plasma chromatogram shows no interference at the retention time of MPA, and is typical of many of the individual blank plasma chromatograms investigated. However, small inter-

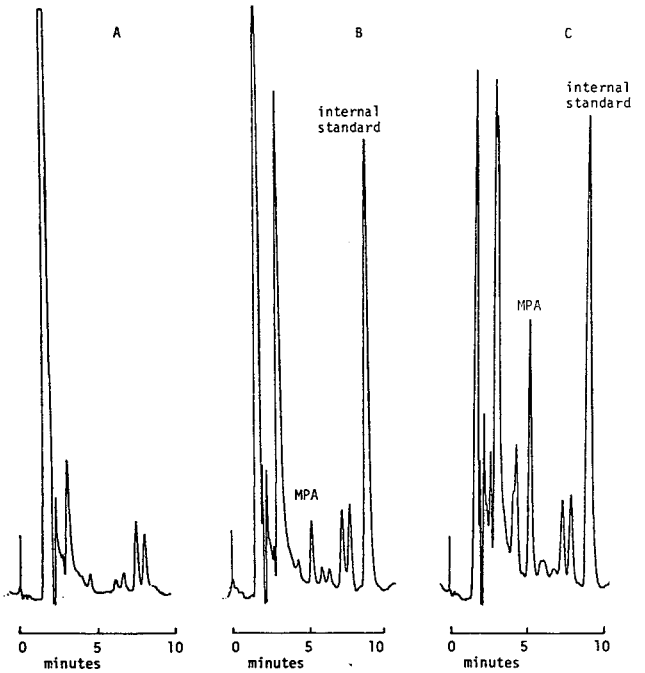

Fig. 1. Typical chromatograms of plasma samples. (A) Pooled plasma; (B) calibration standard containing 25 ng/ml medroxyprogesterone acetate (MPA) and 16β-methylprogesterone (internal standard), 500 ng; (C) patient sample following oral administration of Provera; MPA concentration = 115 ng/ml.

fering peaks equivalent to approximately 5 ng/ml MPA were occasionally found in plasma samples from healthy female subjects. A broad peak eluting after 50—60 min was also present in some plasma samples. The use of lignocaine as a local anaesthetic during blood collection, produced a large interfering peak in the resulting plasma chromatogram. The peak was found to be lignocaine itself, which should therefore not be used in conjunction with this method.

TABLE II

RETENTION TIMES OF ENDOGENOUS STEROIDS

HPLC conditions as described in text.

Compound	Retention time (min)
6α-Methyl-17α-hydroxyprogesterone acetate (MPA)	5.3
16β-Methylprogesterone (internal standard)	9.0
Progesterone	6.8
17-α-Hydroxyprogesterone	4.0
Cortisone	2.5
Corticosterone	3.0
Aldosterone	2.4
Testosterone	4.0
Androstenedione	3.8
Oestradiol	3.5
Cholesterol	Not detected

The similarity between plasma chromatograms from patients receiving MPA therapy and those of healthy subjects, suggests that MPA metabolites were not detected by this method.

Retention times of a selection of endogenous steroids are shown in Table II. None had the same retention times as MPA or 16β-methylprogesterone.

Sensitivity

The absorption spectrum of a methanolic solution of MPA showed the λ_{max} to be at 240 nm (see Fig. 2). The assay sensitivity was therefore optimised by UV monitoring at this wavelength.

Although 5 ng/ml MPA could be reliably detected in spiked pooled plasma, individual plasma samples occasionally contained an interfering peak equivalent to 5 ng/ml MPA, as described above, therefore the sensitivity of the method was limited to 10 ng/ml MPA.

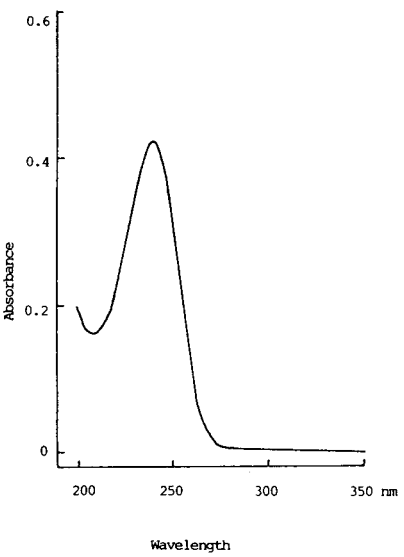

Fig. 2. Absorption spectrum of medroxyprogesterone acetate (MPA), a 10 μg/ml solution in methanol; λ_{max} = 240 nm.

TABLE III

RECOVERY OF MEDROXYPROGESTERONE ACETATE (MPA) IN SOLVENTS OF VARYING POLARITY

Plasma concentration of MPA = 100 ng/ml.

Extraction solvent	Recovery (%)
Dichloromethane	10
Ethyl acetate	37
Cyclohexane	66
n-Pentane	84
Hexane	87
Diethyl ether	91

Recovery

The mean recovery of 100 ng/ml MPA from plasma, at pH 7, was 86 ± 1.5% S.D., n = 5. The effect of extraction pH on MPA recovery was investigated by addition of 1.0 ml of 0.2 M buffers at pH 2, 5, 7, 9 or 12, prior to extraction of 100 ng/ml MPA from plasma. Recoveries (79—86%) were not affected by the pH of extraction over the range studied. Extraction of plasma containing 100 ng/ml MPA with solvents of varying polarity, resulted in recoveries ranging from 10% to 91%, as shown in Table III. MPA was efficiently extracted by pentane, hexane and diethyl ether but hexane was the solvent of choice since fewer endogenous background materials appeared in the chromatograms.

Reproducibility and linearity

Reproducibility data at 25.8, 103 and 258 ng/ml MPA are shown in Table IV. Intra- and inter-assay coefficients of variation (C.V.) were 1.1—7.3% and 1.2—3.5%, respectively.

Calibration curves were linear between 5 and 250 ng/ml, and the mean coefficient of correlation (r) was 0.999 (n = 8).

TABLE IV

INTRA- AND INTER-ASSAY VARIATION

MPA concentration (ng/ml)	Intra-assay C.V.* (%)	Inter-assay C.V.* (%)
25.8	7.3, 6.6	3.1
103	3.8, 1.6	3.5
258	1.2, 1.1	1.2
Mean	3.6	2.6

*Coefficient of variation (C.V.) = 100% × standard deviation/mean (n = 5).

Sample stability

Spiked plasma samples stored at −20°C showed no deterioration over a period of four months.

Drug monitoring in patients

Twenty patient's samples have been analysed using this method. The patients had been receiving oral MPA therapy at 1 g per day, for at least 21 days, for the treatment of breast cancer, and samples were taken 2—4 h after the morning dose of 500 mg MPA. The plasma concentrations of MPA were between 12.6 and 270 ng/ml with a mean value of 104 ng/ml (± 68.9 S.D.).

The plasma profile of MPA was studied in one patient who had been taking oral MPA, 500 mg, once a day, for at least 21 days previously. Plasma concentrations were monitored at 0, 1, 2, 3, 4, 6 and 8 h after dosing (500 mg MPA) and are shown in Fig. 3.

The MPA concentration before dosing (i.e. 24 h after the previous dose) was 5 ng/ml, and the peak plasma concentration, 118 ng/ml, occurred at 3 h after dosing.

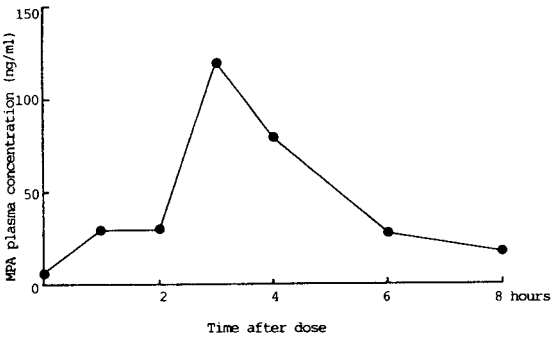

Fig. 3. Plasma profile of medroxyprogesterone acetate (MPA) following a 500-mg oral dose, in a patient who had been taking 500 mg once a day for at least 21 days previously.

DISCUSSION

This HPLC method was developed as a simple, reliable and selective alternative to the current methods [2, 3, 5] available for measuring plasma concentrations of MPA. A solvent extraction followed by HPLC separation and analysis appeared to be the most straightforward approach.

MPA was efficiently extracted, at all pH values, by non-polar solvents (Table III). Hexane extraction at pH 7 gave the cleanest chromatogram and the use of HPLC-grade solvents considerably reduced the background noise. Emulsion formation was avoided by a gentle mixing technique, and contamination was minimised by the use of disposable glassware.

The Spherisorb 5-ODS2 HPLC column gave excellent separation of MPA from the endogenous steroids of similar structure and from the extraneous plasma blank peaks, many of which originated from the hexane.

The main advantage of this method is its rapid yet inexpensive sample preparation. A batch of 30—40 samples can be prepared for HPLC analysis in 2—3 h, and the chromatographic run time is 10 min per sample. Sample extracts are stable for at least 24 h at +4°C, if storage is required before injection.

Calibration curves between 5 and 250 ng/ml show a good correlation (r = 0.999) between peak height ratio and MPA concentration, even at low levels. The reliability of the method is shown in the low C.V. values (3.6% and 2.6%) of the intra- and inter-assay analyses (Table IV).

Plasma concentrations of MPA in patients receiving 1 g per day were between 12.6 and 270 ng/ml in this study, suggesting that the sensitivity of the method, 10 ng/ml, is sufficient for monitoring therapeutic concentrations of MPA. These results show a wide individual variation in plasma concentrations following similar dosing schedules, as reported by other workers [6]. The plasma profile following once daily dosing at 500 mg MPA (Fig. 3) covered a wide range of MPA concentrations (5—118 ng/ml) during the 24-h dosing interval. Since the response of the drug has been related to the plasma concentration [7], routine monitoring would be helpful in the use of this drug.

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REFERENCES

1 F. Pannuti, A.R. Di Marco, A. Martoni, F. Freut, E. Strocchi, P. Burroni, A.P. Rossi and A. Cricca, Role of Medroxyprogesterone in Endocrine-Related Tumors, Raven Press, New York, 1980, p. 73.
2 J.C. Cornette, K.T. Kirton and G.W. Duncan, J. Clin. Endocrinol. Metab., 33 (1971) 459.
3 D.G. Kaiser, R.G. Carlson and K.T. Kirton, J. Pharm. Sci., 63 (1974) 420.
4 H. Adlercreutz, P.B. Eriksen and M.S. Christensen, J. Pharm. Biomed. Anal., 1 (1983) 153.
5 G. Milano, G. Carle, N. Renée, J.L. Boublil and M. Namer, J. Chromatogr., 232 (1982) 413.
6 M. Salimtschik, H.T. Mouridsen, J. Loeber and E. Johansson, Cancer Chemother. Pharmacol., 4 (1980) 267.
7 F. Cavalli, W.L. McGuire, F. Pannuti, A. Pellegrini and G. Robustelli Della Cuna (Editors), Round Table: MPA Pharmacokinetics, Proceedings International Symposium on Medroxyprogesterone Acetate, Geneva, February 24—26, 1982, Excerpta Medica, Amsterdam, 1982.

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Note

Determination of serotonin in plasma by liquid chromatography with electrochemical detection

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Serotonin (5-hydroxytryptamine; 5-HT) is located primarily in the enterochromaffin cells of the intestine, the serotonergic neurons of the brain and the platelets of the blood. Serotonin has been established both as a neurotransmitter in the central nervous system and as a vasoconstrictor, and is implicated in some physiological functions such as sleep regulation [1] and pathological phenomena such as depression [2], carcinoid syndrome [3] and essential hypertension [4].

Various methods have been applied for the determination of serotonin in biological fluids and nervous tissues, including fluorimetric techniques [5—10], gas chromatography—mass spectrometry (GC—MS) [11] and radioimmunological [12, 13] and radioenzymatic methods [14]. However, they suffer from a lack of sensitivity and specificity (fluorimetric techniques) or are expensive (GC—MS). Recently, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has proved to be a sensitive and inexpensive method for measuring catecholamines, indoleamines and their metabolites in tissues and body fluids [15—20].

This paper describes a method with an internal standard for the measurement of serotonin in platelet-poor plasma (PPP) and in platelet-rich plasma (PRP), using reversed-phase HPLC—ED. The method requires two successive liquid extractions to concentrate and purify the samples. We carried out this study on healthy young adults in order to determine normal values for extra-platelet serotonin (from PPP) and intra-platelet serotonin (from PRP).

