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JOURNAL OF CHROMATOGRAPHY

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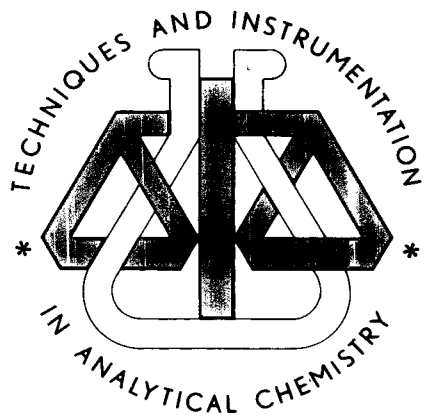

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Biomedical Applications

CHROMBIO. 560

THE USE OF FORMIC ACID IN CARRIER GAS

RAPID METHOD FOR IDENTIFICATION AND DETERMINATION OF PHYTANIC ACID BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—CHEMICAL IONIZATION

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(First received November 27th, 1979; revised manuscript received January 22nd, 1980)

SUMMARY

A rapid gas chromatographic method to determine phytanic acid in plasma from Refsum's disease is described. After a brief alkaline hydrolysis of lipids, the biological sample is directly injected into a glass pre-column; an acid carrier gas (formic acid in nitrogen) is used to displace the long-chain fatty acids from their sodium salts and from their binding to proteins. Formic acid introduced through the column may also be used as a reagent gas for chemical ionization in combined gas chromatography—mass spectrometry; fatty acids (C_{14} to $C_{18:2}$ and phytanic acid) are easily identified by their M + 1 (base peak) and M — 17 peaks. The described procedure is also suitable for studying normal fatty acids from plasma lipids.

INTRODUCTION

The use of an acid carrier gas in gas chromatography (GC) has been shown to

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be useful in the screening of some metabolic diseases by allowing direct injection of plasma volatile fatty acids (VFA) and limiting artefacts due to the adsorption of free VFA on the column [1—3]. Moreover, formic acid in carrier gas may be used as the reagent gas of chemical ionization in combined gas chromatography—mass spectrometry (GC—MS) [4] which allows an easy identification of the acids by their (M + 1) ions. When used with Carbowax 20M as stationary phase, this technique is suitable for the determination of C_2 to C_8 plasma VFA, saturated or not, branched or not, but this column packing is not polar enough to allow a rapid separation of long-chain fatty acids.

As previously described [5], the use of 10% SP 216 PS as stationary phase allows a rapid elution of C_{10} to C_{18} free fatty acids. So it seems interesting to adapt the technique of on-column elution to the analysis of long-chain fatty acids by using SP 216 PS as stationary phase. This procedure, which bypasses the tedious extraction and derivatization steps, should be much more rapid and simple than previously described methods for the identification and estimation of phytanic acid in the diagnosis and care of Refsum's disease [6–8].

Refsum's disease is an inherited lipid storage disease in which phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) accumulates in various organs and fluids; this accumulation is due to the failure of the α-oxidation system which allows the conversion of phytanic acid to the α-hydroxy derivative. Phytanic acid, virtually undetectable in normal plasma, has been found, up to 1.7 mg/ml in the blood plasma of patients with Refsum's disease [9,10].

MATERIALS AND METHODS

Principle

After a rapid alkaline hydrolysis, the biological sample is directly injected into a glass pre-column. Fatty acids are displaced from their salts and protein binding by a formic acid flow saturating the carrier gas.

Reagents

Formic acid (98%) was purchased from Baker (Deventer, The Netherlands), stationary phase SP 216 PS from Sulpelco (Bellefonte, PA, U.S.A.), other chromatographic reagents from Varian Aerograph (Walnut Creek, CA, U.S.A.), phytanic acid from Applied Science Europe (Oud-Beijerland, The Netherlands) and other chemicals from Merck (Darmstadt, G.F.R.).

All volumes were measured with Hamilton syringes.

Gas chromatography

An Intersmat IGC 120 DFL dual-column gas chromatograph equipped with a flame ionization detector (FID) was used without modification. The injector was equipped with a glass pre-column (Insert tube Intersmat) carefully acid-washed, rinsed out, then heated overnight at 270°C in the injector port. This injector was maintained at 270°C and the FID at 200°C.