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EXPERIMENTAL

Chemicals and reagents

Serotonin creatinine sulphate and N-methyl-5-hydroxytryptamine oxalate (internal standard, I.S.) were obtained from EGA-Chimie (Strasbourg, France). The other chemicals were of analytical-reagent grade: chloroform, 1-pentanol, sodium chloride, glycine, disodium hydrogen phosphate dihydrate, citric acid, the disodium salt of ethylenediaminetetraacetic acid (Merck, Nogent, France), methanol and sodium hydroxide (Prolabo, Paris, France).

Standard solutions

Stock solution of serotonin was prepared by dissolving 22.8 mg of serotonin creatinine sulphate in 100 ml of distilled water to give 100 μg/ml free base. A stock solution of the internal standard was prepared by dissolving 14.7 mg in 100 ml of distilled water to give a concentration of 100 μg/ml. These solutions remain stable for one month if stored at 4°C.

Apparatus

The chromatographic system was composed of a Waters 6000A pump (Waters Assoc., Paris, France), and a Rheodyne injection valve (Touzart et Matignon, Vitry sur Seine, France) fitted with a sample loop (20 μl). A guard column filled with μBondapak C_{18} was placed before the C_{18} reversed-phase analytical column (250 × 4.6 mm I.D., particle size 5 μm) (Altex). The column effluent was monitored by using an electrochemical detector (Eldec 102) (Chromatofield, Chateauneuf les Martigues, France). The output of the detector was connected to a Model S recorder (Servogor, St. Etienne, France) with a chart speed of 120 mm/min. The mobile phase was pumped at a flow-rate of 1 ml/min (147 bars).

Operating conditions

The HPLC mobile phase consisted of 0.1 *M* citric acid buffered with 0.1 *M* $Na_2HPO_4 \cdot 2H_2O$ (pH 3.8) containing 12% of methanol and 0.1% of 0.1 *M* EDTA. To minimize the background noise and improve the detection sensitivity, it is important to filter the mobile phase under vacuum through a 0.22-μm Millipore filter and degas it. Chromatography was carried out at room temperature. The potential of the working electrode (glass carbon) was set at +0.6 V versus the Ag—AgCl reference electrode. The applied cell potential was determined by hydrodynamic voltammetry of serotonin and the internal standard in the mobile phase. The sensitivity of the detector was 1 and 20 nA for PPP and PRP, respectively.

Patients

Serotonin levels in PPP and PRP were determined in twenty healthy adults (ten females and ten males) with a mean age of 30 ± 11 years and a mean weight of 57 ± 12 kg.

Blood collection and processing

Volumes of 10 ml of venous blood were collected from individuals in the fasting state at 8.00 a.m. in siliconized glass tubes containing 0.1 ml of EDTA solution (15%, w/v). The samples were mixed and placed in ice—water and the blood was rapidly centrifuged at 100 *g* for 10 min at 4°C to obtain PRP. A platelet count on PRP was obtained using a Model S Plus Coulter Counter (Coultronics, Margency, France). A 1-mg amount of sodium disulphite, used as antioxidant, was added and the PRP stored at −70°C until taken for assay.

A 1.5-ml aliquot of PRP was centrifuged at 6000 *g* for 15 min at 4°C to produce PPP. Subsequently, the supernatant was carefully decanted and transferred into a clean tube containing 1 mg of sodium disulphite and stored.

Sample preparation

Platelet-poor plasma samples. A 1-ml volume of PPP was introduced in a 20-ml glass tube, then 1 ml of buffer (pH 11) containing glycine, 0.1 *M* NaOH and 0.1 *M* NaCl was added. The plasma was adjusted to pH 11 with 0.1 *M* NaOH and 7 ml of chloroform—1-pentanol (60:20, v/v) saturated with water were added and the mixture was shaken mechanically for 10 min. After centrifugation for 10 min at 2000 *g*, the supernatant organic phase (5 ml) was transferred into a 10-ml tube containing 400 μl of 0.1 *M* hydrochloric acid and the mixture was then shaken for 10 min. After centrifugation for 10 min at 2000 *g*, 20 μl of the aqueous phase were injected into the HPLC system.

Calibration graphs were generated by spiking blank PPP (950 μl) with 50 μl of serotonin solution at concentrations of 3.12, 6.25, 12.5, 25, 50, 100 and 200 ng/ml. All the PPP and the standards were previously supplemented with internal standard (50 μl of N-methyl-5-hydroxytryptamine solution at a concentration of 100 ng/ml). The standard solutions were processed in the same manner as the samples.

Platelet-rich plasma samples. The extraction procedure used for assaying PRP was similar to that described above. Only 500 μl of PRP were taken; the calibration graphs were prepared by supplementing blank plasma (450 μl) with 50 μl of serotonin solution at concentrations of 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/ml. All the PRP samples were supplemented with the internal standard (50 μl of solution at a concentration of 2000 ng/ml).

RESULTS AND DISCUSSION

Effects of centrifugation

Plasma serotonin is mostly intra-platelet, a small proportion being "free" extra-platelet. We had to determine the optimal centrifugal speed to obtain good sedimentation of platelets without damaging them. We centrifuged PRP for 15 min at increasing speeds of 1000, 6000, 13 000, 23 000, 33 000, 52 000 and 70 000 *g*. Then we determined the serotonin concentration of the supernatant (Fig. 1). The proportion of extra-platelet serotonin was lowest at 6000 *g*. When a high centrifugal speed was applied the concentration of serotonin in the PPP obtained increased; the platelets were damaged during high-speed centrifugation and serotonin was released. We therefore chose 6000 *g* at 15 min for the routine preparation of PPP.

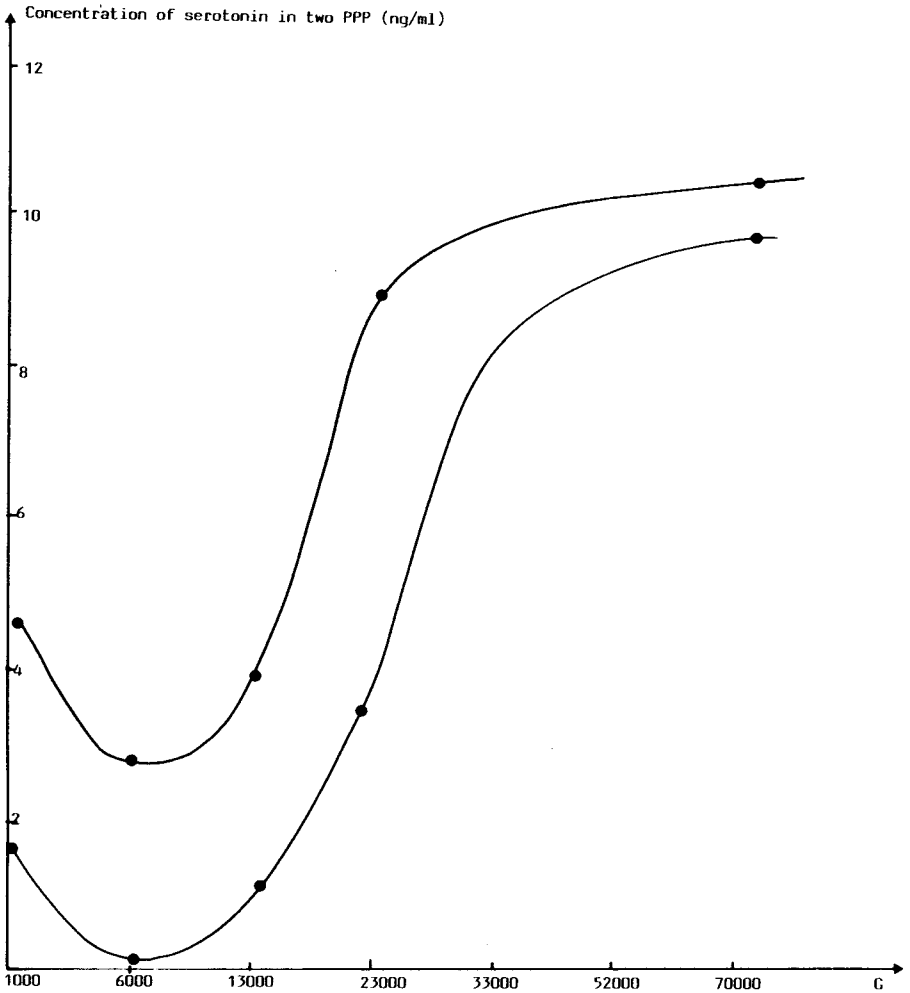

Fig. 1. Effects of centrifugation on the concentration of serotonin in the PPP.

Extraction

Different organic solvents were tried for the extraction of serotonin from biological fluids and the results are presented in Table I. The highest recovery was obtained with chloroform—1-pentanol. Neither the kind nor the molarity of the acid used for the back-extraction had any influence on the recovery.

TABLE I

VARIATION OF RECOVERY WITH THE ORGANIC SOLVENT USED FOR EXTRACTION

Organic solvent	Recovery (%)
Chloroform—1-pentanol	90
Ethylacetate	0
Hexane	5
Chloroform	10

Quantification

Calibration graphs of the serotonin-to-I.S. peak-height ratio versus serotonin concentration were plotted and that for serotonin in plasma was linear throughout the concentration range studied. The correlation coefficient of the linear calibration was 0.9994 for PPP, the equation being $y = 0.3745x - 0.0079$, where y represents the serotonin-to-I.S. peak-height ratio and x the concentration of serotonin. For PRP the correlation coefficient was 0.9995 and the equation was $y = 0.0217x - 0.0042$.

Reproducibility and repeatability

Good intra-day repeatability was obtained; the coefficients of variation for ten assays were 4.9% at 1 ng/ml and 5.3% at 5 ng/ml. The inter-day precision evaluated by analysis over several days ($n = 10$) was 5% at a concentration of 5 ng/ml.

Chromatographic conditions

The best separation of the two chromatographic peaks corresponding to the internal standard and serotonin was obtained with a mobile phase containing 12% of methanol. Under these conditions the retention times were 9 and 11 min, respectively. For a signal-to-noise ratio of 3 the lower limit of quantitation was 200 pg/ml with a sensitivity of 0.2 nA. In each assay, an endogenous peak with the same retention time as the internal standard was carefully screened by means of a blank. In the same way, we checked that other molecules with an indolic nucleus (tryptophan, 5-hydroxytryptophan, 5-hydroxyindolacetic acid, melatonin, tryptamin) did not interfere with serotonin and the internal standard.

Normal values

For PPP the concentrations of plasma serotonin in healthy adults were of 2.71 ± 0.62 ng/ml for men and 1.83 ± 0.90 ng/ml for women. For PRP the concentrations of intra-platelet serotonin were obtained by subtraction of the extra-platelet serotonin concentration. The results, expressed in terms of 10^9 platelets (10^9 pl) were 626 ± 207 ng per 10^9 pl for men and 625 ± 86 ng per 10^9 pl for women.

TABLE II

INTRA-PLATELET AND EXTRA-PLATELET CONCENTRATIONS OF SEROTONIN DETERMINED BY VARIOUS WORKERS

Reference	Techniques	Serum (ng/ml)	PPP (ng/ml)	PRP (ng per 10^9 pl)
6	Fluorimetry			670
7	Fluorimetry		6—19	
8	Fluorimetry		5—12	295—659
9	Fluorimetry		80	150—450
10	Fluorimetry			716—755
12	Radioimmunoassay		6—15	300—380
13	Radioimmunoassay	66—158	0.8—2.4	
14	Radioenzymatic		3.9	
17	HPLC—ED		9.1	
18	HPLC—ED	67—77	3—3.6	

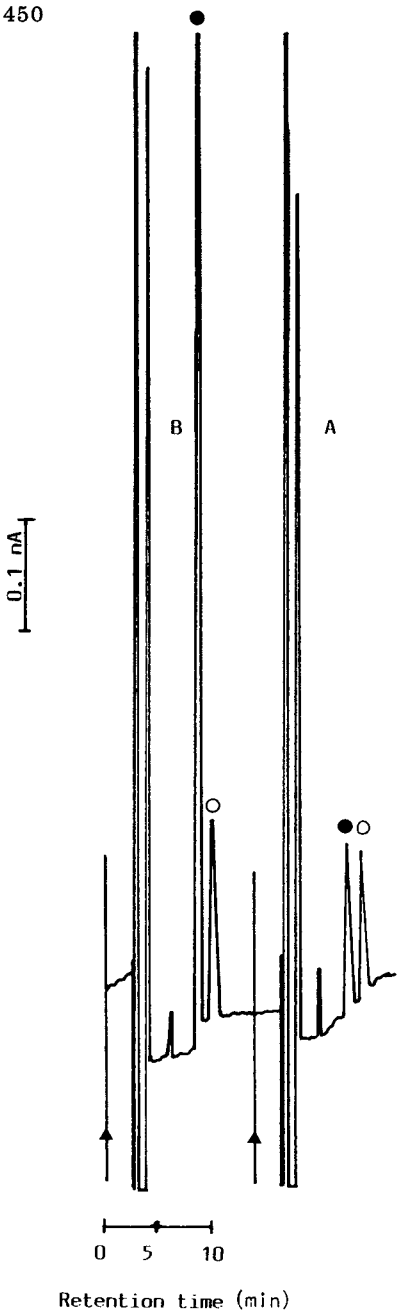

Fig. 2. Chromatograms of (A) normal and (B) pathological plasma samples (PPP). PPP serotonin concentrations: (A) 1.5 ng/ml; (B) 35 ng/ml. Chromatographic conditions: column, μBondapak C_{18}, 250 × 4.6 mm I.D.; mobile phase, 0.1 *M* citric acid buffered with 0.1 *M* $Na_2HPO_4 \cdot 2H_2O$, pH 3.8, containing 12% of methanol and 0.1% of 0.1 *M* EDTA; electrode potential, +0.6 V. ●, Serotonin; ○, internal standard.