The stainless-steel column (2 m × 2 mm I.D.) was packed with 10% SP 216 PS on Supelcoport (100—120 mesh) and was operated isothermally at 170°C. Just before gas entry, the nitrogen flow (40 ml/min) was saturated with acid by flowing without bubbling over 1 ml of pure formic acid contained in a 5-ml

screw-cap vial as previously described [3]. The use of pure anhydrous formic acid was necessary to avoid a premature deterioration of the column, especially with a polar phase such as SP 216 PS.

Combined gas chromatography—mass spectrometry

Combined GC—MS was performed on a Ribermag 10-10 B equipped with a Girdel 30 gas chromatograph and a Ribermag 400 data system computer. GC conditions were identical, except for the carrier gas (helium instead of nitrogen) and column temperature (200°C instead of 170°C). GC—MS interface temperature was 250°C, electron energy 70 eV, formic acid in the carrier gas was used as chemical ionization reagent gas, each mass from *m/e* 100 to *m/e* 400 was integrated during 5 msec.

Procedure

Internal standard, pentadecanoic acid in methanol (5 μl) and 20 μl of 10 *M* sodium hydroxide were added to 100 μl of heparinized plasma obtained from blood drawn at the finger tip. After 2 min at 20°C, the solution was rapidly centrifuged and 1 μl injected into the gas chromatograph. Between injections of biological samples 10 μl of 50% (v/v) formic acid were injected in order to clean the pre-column from residual compounds possibly present. In such conditions, the pre-column was used for up to about 20 injections with protein-containing samples.

RESULTS AND DISCUSSION

As noticed by Skrbic and Cumings [11] phytanic acid in plasma from Refsum's disease is esterified and represents up to 35% of total fatty acids. So it is necessary to hydrolyze phytanic acid from its plasmatic esters, which is rapidly performed under strong alkaline conditions (1.7 *M* sodium hydroxide, final concentration) (Fig. 1). The injection of more than 1 μl of such a solution into the gas chromatograph raises some difficulties with regard to the quantity of formic acid found in the injection port during the injection time and it is necessary to displace the sodium salts of fatty acids. One microlitre gives sufficient sensitivity and creates no problem, but the injection of a larger volume (e.g. 5 μl) of alkaline solution leads to a non-quantitative recuperation of injected material and appearance of ghost peaks due to the displacement of the residual salts during subsequent aqueous injections. The linearity of the detector response has been tested on six concentrations from 0.5 m*M* to 10 m*M* for C_{14}, C_{16} and C_{18} in chloroformic solutions. This study has shown that detector response was quite linear for each acid at the quoted concentrations and the response coefficient was very close from one acid to another (1.33 per mmol for C_{18}, 1.31 per mmol for C_{14}).

Thus, results of phytanic acid determinations are expressed in reference to C_{15}, assuming a similar response coefficient; C_{15} was chosen as an internal standard, because it is chemically related to phytanic acid, practically absent from biological fluids, and is well separated from C_{14} and C_{16}. It was added in the concentration range of phytanic acid in plasma from Refsum's disease. The small systematic error possibly arising from reference to C_{15} is not a real problem in clinical applications.

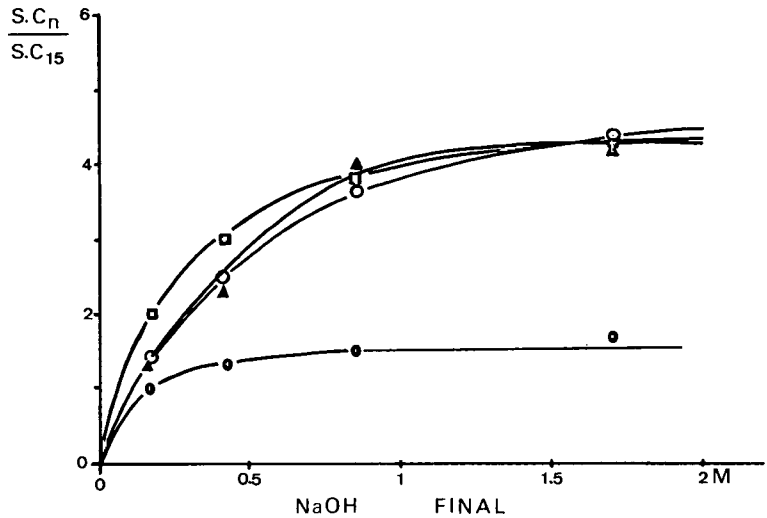

Fig. 1. Influence of final sodium hydroxide concentration on the liberation of fatty acids from normal plasma lipids. Results are expressed as area ratios to internal standard (C_{15}). ■, C_{16}; ●; C_{18}, ○, $C_{18:1}$ and ▲, $C_{18:2}$.

An error by excess in quantification could be made because of the poor separation of C_{17} and phytanic acid on SP 216 PS, but C_{17} is present in a small amount relative to phytanic acid in Refsum's disease.

Standard deviation, studied by ten repetitive determinations on plasma from

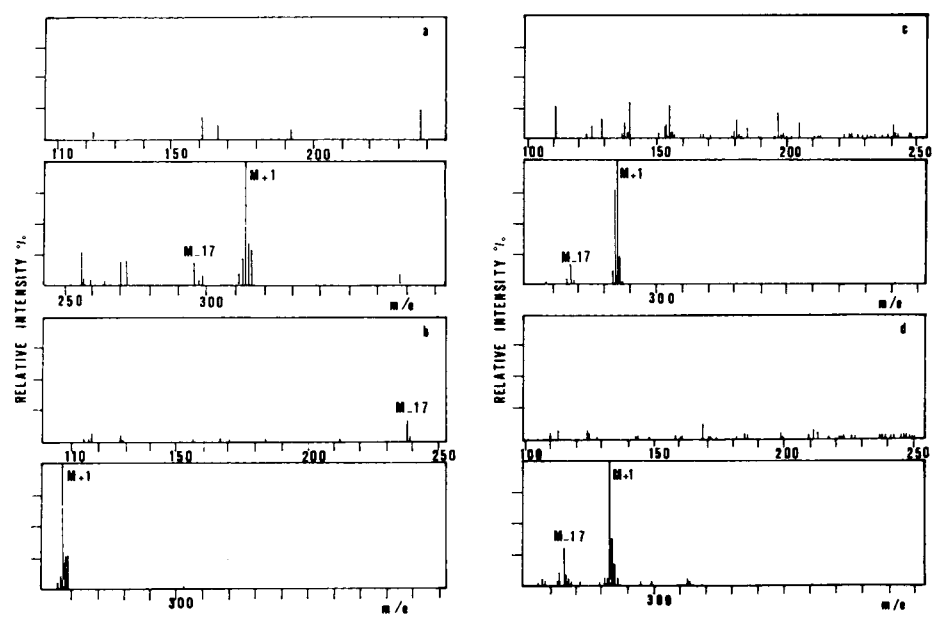

Fig. 2. Chemical ionization (formic acid) mass spectra of four fatty acids. (a) Phytanic acid, M + 1 = 313; (b) palmitic acid C_{16}, M + 1 = 257; (c) stearic acid C_{18}, M + 1 = 285; (d) oleic acid $C_{18:1}$, M + 1 = 283. Spectra were identical with those of authentic acids.

Refsum's disease, was 0.013 at a concentration of 0.265 mg/ml (5%).

As shown by Figs. 2 and 3 the identification of C_{16}, C_{18}, $C_{18:1}$ and phytanic acid may be achieved by formic acid—chemical ionization—mass spectrometry combined to the above-described GC procedure. Characteristic spectra with M + 1 (base peak) and M − 17 peaks were obtained (Fig. 2). They were identical with those of authentic acids. Thus, mass chromatograms of the M + 1 base peak allow unambiguous identification of phytanic acid (Fig. 3B) in plasma from Refsum's disease and the absence of *m/e* 313 (M + 1) base peak in normal plasma (Fig. 3A).