It seemed of interest to compare the serotonin levels in PRP and PPP as assessed by different workers (Table II). The results differed not only with the mode of detection used, but even when the same mode was used by different

workers. These variations were probably due to the difference in the specificities of the techniques and to the lack of standardization of sample preparation, PPP, PRP, serum, centrifugal speed [21], intra-platelet serotonin level expressed as ng/ml or ng per 10^9 pl and the choice of anticoagulant.

The serotonin levels in PPP obtained in this work are in accord with those of other workers [13, 14, 17, 18] using different techniques, but the values are lower than all the other results but one [13]. The values in PRP are too difficult to compare owing to the lack of standardized methods of preparation, as mentioned above. We can nevertheless make a comparison with the closely related techniques described by Koch and Kissinger [18] and Tagari et al. [17]. What is interesting in Koch and Kissinger's technique is enrichment on a pre-column, which increases the sensitivity and consequently permits smaller test samples to be used. However, it requires very expensive and heavy equipment, and is therefore difficult to use. Moreover, the use of an internal standard confers greater reliability and better reproducibility.

CONCLUSION

The technique described for the assay of intra-platelet and extra-platelet serotonin is highly sensitive (detection limit 200 pg/ml), reproducible (intra-day coefficients of variation 4.9% and 5.4%; inter-day coefficient of variation 5%) and specific. The assay of extra-platelet serotonin seems to be an important tool for the clinical diagnosis of carcinoid tumours (Fig. 2); moreover, it allows a biological approach to depression and the effects of some anti-depressants.

REFERENCES

1 W. Wejemann, N. Weiner, M. Rotsch and E. Schulz, J. Neural Transm. Suppl., 18 (1983) 287.
2 J. Barchas and E. Usdin (Editors), Serotonin and Behavior, Academic Press, New York, 1973.
3 D.G. Grahame-Smith, in S.C. Trielove and E. Lee (Editors), Topics in Gastroenterology, Blackwells, London, 1977, p. 285.
4 P.M. Vanhoutte, in F. de Clerck and P.M. Vanhoutte (Editors), 5-Hydroxytryptamine in Peripheral Reactions, Raven Press, New York, 1982, p. 163.
5 S. Udenfriend (Editor), Fluorescence Assay in Biology and Medicine, Academic Press, New York, 1969.
6 G.T. Vatassery, M.A. Sheridan and A.M. Krezowski, Clin. Chem., 27 (1981) 328.
7 N. Crawford, Clin. Chim. Acta, 8 (1963) 39.
8 P. Frattini, M.L. Cucchi, G. Santagostino and G.L. Corona, Clin. Chim. Acta, 92 (1979) 353.
9 A. Parbtani and J.S. Cameron, Thromb. Res., 15 (1979) 109.
10 D.R. Shuttelworth and J.O. Rien, Blood, 57 (1981) 505.
11 F. Articas and E. Gelpi, Anal. Biochem., 92 (1979) 233.
12 J.M. Kellum and B.M. Jaffe, Gastroenterology, 70 (1976) 516.
13 F. Engbaek and B. Voldy, Clin. Chem., 28 (1982) 624.
14 M.N. Hussain and M.J. Sole, Anal. Biochem., 111 (1981) 105.
15 G.S. Mayer and R.E. Shoup, J. Chromatogr., 255 (1983) 533.
16 L. Semerdjian-Rouquier, L. Bossi and B. Scatton, J. Chromatogr., 218 (1981) 663.
17 P.C. Tagari, D.J. Boulin and C.L. Davies, Clin. Chem., 30 (1984) 131.
18 D.D. Koch and P.K. Kissinger, Anal. Biochem., 52 (1980) 27.
19 M. Warnhoff, J. Chromatogr., 307 (1984) 271.
20 C.F. Saller and A.I. Salama, J. Chromatogr., 309 (1984) 287.
21 R.C. Arora and H.Y. Meltzer, Biol. Psychiatry, 17 (1982) 115?.

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Note

Fractionation of rat α-fetoprotein by high-performance liquid chromatography

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α-Fetoprotein (AFP) is a major plasma glycoprotein of the foetus [1]. It disappears after birth and is normally absent in adults [2, 3], although it reappears in association with certain pathological conditions [4—7]. Its function in the foetus is unknown, but it is thought to act as a binding protein for such ligands as steroid hormones, fatty acids and bilirubin, as well as having a role in immunoregulation [8].

It has been well documented that AFP is heterogeneous, comprising sub-populations of differing size, charge and lectin-binding behaviour (for a review see ref. 9). An extensively studied case of this heterogeneity is the existence of two charge variants of rat AFP (RAFP), distinguished by their migration as "fast" and "slow" bands in polyacrylamide gel electrophoresis (PAGE) [10—12].

The molecular basis of this difference, whether due to differences in primary structure, conformation or carbohydrate contents, remains unclear [9]. It is also unclear whether there are functional differences in ligand binding or immunoregulation by these variants. Elucidation of this heterogeneity, especially in the light of the possibility of changes in the function of AFP

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during development, should contribute greatly to our understanding of the biological role of this glycoprotein.

We have recently developed a high-performance liquid chromatographic (HPLC) methodology for the purification of AFP from rat amniotic fluid and foetal extract [13]. In this purification procedure, we have consistently observed heterogeneity of the rat AFP on the anion-exchange column. We have further studied this observation and we report here our results and compare the HPLC fractionation of RAFP with its electrophoretic heterogeneity as shown by PAGE.

MATERIALS AND METHODS

Materials

Rat amniotic fluid was obtained from Wistar rats (Charles River, St. Constant, Canada) of 17—19 days' gestational age. All reagents used were of analytical or reagent grade and purchased from local suppliers.

Instrumentation

HPLC was performed on a Pharmacia liquid chromatography system equipped with two pumps and capable of generating a gradient or step elution profile. Sample injections were carried out using a V-7 valve, and chromatograms were recorded by monitoring the absorbance of the eluent at 280 nm using a single-path UV-1 monitor fitted with a 10-mm path cell. The absorbance unit full scale (a.u.f.s.) was set between 0.02 and 0.1, as appropriate. Fractions were collected with a Pharmacia FRAC-100 fraction collector. The HPLC system was operated at room temperature.

Fractionation of RAFP

RAFP was purified from rat amniotic fluid by the HPLC method described previously [13]. Fractionation of RAFP was carried out on the HPLC system equipped with a Pharmacia Mono Q SI anion-exchange column (50 × 5 mm I.D., 10 μm particle size). Buffers A and B were 6.5 m*M* 1,3-bis[tris-(hydroxymethyl)methylamino]propane (Bis-Tris propane) (pH 9.5) containing 0.25 *M* and 0.50 *M* sodium chloride, respectively. A preprogrammed linear gradient was used for the chromatography, and the appropriate peak fractions were collected, dialyzed against distilled water and lyophilized.

Polyacrylamide gel electrophoresis

Slab polyacrylamide gel electrophoresis was performed on a PROTEAN double-slab electrophoresis cell (Bio-Rad Labs., Mississauga, Canada) using 10% and 5% gels as the stacking and separating gels, respectively. The gels were fixed with trichloroacetic acid and then stained with Coomassie blue. Bands in the gels were quantified by scanning with a DCD-16 digital densitometer (Gelman, Ann Arbor, MI, U.S.A.).

Protein assay

Total protein concentrations were determined by the Bio-Rad protein assay method [14] with bovine serum albumin as a standard. RAFP concentrations

were assayed by the radial immunodiffusion method of Mancini et al. [15].

RESULTS AND DISCUSSION

Following our HPLC purification of RAFP, we applied the resolving power of HPLC to the resolution of RAFP subpopulations. We found that strong anion-exchange HPLC of pure RAFP resolves two major fractions, corresponding to the well known charge variants of RAFP.

Fig. 1 shows the HPLC fractionation of pure RAFP on a Mono Q HR5/5 strong anion-exchange column. In the region associated with RAFP, two major peaks, 1 and 2, were resolved; integration showed that these peaks comprised ca. 59% and 30%, respectively, of the total RAFP. These peaks were isolated and rechromatography showed that they are genuine peaks with different elution volumes (Fig. 2).

Fig. 3 shows the characterization of the fractions by PAGE. Standard RAFP (track S) shows the typical fast and slow variants; peak 1 (track 1), comprising ca. 59% of the total RAFP, is essentially pure and co-migrates with the slow variant. The slow migration of this fraction toward the cathode in PAGE is consistent with its rapid elution from the cationic HPLC column. Peak 2 (track 2), on the other hand, is highly enriched with a molecular species (comprising ca. 80% of the fraction) which co-migrates with the fast RAFP variant. The minor (20%) band co-migrates with the slow variant. This appparent

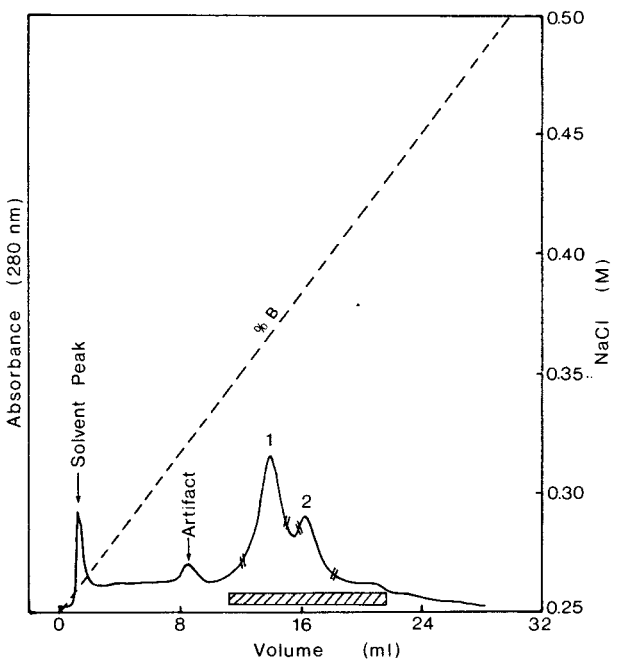

Fig. 1. HPLC fractionation of 1 mg of RAFP on a pre-packed HR5/5 Mono Q SI column. Buffer A, 6.5 m*M* Bis-Tris propane (pH 9.5) containing 0.25 *M* sodium chloride; buffer B, 6.5 m*M* Bis-Tris propane (pH 9.5), containing 0.5 *M* sodium chloride; flow-rate, 1 ml/min; a.u.f.s., 0.1. The presence of RAFP in the fractions is shown by the hatched zones. The RAFP peaks collected are indicated by hash marks in the elution profile.

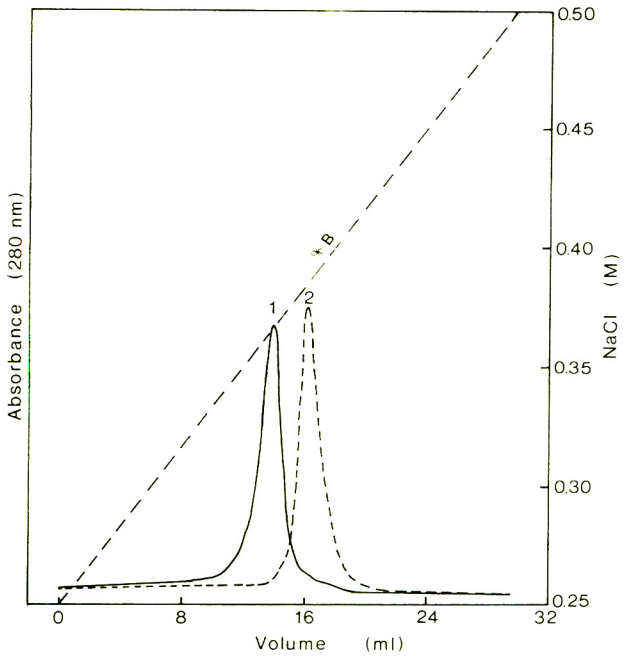

Fig. 2. HPLC re-injection of peaks 1 and 2 isolated in Fig. 1. Buffers A and B, as in Fig. 1. Flow-rate, 1 ml/min, a.u.f.s., 0.02.