Results in Refsum's disease; comparison with other procedures

The described technique was first tested to quantify the plasmatic non-

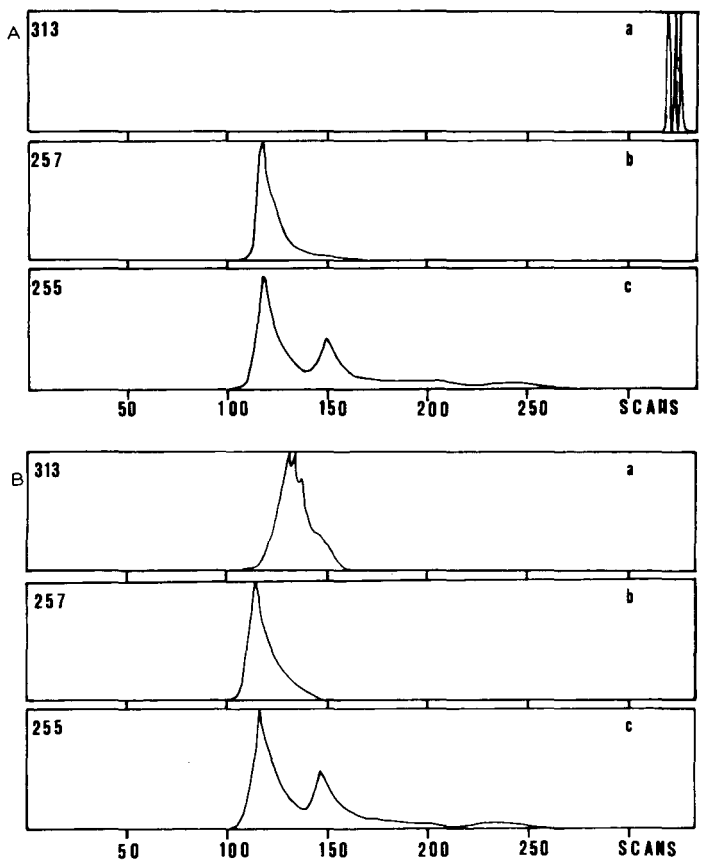

Fig. 3. Mass chromatograms of the fatty acids hydrolysed by 1.7 *N* sodium hydroxide. For GC—MS conditions see text. (A) Normal plasma and (B) Refsum's disease plasma. To achieve a more rapid separation, mass chromatograms in the GC—MS system were performed at 200°C whereas simple chromatographic separations had been realized at 170°C; the elution time of phytanic acid relative C_{16} and $C_{16:1}$ was modified by this temperature difference. Formic acid was used as carrier gas and as a reagent gas in chemical ionization. Each mass corresponds to the M + 1 base peak of a fatty acid. Each chromatogram is normalized on the largest peak so that 313 peak of Fig. 3A is practically non-existent and does not correspond to phytanic acid.

esterified fatty acid (NEFA) but it is not sensitive enough under the described conditions to allow an estimation of each class of NEFA. In Refsum's disease, Fig. 4 shows that phytanic acid is undetectable in the NEFA plasma fraction.

The phytanic acid content of hydrolyzed plasma lipids of Refsum's disease (three patients) (Fig. 5) was compared by two procedures (Table I), the above described on-column injection method and the classical extraction and methylation procedure. Samples were treated by chloroform—methanol (2:1, v/v), and the extract evaporated and methylated [8]. After concentration, the resulting solution was injected onto 10% polyethylene glycol adipate on diatomite (100—120 mesh). All results were expressed with reference to C_{15}.

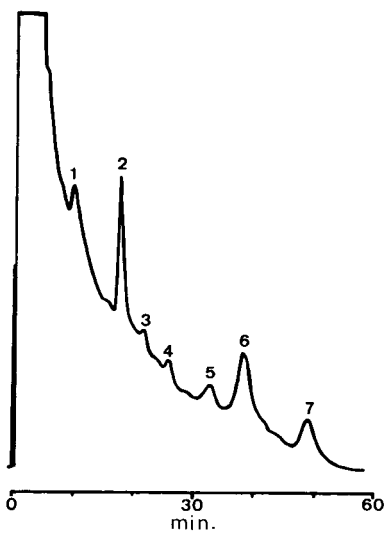

Fig. 4. Non-esterified fatty acids profile in plasma from Refsum's disease. Peaks: 1 = C_{14}; 2 = C_{16}; 3 = $C_{16:1}$; 4 = C_{17} and phytanic acid; 5 = C_{18}; 6 = $C_{18:1}$; 7 = $C_{18:2}$. For chromatographic procedure, see text. Column temperature, 170°C.

TABLE I

COMPARISON OF TWO METHODS, EXTRACTION—METHYLATION AND ON-COLUMN ELUTION DIRECT PROCEDURE, FOR THE ESTIMATION OF PHYTANIC ACID

All results were referred to C_{15} (internal standard).

Sample		Phytanic acid (μg/ml)	
		Extraction—methylation, fresh plasma	Direct procedure, deep frozen plasma
M.G.	17/4	65	90
	31/10	51	65
M.R.	10/10	205	265*
	31/10	50	60
M.S.	untreated	920	870**

*n = 10; S.D. = ± 0.013.

**Fresh plasma.

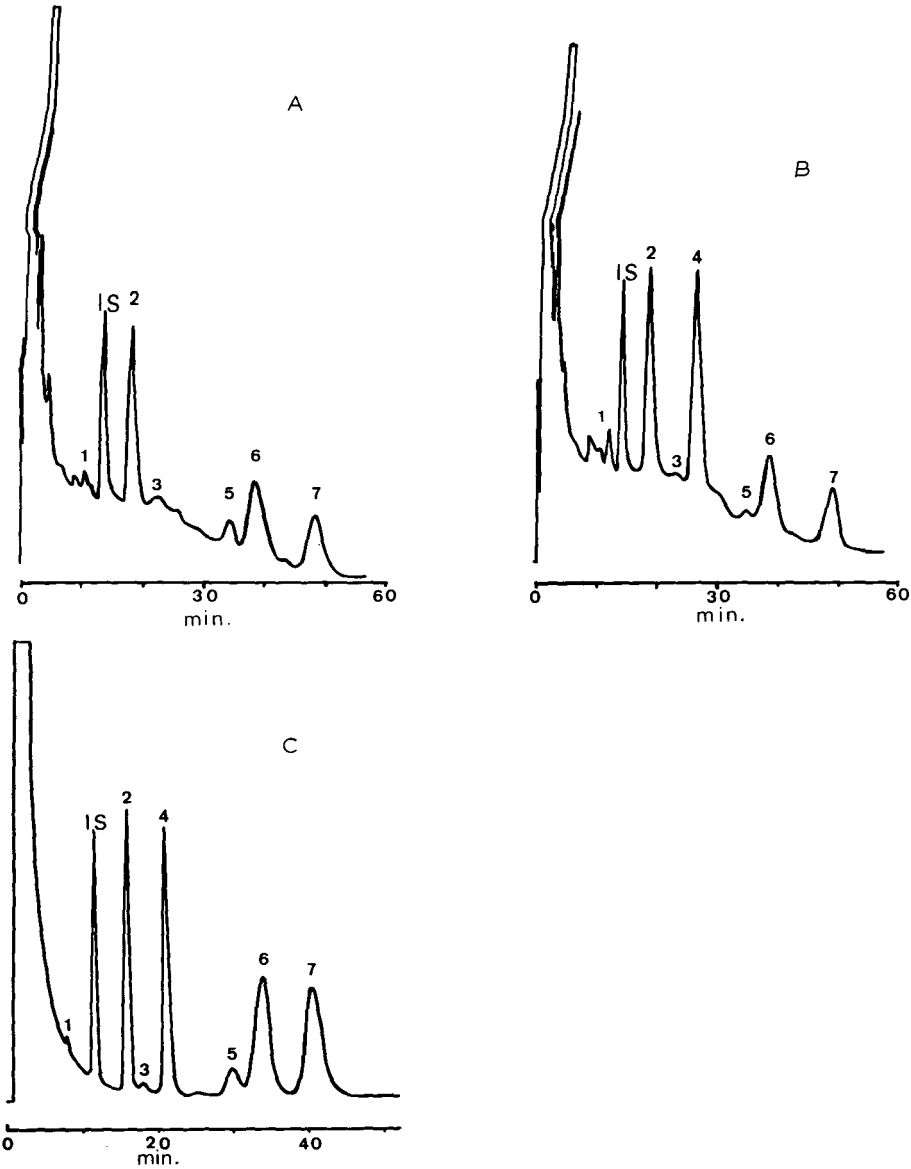

Fig. 5. Plasma fatty acids profile after alkaline hydrolysis. (A) Normal plasma after alkaline hydrolysis direct injection. (B) Plasma from Refsum's disease after alkaline hydrolysis. Direct injection (phytanic acid, 870 μg/ml). (C) Plasma from Refsum's disease (same sample as B) after extraction and methylation (phytanic acid, 920 μg/ml). For chromatographic details see text. Column temperature, 170°C. Peaks: 1 = C_{14}; 2 = C_{16}; 3 = $C_{16:1}$; 4 = C_{17} and phytanic acid; 5 = C_{18}; 6 = $C_{18:1}$; 7 = $C_{18:2}$; IS = internal standard, pentadecanoic acid in methanol.

The fatty acid composition of plasma lipids of one patient (untreated Refsum's disease) was also compared by the two procedures (Table II) which gave similar results and confirmed the high percentage of phytanic acid already noticed by Skrbic and Cumings [11].

CONCLUSION

The described method presents several advantages. First, it allows a very

TABLE II

FATTY ACID COMPOSITION OF PLASMA LIPIDS FROM UNTREATED REFSUM'S DISEASE

Fresh plasma of patient M.S. Total fatty acids: extraction procedure, 920 μg/ml and direct procedure, 870 μg/ml. All results were referred to C_{15} internal standard.