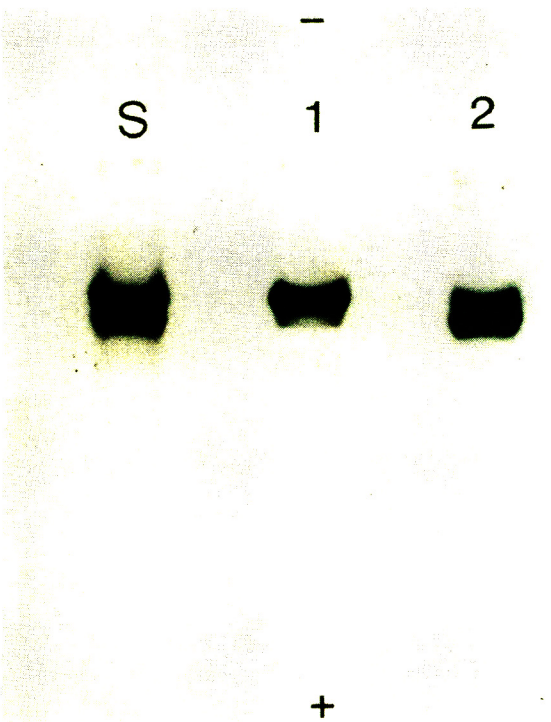

Fig. 3. Polyacrylamide gel electrophoresis of peak 1 (track 1) and peak 2 (track 2) isolated in Fig. 1, with purified RAFP (track S) as standard.

contamination of peak 2 by slow RAFP may be due to tailing from the larger peak 1, although this seems unlikely since fractions taken throughout peak 2 show the same PAGE pattern. The electrophoretic heterogeneity of this peak, therefore, seems genuine and requires further study.

To the best of our knowledge, this is the first method to allow the simple and rapid purification of large amounts of the major (slow) charge variant of RAFP. Previously this has been done only by preparative PAGE, which is rather tedious and which can accommodate only small amounts of protein. The slow variant seems the more interesting to study: it is more abundant than the fast AFP in the rat foetus [9] and it shows most of the oestrogen binding affinity of RAFP [12]. This simple, rapid and high-yield purification method should greatly aid the study of this RAFP subpopulation.

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REFERENCES

1 A. Adinolfi, M. Adinolfi and M.H. Lessof, J. Med. Genet., 12 (1975) 138.
2 E. Ruoslahti and M. Seppälä, Nature (London), 235 (1972) 161.
3 S. Sell and D. Gord, Immunochemistry, 10 (1973) 439.
4 G.I. Abelev, Transplant Rev., 20 (1974) 3.
5 S. Sell and H.T. Wepsic, Progr. Exp. Tumor Res., 19 (1974) 297.
6 P. Alexander, Nature (London), 235 (1972) 137.
7 E. Ruoslahti, H. Pihko and M. Seppälä, Transplant Rev., 20 (1974) 38.
8 E. Ruoslahti and M. Seppälä, Advan. Cancer Res., 29 (1975) 275.
9 C.J.P. Smith and P.C. Kelleher, Biochim. Biophys. Acta, 605 (1980) 1.
10 H. Watabe, Int. J. Cancer, 13 (1974) 377.
11 J.P. Kerckaert, B. Bayard, S. Quief and G. Bisette, FEBS Lett., 53 (1975) 234.
12 C. Benassayag, G. Vallette, N. Cittanova, E. Nunez and M.F. Jayle, Biochim. Biophys. Acta, 412 (1975) 295.
13 L.T. Wong, Z.J. Xu and C.J.C. Hsia, J. Chromatogr., 338 (1985) 410.
14 M. Bradford, Anal. Biochem., 72 (1976) 248.
15 G. Mancini, A.O. Carbonara and J.F. Heremans, Immunochemistry, 2 (1965) 235.

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Note

Two-step purification of human α_1-acid glycoprotein

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Serum α_1-acid glycoprotein (AGP) or orosomucoid concentrations are known to increase during acute inflammatory states such as cancer [1—4], tuberculosis [5], head injury [6], surgery [7] and myocardial infarction [8—10], so this protein can be classified in the group of plasma proteins known as acute phase reactants. It is characterized by a high carbohydrate content (42%), which includes a large number of sialyl residues, a very low isoelectric point (p*I* 2.7) [11] and a high solubility.

To study the variations in sialic acid content during pathological states, it is necessary to obtain the glycoprotein in a pure form and that no desialylation occurs during the purification steps. Most of the techniques for the isolation of human α_1-acid glycoprotein use as a first step an acidic precipitation in order to remove most of the plasma proteins, followed by chromatography on DEAE- and/or CM-cellulose, generally using a strongly acidic buffer. Under these conditions, some variations in the sialic acid content may be observed.

We describe here a two-step purification of α_1-acid glycoprotein from normal plasma that prevents alterations due to exposure to strongly acidic conditions.

EXPERIMENTAL

Human plasma, obtained from healthy blood donors, was kept frozen at $-40\,^{\circ}C$ until used.

Isolation procedure

A 10-ml volume of plasma was dialysed overnight against 20 m*M* citrate—phosphate buffer (pH 4.0). After centrifugation at 500 *g* for 10 min, the sample was applied on a column (9 × 2.1 cm) of DEAE-Trisacryl (Industrie Biologique

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Française, Villeneuve-la-Garenne, France) equilibrated with the same buffer. The column was washed with the equilibrating buffer until no absorbance at 280 nm was recorded, then with the same buffer containing 100 m*M* disodium phosphate. The flow-rate was 140 ml h^{-1} and 2-ml fractions were collected. The remaining contaminating proteins were then eluted by addition of 1 *M* sodium chloride to the buffer (Fig. 1a). The whole procedure was run at 4°C.

After detection by radial immunodiffusion, the fractions containing α_1-acid glycoprotein were pooled, dialysed overnight against distilled water and then concentrated by ultrafiltration on Schleicher & Schüll Ultragains (UH 100).

The concentrated sample was then applied on a column (12.5 × 2.1 cm) of CM-Trisacryl equilibrated with 20 m*M* citrate—phosphate buffer (pH 4.0). The column was washed with the equilibrating buffer, then with the same buffer containing 1 *M* sodium chloride. The elution was run at 4°C at a flow-rate of 140 ml h^{-1}.

The fractions eluted as a single symmetrical peak with the equilibrating buffer (Fig. 1b) contained the purified α_1-acid glycoprotein. The fractions were pooled, dialysed overnight against distilled water and freeze-dried.

Polyacrylamide gel electrophoresis

Electrophoresis was performed using 7% acrylamide plates (Cellacryl, Sebia, Issy-les-Moulineaux, France), with migration for 90 min at 20 V cm^{-1} in 25 m*M* Tris—glycine buffer (pH 8.3). Proteins were stained with Coomassie blue.

Two-dimensional immunoelectrophoresis

This technique was used to control the purity of isolated α_1-acid glycoprotein and was performed on 1% acrylamide gel plates. For the first dimension, the migration was conducted in 25 m*M* Tris—glycine buffer (pH 9.2) for 45 min at 20 V cm^{-1}. For the second dimension, a rabbit antiserum to human serum proteins (Dako, Copenhagen, Denmark) was incorporated in the gel before running for 3 h at 17 V cm^{-1} in the above buffer. The plates were then washed, dried and stained with Coomassie blue.

Quantitative determination of sialic acid and α_1-acid glycoprotein

Sialic acid was determined according to the method of Warren [12] using 2-thiobarbituric acid after mild hydrolysis with 0.05 *M* sulphuric acid for 1 h at 80°C. α_1-Acid glycoprotein was determined by radial immunodiffusion (M-Partigen plates, Behring, Marburg, F.R.G.).

RESULTS AND DISCUSSION

The proposed two-step ion-exchange chromatography leads to purified α_1-acid glycoprotein in a 50% yield. Individual recoveries and yields are summarized in Table I.

Fig. 1 shows the elution profiles obtained in the two chromatographic steps. α_1-Acid glycoprotein is eluted in the bound fraction from the DEAE-Trisacryl column together with albumin, α_1-antitrypsin, haptoglobin and α_2-macroglobulin (Fig. 2). These proteins are further retained on the CM-Trisacryl column, except α_1-acid glycoprotein, which was unbound and eluted in a pure

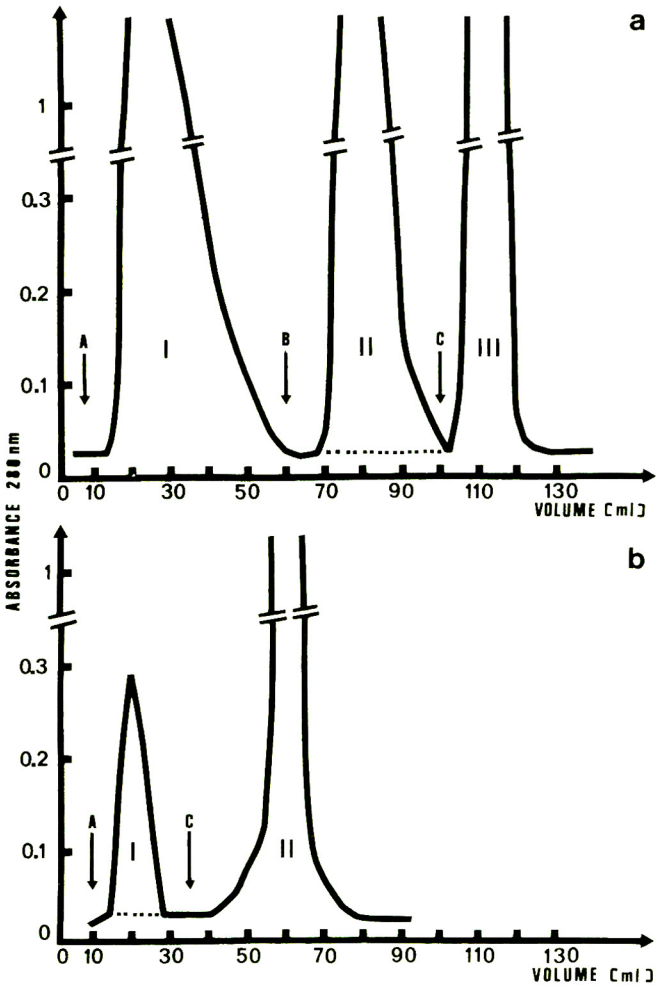

Fig. 1. Stepwise elution of human α_1-acid glycoprotein from (a) DEAE- and (b) CM-Trisacryl columns. (a) A 10-ml volume of human serum, dialysed against 20 m*M* citrate—phosphate buffer, was applied to a DEAE-Trisacryl column (9 × 2.1 cm) equilibrated with the same buffer. Elution was carried out successively with the equilibrating buffer (A), buffer A containing 100 m*M* disodium phosphate (B) and buffer A containing 1 *M* sodium chloride (C). The positions of the changes in elution buffer are indicated by the arrows. The fractions eluted with buffer B (peak II) contain mainly α_1-acid glycoprotein. (b) Fractions containing α_1-acid glycoprotein were applied to a CM-Trisacryl column (12.5 × 2.1 cm). Elution was carried out with buffers A and C. The first peak (I) eluted with buffer A contains pure α_1-acid glycoprotein.

form. No contaminants would be detected by using rabbit antiserum to human serum proteins (Fig. 3). The detection limit was 0.1 μg.

The purification procedure appears to have some advantages over previously reported two-step techniques. It does not involve denaturing steps such as preliminary acidic precipitation and/or exposure to strongly acidic buffers during chromatography. Therefore, it provides an α_1-acid glycoprotein without desialylation. When control α_1-acid glycoprotein (Sigma, St. Louis, MO, U.S.A.)

TABLE I

α_1-ACID GLYCOPROTEIN RECOVERIES AND YIELDS AFTER THE DIFFERENT PURIFICATION STEPS

Material	Volume (ml)	α_1-AGP* (mg)	Overall yield (%)
Dialysed human serum	10	9	100
Eluate from DEAE-Trisacryl column	50	5.4	60
Eluate from CM-Trisacryl column	15	4.5	50

*α_1-AGP concentrations were measured by radial immunodiffusion.

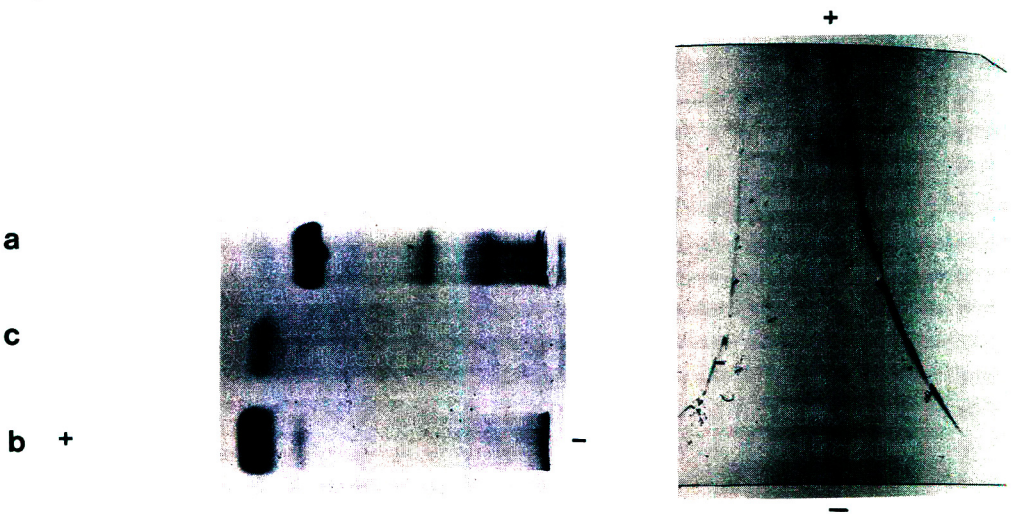

Fig. 2. Polyacrylamide gel electrophoresis at various stages of purification of α_1-acid glycoprotein. (a) Human serum; (b) peak II from the DEAE-Trisacryl column; (c) α_1-acid glycoprotein after the two chromatographic steps. Electrophoresis was carried out on polyacrylamide plates (Cellacryl) with 25 m*M* Tris—glycine buffer (pH 8.3). A current of 20 V cm^{-1} was applied for 90 min. Proteins were stained with Coomassie blue. Peak II from the DEAE-Trisacryl column (b) contains α_1-acid glycoprotein together with albumin, haptoglobin and α_2-macroglobulin. The two-step chromatographic fraction (c) is pure.

Fig. 3. Two-dimensional immunoelectrophoresis on agarose gel of purified α_1-acid glycoprotein. Electrophoresis in the first dimension was carried out on 1% agarose gel plates (9.5 × 1.5 × 0.1 cm) with 25 m*M* Tris—glycine buffer (pH 9.2). A current of 20 V cm^{-1} was applied for 45 min. Electrophoresis in the second dimension was carried out on a gel plate (9.5 × 9 × 0.1 cm) in which rabbit antiserum to human serum proteins was incorporated before migration. A current of 17 V cm^{-1} was applied for 3 h. The gel was then washed and dried and proteins were stained with Coomassie blue.

was used, the percentage of sialic acid was the same before and after chromatography (10.5%).

Other techniques using ion exchangers, particularly that of Bezkorovainy and Winzler [13], involved DEAE-Sephadex chromatography during which α_1-acid glycoprotein was eluted with a strongly acidic buffer (pH 2.8). Under these conditions, desialylation occurred and the sialic acid content of the purified glycoprotein was only 8% [14].

In the procedure described, exposure to acidic pH can be avoided by eluting with buffered 100 m*M* disodium phosphate solution. Citrate and phosphate ions were chosen because they have a good affinity for DEAE-Trisacryl and the separation obtained was better than that using chloride ions.

Some techniques use only one chromatographic step [15—19], but they need preliminary fractionation of plasma, which can be denaturing such as acidic precipitation [15, 16] or salt precipitation [18, 19].

We must also point out the importance of our choice of DEAE- and CM-Trisacryl as chromatographic ion exchangers. It allows high flow-rates, hence limiting the denaturation risks. This seems to be a decisive advantage in comparison with the recently published three-step purification of α_1-acid glycoprotein [20] in which chromatography is followed by a preparative isoelectric focusing step.

The two-step purification described here provides an α_1-acid glycoprotein without apparent desialylation. Hence it would be a valuable tool in studying pathological variations in the sialic acid content of this glycoprotein.

REFERENCES

1 A.M. Baskies, P.B. Chretien, J.F. Weiss, R.W. Makuch, R.A. Beveridge, W.J. Catalona and H.E. Spiegel, Cancer, 45 (1980) 3050—3060.
2 C.Y.T. Chu, L.T.Y. Lai and H.P. Pokala, J. Natl. Cancer Inst., 68 (1982) 75—79.
3 P.G. Gobbi, G. Merlini, G.A. Parrinello, P. Cavalli and E. Ascari, Acta Haematol., 67 (1982) 255—262.
4 P.A. Ganz, W.E. Shell and Z.A. Tökes, J. Natl. Cancer Inst., 71 (1983) 25—30.
5 P.P. Asses and G.D. Tracopoulos, Amer. J. Clin. Pathol., 76 (1981) 437—441.
6 A. Bourguignat, A. Albert, G. Férard, A. Tulasne, I. Kempf and P. Métais, Clin. Chem., 29 (1983) 1904—1907.
7 C.M. Colley, A. Fleck, A.W. Goode, B.R. Muller and M.A. Myers, J. Clin. Pathol., 36 (1983) 203—207.
8 B. Fellahi, P. Bardos, J.P. Muh, H. Mouray and J. Weill, Ann. Biol. Clin., 31 (1973) 421—429.
9 S.J. Smith, G. Bos, M.R. Esseveld, H.G. Van Eijk and J. Gerbrandy, Clin. Chim. Acta, 81 (1977) 75—85.
10 J.P. Chapelle, A. Albert, J.P. Smeets, C. Heusghem and H.E. Kulbertus, Clin. Chim. Acta, 115 (1981) 199—209.
11 R.W. Jeanloz, in A. Gottschalk, (Editor), Glycoproteins, Elsevier, Amsterdam, 2nd ed., 1972, pp. 565—611.
12 L. Warren, J. Biol. Chem., 234 (1959) 1971—1975.
13 A. Bezkorovainy and R.J. Winzler, Biochim. Biophys. Acta, 49 (1961) 559—565.
14 E. Athineos, J.C. Kukral and R.J. Winzler, Arch. Biochem. Biophys., 106 (1964) 338—342.
15 S. Delong Bolmer and E.A. Davidson, Biochemistry, 20 (1981) 1047—1054.
16 G. Biserte, R. Havez, J. Laturaze and A. Hayem-Lévy, Pathol. Biol., 9 (1961) 1081—1088.
17 K. Schmid, M.B. McNair and F. Burgi, J. Biol. Chem., 230 (1958) 853—864.
18 P.A. Charlwood, M.W.C. Hatton and E. Regoeczi, Biochim. Biophys. Acta, 453 (1976) 81—92.
19 I. Nicollet, J.P. Lebreton, M. Fontaine and D. Hiron, Biochim. Biophys. Acta, 668 (1981) 235—245.
20 P. Laurent, L. Mirbel, J. Bienvenu, C. Vallve and P. Arnaud, FEBS Lett., 168 (1984) 79—83.

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Note

Determination of meprobamate as an *n*-butylboronate ester derivative in serum by gas—liquid chromatography

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Meprobamate, 2-methyl-2-propyl-1,3-propanediol dicarbamate, has been used for many years as a tranquillizer and a muscle relaxant. Previously described methods for the analysis of meprobamate in biological material are based on colorimetry [1, 2] and gas—liquid chromatography (GLC) [3, 4]. Colorimetric methods lack both specificity and sensitivity for measurements of therapeutic concentrations. The GLC methods available involve either direct injection of the sample with the problem of thermal decomposition, or a silylation step with limited reproducibility.

To improve the chromatographic properties of such polar bifunctional compounds as 1,2- and 1,3-diols, the formation of cyclic esters with boronic acids has been recommended and used for the separation of diols from organic natural products [5, 6] and for the quantitation of ethylene glycol in blood [7]. We have applied this reaction in the determination of serum concentrations of meprobamate. The procedure is based on alkaline hydrolysis of meprobamate to the corresponding propanediol, followed by esterification by *n*-butylboronic acid to form 2-methyl-2-propyl-1,3-propanediol *n*-butylboronate.

EXPERIMENTAL

Materials

Meprobamate was of pharmaceutical purity. The internal standard (2-methyl-2-ethyl-1,3-propanediol) was obtained from K & K Labs. (Plainview, NY, U.S.A.), and *n*-butylboronic acid from Fluka (Buchs, Switzerland). All

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other chemicals used were of analytical-reagent grade and supplied by E. Merck (Darmstadt, F.R.G.).

Apparatus

A Varian 3700 gas chromatograph equipped with a flame ionization detector was used. The glass column (180 × 0.2 cm I.D.) was packed with 10% OV-17 on Chromosorb W HP, 100—120 mesh. The oven temperature was set at 140°C, with the injector operating at 190°C and the detector at 210°C. Nitrogen (25 ml/min) was used as carrier gas.

Methods

To evaluate the reaction time, two 1-ml serum samples, containing 25 and 250 μmol/l meprobamate, respectively, were each mixed with 1 ml of 12.5 *M* sodium hydroxide. The two serum samples were hydrolysed and extracted as described below. At different reaction times (5—60 min) aliquots of 1 μl were injected onto the chromatograph.

Determination of meprobamate in serum was carried out as follows. A 1-ml serum sample or standard sample (25—250 μmol/l) was hydrolysed by addition of 1 ml of 12.5 *M* sodium hydroxide and kept at 100°C for 10 min. To this mixture were added 5 ml of chloroform containing the internal standard (24 μmol/l). The tube was shaken for 10 min, and after centrifugation at 2500 *g* for another 10 min 4 ml of the organic phase were transferred to a clean test-tube, dried over anhydrous sodium sulphate, and evaporated to dryness under nitrogen. The residue was suspended in 25 μl of 50 m*M* *n*-butylboronic acid in dimethylformamide, and 1 μl of the organic phase was injected onto the chromatograph.

RESULTS AND DISCUSSION

In our investigation we used the quantitative hydrolysis and extraction procedures of Martis and Levy [4], and our results were in agreement with theirs. Esterification of the meprobamate hydrolysis product and the internal standard was completed in 15 min at room temperature. Both boronate ester derivatives were stable for more than 24 h under the reaction conditions used. As internal standard 2-methyl-2-ethyl-1,3-propanediol was selected owing to its structural similarity to meprobamate. Typical gas chromatograms of a serum sample, a blank serum and a serum sample with addition of meprobamate are shown in Fig. 1.

For the determination of concentrations in serum, 1 μl of the reaction mixture was injected directly into the chromatograph without further purification or concentration. A linear standard graph between peak height ratios and meprobamate concentration was obtained ($r = 0.998$) for serum concentrations ranging from 25 to 250 μmol/l. The precision of the method was tested at two concentrations, 25 and 200 μmol/l. Mean values of 26.4 ± 0.9 ($n = 10$) and 204.6 ± 8.2 ($n = 10$) were found, corresponding to a coefficient of variation of 3.6% and 4.0%, respectively. The mean recovery of meprobamate after addition of 25 and 200 μmol/l of pure drug to human serum was found to be 95% and 94%, respectively, in comparison with the

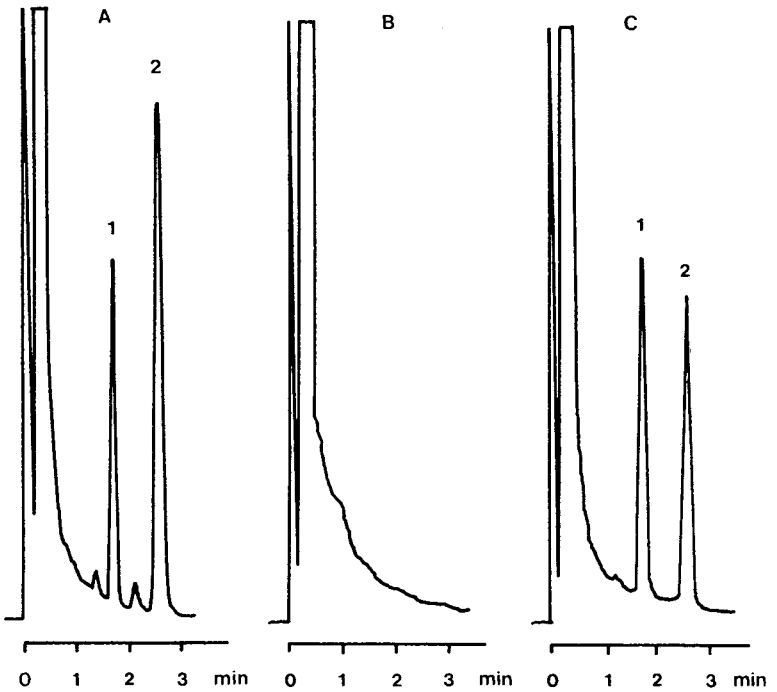

Fig. 1. Gas chromatograms from the analysis of (A) a patient's serum containing meprobamate (380 μmol/l), (B) a blank serum and (C) a patient's serum containing meprobamate (75 μmol/l) with pure substance added (100 μmol/l) (175 μmol/l meprobamate in total). Peaks: 1 = 2-methyl-2-ethyl-1,3-propanediol *n*-butylboronate (internal standard) (120 μmol/l); 2 = meprobamate (2-methyl-2-propyl-1,3-propanediol *n*-butylboronate).

yield found in methanolic standard solutions. With the proposed method, meprobamate was detected in serum at concentrations down to 1.0 μmol/l.

REFERENCES

1 S.L. Kanter, Clin. Chim. Acta, 8 (1963) 2.
2 J.W. Poole, G.M. Irwin and S. Young, J. Pharm. Sci., 60 (1971) 1850.
3 L. Martis and R.H. Levy, J. Pharm. Sci., 61 (1972) 1341.
4 L. Martis and R.H. Levy, J. Pharm. Sci., 63 (1974) 834.
5 C.J.W. Brooks and J. Watson, J. Chem. Soc., Chem. Commun., (1967) 952.
6 C.J.W. Brooks and I. Maclean, J. Chromatogr. Sci., 9 (1971) 18.
7 D.W. Robinson and D.S. Reive, J. Anal. Toxicol., 5 (1981) 69.

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Note

Sensitive analysis of butyrophenone neuroleptics by high-performance liquid chromatography with ultraviolet detection at 254 nm

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Haloperidol, spiroperidol and trifluperidol are used clinically as neuroleptics as well as pharmacological tools in the laboratory. Several methods are already available for the detection and assay of a variety of neuroleptics in plasma. Gas—liquid chromatography requires extensive sample purification but has the advantages of selectivity and accuracy when the internal standard ratio method is used [1, 2]. Radio-ligand displacement assays have high sensitivity and the widest application to different chemical classes but such methods require access to appropriate animal tissues as a source of specific-binding protein [3—5] or a specific antibody [6] and make use of radio-labelled materials. These techniques also lack selectivity, being unable, for example, to differentiate between the neuroleptic parent compound and a pharmacologically active metabolite with the result that drug levels can be overestimated [6]. In addition it is not easy to correct for the efficiency of recovery in a particular sample.

High-performance liquid chromatographic (HPLC) methods have been described for the analysis of haloperidol. Korpi et al. [7] applied isocratic reversed-phase HPLC and electrochemical detection on a glassy carbon electrode at +0.9 V to the assay of haloperidol and a metabolite. Electrochemical detectors are not, however, in wide use and routine high-sensitivity operation at high electrode voltages can be difficult. The strong ultraviolet (UV) absorption of butyrophenones (e.g. haloperidol, λ_{max} 247 nm; E =

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12 000) offers the possibility of UV detection to assay these compounds to the low nanogram range [8, 9]. In this work variable-wavelength detectors were used which are inherently more noisy than fixed-wavelength detectors. None of the HPLC methods describes the analysis of haloperidol or other butyrophenones from tissue samples. This paper describes a method for haloperidol analysis in brain samples with similar sensitivity to the other HPLC methods. UV detection at 254 nm is used since this is most often found in the detector on liquid chromatographs. Furthermore, other butyrophenones (spiroperidol, trifluperidol) can be measured in the same way and the application of trace enrichment to reduce the detection limits and simplify sample preparation is described.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Spectra-Physics SP-8000 unit fitted with an SP-8310 UV detector (254 nm) or Micromeritics 760 variable-wavelength detector, an SP4000 plotter/integrator and a Waters U6K injector with a 2-ml loop. Columns were thermostatted at 30°C unless otherwise stated. A Waters μBondapak C_{18} (300 × 3.9 mm, 10 μm particle size) and a μBondapak Phenyl (300 × 3.9 mm, 10 μm particle size) columns were used and the mobile phases were 0.1% (v/v) trifluoroacetic acid (TFA) with 30% or 40% (w/v) acetonitrile (Burdick & Jackson Labs.).

A Varian 2200 UV—VIS spectrophotometer was used to obtain UV absorption spectra of 10 μg/ml solutions of butyrophenones in 0.05% (v/v) lactic acid. The spectrum of the dilute lactic acid solvent was subtracted from the drug spectra.

Drug administration

Butyrophenones (kindly supplied by Dr. Pierre Laduron, Janssen Pharmaceutica, Belgium) were dissolved in lactic acid, then diluted with distilled water to a final concentration of 5 mg/ml in 0.05% (v/v) lactic acid and stored at 4°C. Under these conditions, the solutions of butyrophenones are stable for more than one year [10]. Further dilutions from these stock solutions were made as necessary.

Male Long Evans Hooded (LEH) rats (200—300 g) received intraperitoneal (i.p.) injections of haloperidol dissolved in dilute lactic acid—methylparaben solution (2 ml/kg). At the appropriate time the animals were killed by decapitation, the brains dissected out and immediately frozen in isopentane on dry ice. Frozen brains were stored at −70°C until required for assay.

Sample extraction

Brains or parts thereof were homogenised in 8 vols. of cold aqueous acid (see Results for details) containing trifluperidol (50 or 500 ng) as internal standard. Samples were placed on ice for 10 min and then centrifuged at 5000 *g* for 30 min at 4°C. Aliquots of the supernatant (usually 1 ml) were made alkaline by the addition of 200 μl of 2 *M* sodium hydroxide per ml and then extracted with 2 vols. of heptane—isoamyl alcohol (97:3) by vigorous vortexing

[1]. The layers were separated by centrifugation and the organic phase transferred to a centrifuge tube containing 200 μl of 5 m*M* sulphuric acid for each 3 ml of organic phase. The butyrophenones were back-extracted into the aqueous phase and the organic layer was aspirated after centrifugation. Portions of the extract, usually 50 μl, were injected onto the liquid chromatograph. The internal standard peak height ratio method was used to calculate haloperidol content which was expressed relative to the wet weight of the tissue.

RESULTS

The UV absorption spectra of the three butyrophenones, haloperidol, spiroperidol and trifluperidol are given in Fig. 1. It can be seen that all three of these compounds absorb strongly in the region of 245—250 nm. The molar absorptivity at 254 nm was calculated to be 11 850, 16 010 and 9980 for the three drugs, respectively. All three show strong absorption below 210 nm and the spectrum of haloperidol also shows a shoulder centered around 220 nm.

The UV spectra suggest that these butyrophenones could be detected with high sensitivity with the 254-nm mercury line lamps most often found in HPLC—UV detectors. This prediction is confirmed by the chromatograms in

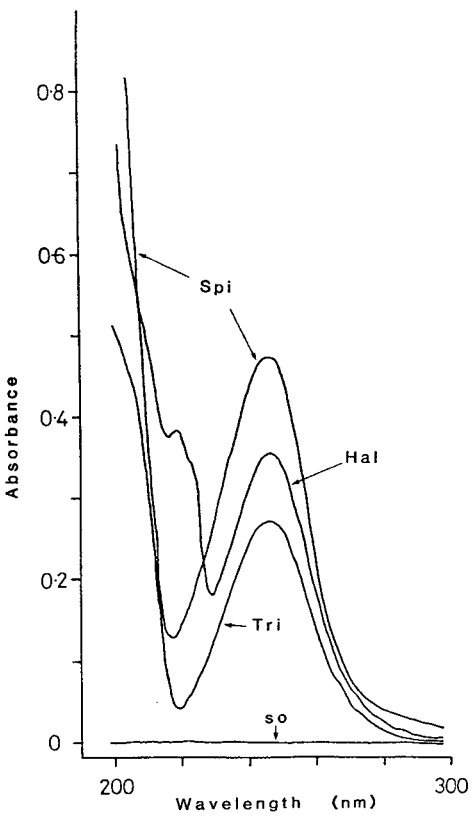

Fig. 1. UV absorption spectra of haloperidol (Hal), spiroperidol (Spi), and trifluperidol (Tri) and the solvent (so, 0.05% lactic acid). The concentration of butyrophenones was 10 μg/ml. The absorption spectrum of the solvent has been subtracted from all spectra.

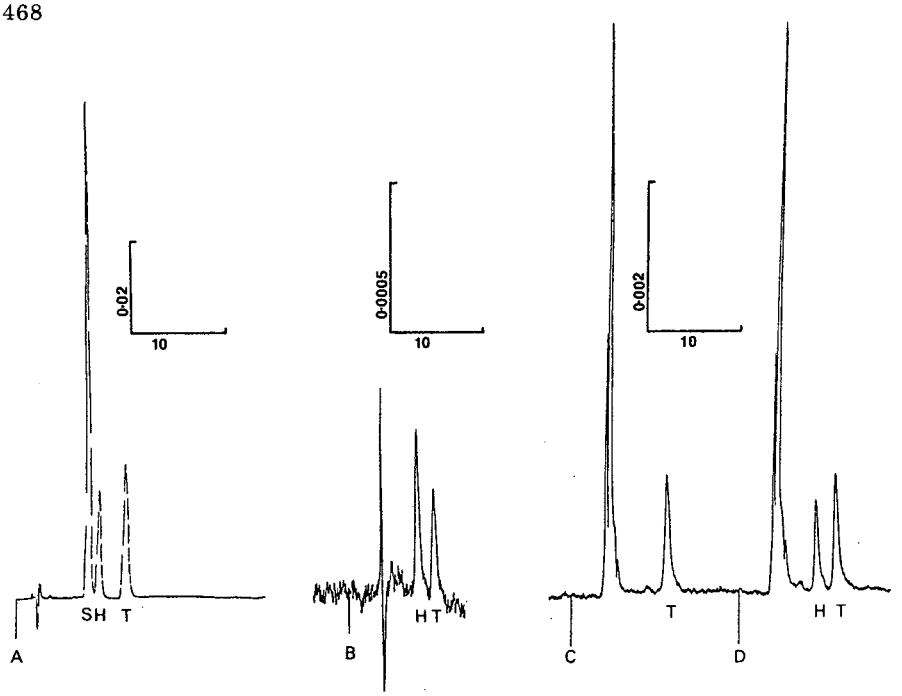

Fig. 2. Chromatograms of butyrophenones with detection at 254 nm. (A) Isocratic elution on a μBondapak C_{18} (300 × 3.9 mm) of 1.2 μg spiroperidol (S); 0.4 μg haloperidol (H); and 0.8 μg trifluperidol (T). Mobile phase, 0.1% TFA—30% acetonitrile; flow-rate, 1.2 ml/min; temperature, 23°C. (B) Chromatogram of 10 ng each of haloperidol (H) and trifluperidol (T). Conditions as in A except flow-rate (0.9 ml/min) and temperature (35°C). (C) Chromatogram of brain extract from vehicle-treated rat. Conditions as in B. T = trifluperidol, internal standard. (D) Chromatogram of brain extract from rat treated with 3 mg/kg haloperidol i.p. 2 h before sacrifice. H = haloperidol; T = trifluperidol, internal standard. Injection volume, 50 μl; Horizontal bar, 10 min; vertical bar absorbance (254 nm).

Fig. 2 where it can also be seen that the three drugs can be separated by isocratic elution on a μBondapak C_{18} column (Fig. 2A) and as little as 10 ng of each detected in a single injection (Fig. 2B). The sensitivity, calculated as twice baseline noise, was 1.5 ng for spiroperidol, 2 ng for haloperidol and 3 ng for trifluperidol. The response of the detector was linear from 10 ng to 10 μg.

The retention times of the three compounds were similar when the mobile phase contained either 0.1% TFA or 50 m*M* sodium acetate, pH 5 and with the same proportion of acetonitrile as organic modifier on the μBondapak C_{18} column. Chromatography on a μBondapak Phenyl column required a higher proportion of acetonitrile in the mobile phase for equivalent retention times but then there was no baseline resolution between spiroperidol and haloperidol. Better resolution on the phenyl-bonded phase was possible by reducing the proportion of acetonitrile to increase retention times or using gradient elution from 30% to 50% acetonitrile over 30 min. Similar results were obtained when methanol was used instead of acetonitrile as the organic modifier, except that the column temperature had to be increased or a higher proportion in the mobile phase was required.

Attempts were made to increase the sensitivity of detection by using low UV wavelengths. The peak height response to each compound was as predicted

by their UV absorption spectra. There was a maximum response at 247 nm; at 210 nm and below, peak height increased with decreasing wavelength but baseline noise also increased. For example, at 206 nm the peak response had doubled over that at 254 nm while baseline noise had increased four-fold. In anticipation of using the 214-nm zinc line, this wavelength was also evaluated. As would be predicted from Fig. 1, the response to haloperidol increased relative to 254 nm while the others decreased.

Extraction of butyrophenones from tissues was first attempted by precipitation of proteins with either 5% trichloroacetic acid (TCA) or 0.1 *M* perchloric acid (PCA), followed by organic extraction as described under Experimental. With either of these precipitants, however, there was poor recovery (< 10%) when drugs were added to brain tissues before homogenisation (Table I). Recovery of haloperidol or trifluperidol from TCA or PCA solutions was considerably higher than from brain homogenates. Protein precipitation, either by boiling the brain homogenate or with zinc sulphate—sodium hydroxide, also gave poor (< 10%) recoveries of butyrophenones. If the brains were homogenised in dilute hydrochloric acid, drug recovery was about 50% (Table I), so that this method was adopted for further investigation. The data in Table I also show that the recovery of haloperidol and trifluperidol closely follow each other so that one drug can be used as the internal standard to correct for the extraction efficiency of the other. Spiroperidol is also extracted in parallel with haloperidol and trifluperidol. The recovery from brain homogenate of added haloperidol (40—200 ng) was 44 ± 2% and for the amount of haloperidol calculated to be present in replicate estimations of the same brain homogenate, the coefficient of variation was 6.7% (n = 5).

Representative chromatograms of brain extracts from rats that had been treated with vehicle or haloperidol (3 mg/kg, i.p.) 2 h before sacrifice are

TABLE I

RECOVERY OF HALOPERIDOL AND TRIFLUPERIDOL FROM BRAIN HOMOGENATES BY SEVERAL ORGANIC EXTRACTION PROCEDURES

Rat brain was homogenized in 4 vols. of distilled water and haloperidol and trifluperidol were added to 500 ng/ml. An equal volume of double-concentrated acidic reagent was added and then extracted as described in the text. For "reagent alone", distilled water was used instead of the brain homogenate.

Reagent	Molarity	Percentage recovery (mean ± S.D.)			
		From acid alone		From brain homogenate	
		Haloperidol	Trifluperidol	Haloperidol	Trifluperidol
Trichloroacetic acid	0.3	67 ± 2	68 ± 4	10 ± 2	13 ± 3
Perchloric acid	0.1	63 ± 1	67 ± 3	25 ± 4	26 ± 2
Hydrochloric acid	0.01	73 ± 4	69 ± 5	45 ± 1	44 ± 1
Hydrochloric acid	0.05	—	—	50 ± 3	50 ± 4
Hydrochloric acid	0.1	—	—	49 ± 6	48 ± 3

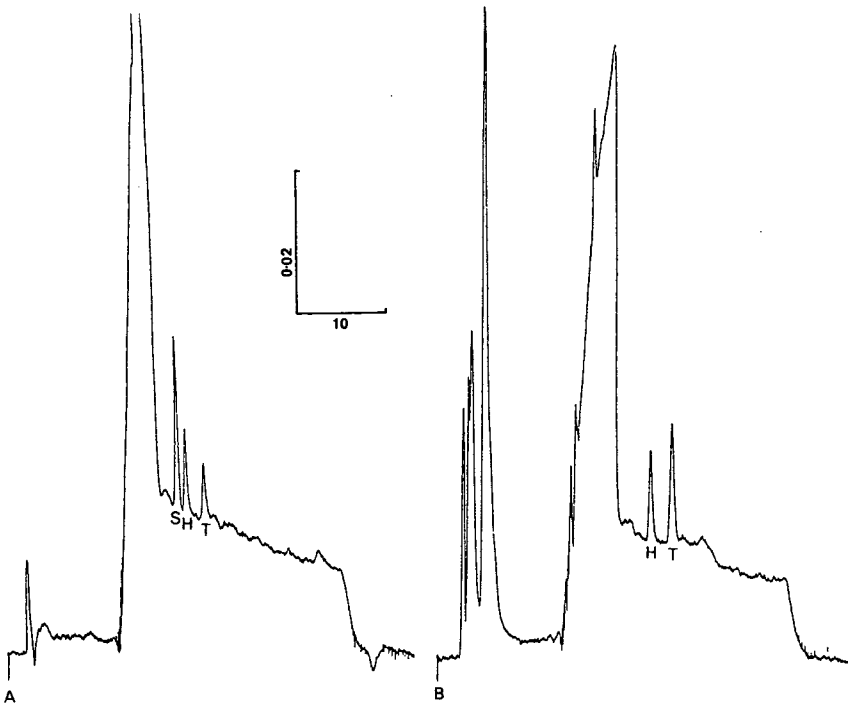

Fig. 3. (A) Step-gradient chromatogram of 25 ng each of spiroperidol (S), haloperidol (H) and trifluperidol (T). See text for details. (B) Step-gradient chromatogram of brain extract from a rat treated with 0.25 mg/kg haloperidol (H) i.p. T = trifluperidol, internal standard. Horizontal bar, 10 min; vertical bar, absorbance 254 nm.

shown in Fig. 2C and D, respectively. The trifluperidol internal standard peak was present in both chromatograms while haloperidol was present only in the extract from the drug-treated animal. The mean (± S.E.M.) haloperidol content of four different brains from animals receiving the same treatment was 2.28 ± 0.25 μg/g of tissue. These results were obtained with extracts that represented a small proportion of the total brain tissue. By the use of trace enrichment and step-gradient chromatography it was possible to easily quantify brain haloperidol 2 h after a dose of 0.25 mg/kg. In Fig. 3A, the column was equilibrated with 0.1% TFA and an injection containing 25 ng of spiroperidol, haloperidol and trifluperidol in 1.5 ml of 5 m*M* sulphuric acid was made onto the column. After 5 min the mobile phase was switched to 40% acetonitrile in 0.1% TFA and chromatography continued for a further 25 min. All three butyrophenones were resolved by this procedure and readily detected at 254 nm. For Fig. 3B, half of the brain was homogenized in 8 ml of 10 m*M* hydrochloric acid and then extracted as described above after the addition of 250 ng of trifluperidol as internal standard. The final sulphuric acid extract had a volume of 1.5 ml. A portion of this (750 μl) was diluted to 1.5 ml and chromatographed as for the standard in Fig. 3A. Fig. 3B then, represents the haloperidol content of only one quarter of the brain, calculated to be 0.19 μg/g of tissue for this sample and 0.18 μg/g of tissue for a second animal treated in

the same way. The extraction efficiency for the internal standard was 44%. The detection limit for a 1-g tissue sample would be 10 ng for spiroperidol and 25 ng for haloperidol if the whole extract (1.5 ml) was injected.

DISCUSSION

The work reported in this paper has demonstrated that butyrophenone neuroleptics can be quantified with high sensitivity by UV detection at 254 nm after reversed-phase HPLC. The sensitivity of the method would only be marginally improved by detection at 247 nm or lower (200—210 nm) wavelengths, and would then be accompanied by a reduction in the signal-to-noise ratio (i.e. increased noise relative to that of the high energy mercury line emission at 254 nm). Detection at 254 nm also allows the use of methanol in the mobile phase (when an octyl or hexyl reversed-phase column would be preferred) avoiding the higher cost and toxicity of acetonitrile, and reduces the possibility of interference from tissue contaminants and other drugs [7]. The use of the 214-nm line of a zinc lamp may be applicable to haloperidol analysis and also aid in its chemical characterisation by the 254 nm/214 nm response ratio.

The primary intention for this work was to devise a method for quantifying tissue butyrophenone content. It was initially hoped to extract tissues by using efficient protein precipitants and then inject the protein-free extract directly onto the liquid chromatograph, but recoveries were low. The poorer recovery in the presence of brain tissue may also be the result of the partitioning of these extremely lipophilic compounds into the precipitated membranes. Recovery from brain was increased by the use of dilute hydrochloric acid, but the resultant extract was too impure for direct injection routinely so that further purification became necessary.

The solvent extraction reported by Forsman et al. [1] for gas chromatographic analysis gave adequate recovery and the chromatograms were not complicated by any tissue-derived peaks (Fig. 2C and D). Haloperidol levels in rat brain areas after doses of 1—3 mg/kg were readily quantified with this method. The trace enrichment method reduces the detection limit for tissue haloperidol. From the data in Fig. 3B it can be shown that small brain areas (e.g. one twentieth of a rat brain, approximately 75 mg tissue) could be successfully analysed by increasing the proportion of the extract to be injected, or butyrophenone content could be measured after even lower doses (e.g. 0.05 mg/kg) had been administered. Since the sensitivity of this method is very similar to the gas chromatographic method [1, 2], it follows that plasma samples can also be analysed for butyrophenones by reversed-phase HPLC with UV detection [3, 4]. HPLC methods have the advantage of being less dependent upon extremely low injection volumes so that some of the volume-reducing extraction steps and solvent evaporations can be omitted when isocratic chromatography is employed and hence reduce extraction losses. The step-gradient modification is even less dependent on the use of small injection volumes.

In conclusion, it has been shown that haloperidol can be measured in tissue samples by a combination of reversed phase chromatography and highly

sensitive UV detection. This method can be extended to spiroperidol, trifluperidol and probably other butyrophenones with minor modifications. It is suggested that this method could be applied to the analysis of butyrophenones in plasma and that the sensitivity could be further increased by using larger plasma samples and trace enrichment.

ACKNOWLEDGEMENT

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REFERENCES

1 A. Forsman, E. Martensson, G. Nyberg and R. Ohman, Naumyn-Schmiedeberg's Arch. Pharmacol., 286 (1974) 1367.
2 G. Bianchetti and P.L. Morselli, J. Chromatogr., 153 (1978) 203.
3 I. Creese and S.H. Snyder, Nature (London), 270 (1977) 180.
4 R.C. Smith, G. Vroulis, C.H. Misra, J. Schooler, C. DeJohn, P. Korivi, D.E. Leelavathi and D. Arzu, Commun. Psychopharmacol., 4 (1980) 451.
5 B.M. Cohen, M. Herschel and E. Miller, Neuropharmacology, 19 (1980) 663.
6 R.E. Poland and R.T. Rabin, Life Sci., 29 (1981) 1837.
7 E.R. Korpi, B.H. Phelps, H. Granger, W.-H. Chang, M. Linnoila, J.L. Meek and R.J. Wyatt, Clin. Chem., 29 (1983) 624.
8 K. Miyazaki, T. Arita, I. Oka, T. Koyama and I. Yamashita, J. Chromatogr., 223 (1981) 449.
9 P.I. Jatlow, R. Miller and M. Swigar, J. Chromatogr., 227 (1982) 233.
10 P.J.A.W. Demoen, J. Pharm. Sci., 50 (1961) 350.

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Note

Determination of gabapentin in plasma and urine by high-performance liquid chromatography and pre-column labelling for ultraviolet detection

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(First received November 30th, 1984; revised manuscript received February 5th, 1985)

Gabapentin [1-(aminomethyl)cyclohexaneacetic acid] (Fig. 1) is a new γ-aminobutyric acid (GABA) analogous substance [1] with penetration of the blood brain barrier and anticonvulsant activities, mainly in seizures elicited by interference with gabaergic transmission [2] or in seizures provoked by excitatory amino acids [3]; for a review, see ref. 4. It is well absorbed and excreted completely unchanged by humans [5] and shows no protein binding.

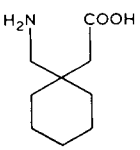

Fig. 1. Chemical structure of gabapentin.

For pharmacokinetic investigations, an assay method for gabapentin in biological fluids was required. This paper reports the sensitive and selective determination of gabapentin in the submicrogram and nanogram range in plasma and urine by means of high-performance liquid chromatography (HPLC). The method is based on the detection of amino acids by pre-column derivatization with 2,4,6-trinitrobenzenesulphonic acid (TNBS) [6, 7] utilising UV photometric detection. The applicability of the method to pharmacokinetic studies is demonstrated.

EXPERIMENTAL

Chromatography

A Series 2/2 high-performance liquid chromatograph equipped with a Rheo-

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dyne 7125 injection valve (Perkin-Elmer, Überlingen, F.R.G.) was used in combination with a Model SF 773 UV detector with a 12-μl flow cell (Kratos, Karlsruhe, F.R.G.). The analyses were performed on a 25 cm × 4 mm I.D. stainless-steel column packed with 10-μm LiChrosorb RP-18 (Knauer, Berlin, F.R.G.).

Chromatograms were recorded on a 0.001—10 V recorder (Hitachi—Perkin-Elmer, Überlingen, F.R.G.). Peak areas were determined by an Autolab System I computing integrator (Spectra-Physics, Darmstadt, F.R.G.).

All analyses were performed using a mobile phase consisting of 58% acetonitrile in water containing 0.5% acetic acid. The flow-rate was 1.0 ml/min. The chromatography was carried out at ambient temperature.

Reagents and standards

Gabapentin and the internal standard, 1-(aminomethyl)cycloheptaneacetic acid (Gö 3609), were obtained from the Chemistry Department, Gödecke Research Institute, Freiburg, F.R.G.

All chemicals were of the highest grade commercially available. They were purchased from E. Merck (Darmstadt, F.R.G.), except for TNBS, which was supplied by Serva (Heidelberg, F.R.G.).

For aqueous solutions, water was purified by reverse osmosis and additionally passed through a water purification system for adsorption of organic substances (Millipore, Neu Isenburg, F.R.G.).

Crystalline forms of 2,4,6-trinitrophenylgabapentin and 2,4,6-trinitro-1-(aminomethyl)cycloheptaneacetic acid were prepared by a procedure similar to that of Caudill et al. [7].

A stock standard solution of gabapentin was prepared by dissolving an appropriate amount of gabapentin in water. Working standards were prepared freshly in drug-free plasma from the stock solution to yield concentrations from 50 ng/ml to 10 μg/ml.

Sample preparation

The derivatization procedure is a modified version of that described by Caudill et al. [7] for the determination of GABA. A 0.5-ml aliquot of standard, control or patient plasma was placed in a 1.5-ml Eppendorf centrifuge tube and an appropriate amount of the internal standard, dissolved in 10 μl water, was added. This was followed by the addition of five drops of 2 *M* perchloric acid to deproteinize the sample. The tube was vortexed vigorously for a few seconds and then centrifuged for 2 min in an Eppendorf microcentrifuge at 15 000 *g* to precipitate the proteins. The supernatant was collected in stoppered conical glass tubes (100 × 14 mm) and 0.5 ml of 1 *M* sodium hydrogen carbonate and 50 μl of a 2 *M* aqueous solution of the derivatizing agent TNBS were added. The pH was adjusted to 8.5 with 0.1 *M* sodium hydroxide solution and the reaction was allowed to progress for 30 min at room temperature. The reaction was quenched by the addition of two drops of 25% hydrochloric acid. Toluene (3 ml) was added to the acidified sample and the mixture shaken for 10 min, followed by centrifugation for 2 min at 5000 *g*. The upper organic phase was transferred into a 5-ml tapered flask and evaporated to dryness on a rotary evaporator at 40°C. The residue was reconstituted with 100 μl of 0.2 *M* sodium

borate buffer (pH 8.5) and washed (vortexing for 1 min) with 1 ml of cyclohexane containing 10% of toluene. A volume of 10—50 µl of the sodium borate buffer solution containing the TNP derivatives was injected directly on to the HPLC column.

When analysing urine samples for their gabapentin content, 10—100 µl of urine were fortified with 2 µg of internal standard dissolved in 10 µl of water, and processed as described above for the plasma samples after the deproteinization step.

Stability studies were also conducted using a pool of plasma spiked with known amounts of gabapentin. Aliquots of these samples were frozen at −18°C and analysed over a period of six months. No significant changes in the gabapentin content were seen.

The within-run precision was evaluated by assaying a prepared gabapentin plasma pool.

RESULTS AND DISCUSSION

The direct isolation of gabapentin from an aqueous matrix was hampered by its hydrophilic nature. Therefore, derivatization was carried out in order to facilitate the extraction of the substance and to aid its sensitive detection by attachment of a chromophoric group.

TNBS proved to be a suitable derivatizing reagent, resulting in the formation of the corresponding HPLC-detectable TNP derivatives of gabapentin and the

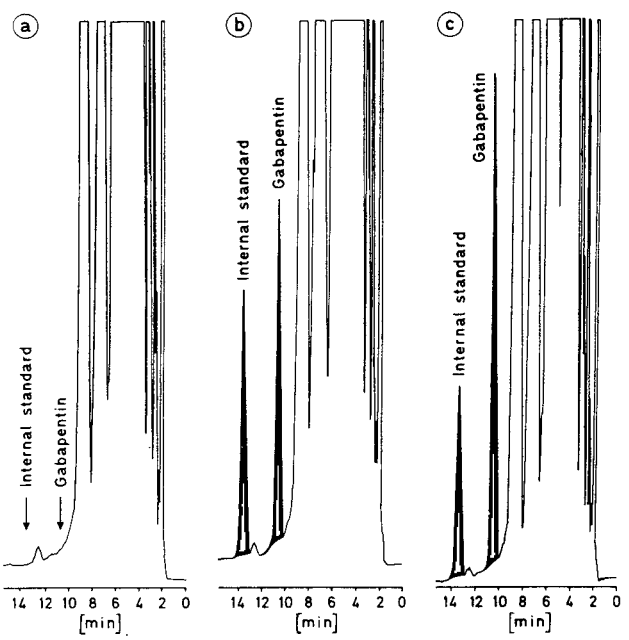

Fig. 2. Chromatograms of human plasma samples obtained after derivatization with 2,4,6-trinitrobenzenesulphonic acid. (a) Blank plasma (0.5 ml). (b) Plasma sample, supplemented with 500 ng of gabapentin and 400 ng of internal standard per 0.5 ml. (c) Plasma sample, 10 h after oral administration of 200 mg of gabapentin to a human volunteer (amount equal to 1844 ng/ml gabapentin).

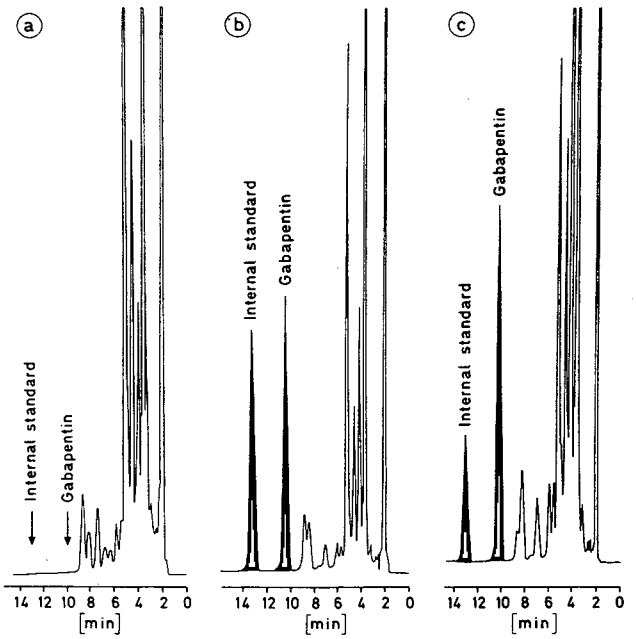

Fig. 3. Chromatograms of human urine samples obtained after derivatization with 2,4,6-trinitrobenzenesulphonic acid. (a) Blank urine (50 μl). (b) Urine sample, fortified with 2 μg of gabapentin and 2 μg of internal standard per 50 μl. (c) Urine sample, 12—15 h fraction after oral administration of 200 mg of gabapentin to a human volunteer (amount equal to 4.8 μg of gabapentin per 50 μl).

internal standard. Amino acids or biogenic amines, present in large amounts in plasma and urine, also reacted with TNBS, forming intensely yellow-coloured compounds. However, these reaction products appeared at the beginning of the chromatogram, were well separated and did not interfere with the compounds of interest.

Typical chromatograms of plasma and urine samples are shown in Figs. 2 and 3. The retention times for 2,4,6-trinitrophenylgabapentin and 2,4,6-trinitrophenyl-1-(aminomethyl)cycloheptaneacetic acid (internal standard) were 10.3 and 13.2 min, respectively. The recovery and derivatization yield of gabapentin and the internal standard with the above method were ca. 90% when comparing the peak heights with directly injected TNP-gabapentin and internal standard derivatives. The minimum detectable concentrations were determined to be about 10 ng/ml in plasma. The peak-area ratios for gabapentin and the internal standard were linearly related ($r = 0.999$) to the amount of gabapentin added to blank plasma over a range from 20 ng/ml to 10 μg/ml.

The reproducibility of the calibration graphs was assessed by assaying triplicate plasma standards over a one-day period. The relative standard deviations of the peak-area ratios between the lowest and highest concentrations were found to range from 8.6% to 0.5%, respectively. These data are summarized in Table I. To enhance the precision when analysing lower concentrations of gabapentin (below 400 ng/ml in plasma), only 150 ng of internal standard were used.

TABLE I

WITHIN-DAY PRECISION AND ACCURACY
Peak-height ratios of gabapentin versus internal standard after three calibration runs with spiked human plasma ($n = 3$).

Amount (ng per 0.5 ml)	Amount of internal standard used (ng)	Mean peak-height ratio	Coefficient of variation (%)
10	150	0.113	8.67
20		0.207	4.73
50		0.503	9.80
100		0.987	0.58
200		2.037	1.58
500	1200	0.4730	2.43
1000		0.9270	0.81
2000		1.8900	2.01
5000		4.6200	0.43

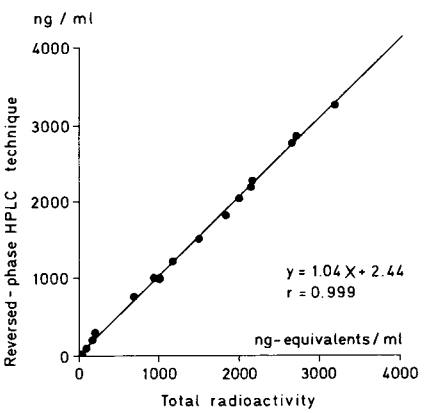

Fig. 4. Comparison of HPLC data and total radioactivity. Gabapentin concentrations in a series of human plasma samples following an oral dose of 200 mg of ^{14}C-labelled compound, determined both by HPLC and by total radioactivity monitoring.

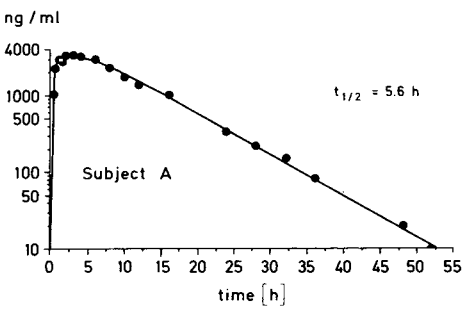

Fig. 5. Plasma levels of gabapentin following an oral dose of 200 mg to a human volunteer, obtained by HPLC.

Plasma and urine samples, obtained in the course of a metabolic study in human volunteers using ^{14}C-labelled gabapentin [5], were assayed by HPLC. These samples had been analysed by liquid scintillation counting on a previous occasion [5]. As gabapentin does not undergo metabolic transformation in humans, the total ^{14}C radioactivity data correspond to the unchanged compound in plasma. The results of both the HPLC and radioactivity detection are presented in Fig. 4 and exhibit an excellent correlation (slope = 1.04, $r = 0.999$) between the methods over a concentration range from 20 ng/ml to 3.5 μg/ml.

Fig. 5 shows a characteristic plasma level versus time profile following oral administration of 200 mg of gabapentin to a male volunteer.

REFERENCES

1 G. Satzinger, J. Hartenstein, M. Herrmann and W. Heldt, Ger. Offen., DE 2,460,891, 1974.
2 G.D. Bartoszyk, E. Fritschi, M. Herrmann and G. Satzinger, Naunyn-Schmiedeberg's Arch. Pharmacol., 322 (1983) R94.
3 G.D. Bartoszyk, Naunyn-Schmiedeberg's Arch. Pharmacol., 324 (1983) R24.
4 Anonymous, Drugs Future, 9 (1984) 418.
5 A. von Hodenberg and K.-O. Vollmer, Naunyn-Schmiedeberg's Arch. Pharmacol., 324 (1983) R74.
6 D.J. Edwards, in K. Blau and G.S. King (Editors), Handbook of Derivatives for Chromatography, Heyden, London, 1977, p. 395.
7 W.L. Caudill, G.P. Houck and R.M. Wightman, J. Chromatogr., 227 (1982) 331.

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