	Total fatty acids (%)	
	Extraction—methylation	Direct
C_{14}	1	2
C_{15}	22	23
Phytanic acid + C_{17}	30	31
C_{18}	5	5
$C_{18:1}$	20	22
$C_{18:2}$	22	17

simple and rapid estimation of phytanic acid in Refsum's disease; no prior manipulations, neither extraction nor derivatization are necessary; a very small sample (1 μl) of alkalinized plasma is directly injected into the pre-column and the whole experiment does not last more than 60 min. Second, it appears to be also suitable for the determination of the total fatty acid composition of plasma lipids. Third, though not very sensitive, it may eventually be used without hydrolysis for a rapid estimation of the main plasmatic non-esterified fatty acid (formic acid does not break the ester bonds of lipids). Finally, formic acid in the carrier gas may also be used to achieve chemical ionization in combined mass spectrometry. Thus, if such an equipment is available, the identification of fatty acids may be easily confirmed, and the sensitivity largely increased by using mass chromatography or fragmentography of the main characteristic peaks (M + 1, M − 17).

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The authors thank Mr. Hardy and Ribermag for fruitful help in the GC—MS analysis and Miss D. Bresson for the preparation of the manuscript.

REFERENCES

1 R.G. Ackman and R.D. Burgher, Anal. Chem., 35 (1963) 647—652.
2 J.C. Cochrane, J. Chromatogr. Sci., 13 (1975) 440—447.
3 B. Pileire, Clin. Chim. Acta, 88 (1978) 321—327.
4 Ph. Beaune, B. Pileire, F. Roccicciolli, M. Hardy, J.P. Leroux and P. Cartier, in A. Frigerio and M. McCamish (Editors), Recent Developments in Mass Spectrometry in Biochemistry and Medicine 6, Elsevier, Amsterdam, 1980, in press.
5 D. Sampson and W.J. Hensley, Clin. Chim. Acta, 61 (1975) 1—8.
6 J.T. Dulaney, M. Williams, J.E. Evans, C.E. Costello and E.H. Kolodny, Biochim. Biophys. Acta, 529 (1978) 1—12.
7 G. Phillipou and A. Poulos, Clin. Chim. Acta, 72 (1976) 319—325.
8 E. Klenk and W. Kahlke, Hoppe-Seyler's Z. Physiol. Chem., 333 (1963) 133—139.
9 D. Steinberg, in J.B. Stanbury, Wyngaarden and D.S. Fredrickson (Editors), Metabolic Basis of Inherited Disease, 1978, pp. 688—706.
10 P. Laudat, Biochimie, 54 (1972) 735—738.
11 T.R. Skrbic and J.N. Cumings, Clin. Chim. Acta, 23 (1969) 17—21.

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Biomedical Applications

CHROMBIO. 553

SIMPLE AND SENSITIVE PROCEDURE FOR THE ASSAY OF SEROTONIN AND CATECHOLAMINES IN BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTION

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(First received October 23rd, 1979; revised manuscript received December 27th, 1979)

SUMMARY

A simple, rapid and specific method for the determination of serotonin and catecholamines in brain is described. After tissue homogenisation, catecholamines are isolated by adsorption onto alumina and elution with perchloric acid. Serotonin is isolated by extraction into *n*-heptanol and back-extraction into acid. High-performance liquid chromatography of the acid extracts is performed with a C_{18} reversed-phase column and simple mobile phases. Detection is by the intrinsic fluorescence of the amines on excitation at 200 nm. Detection limits are 100 pg for norepinephrine, 300 pg for dopamine and 20 pg for serotonin. The results are found to correlate well with a catechol O-methyl transferase radioenzymatic assay for catecholamines and a ninhydrin derivatisation procedure for serotonin.

INTRODUCTION

Catecholaminergic and serotoninergic neurones have been shown to be involved in the central nervous control of cardiovascular reflexes but their exact role in this and the pathogenesis of hypertension remains unclear [1, 2]. Studies in this Institute aimed at elucidating these pathways have involved the study of cardiovascular reflexes after the depletion of these neurotransmitters by 6-hydroxydopamine and 5,6-dihydroxytryptamine [3—5]. In order to assess the degree of depletion of these transmitters and their possible role in the observed cardiovascular responses, reliable specific and sensitive assays are required for routine use.

We have found that the combination of adsorption on alumina for catecholamines and a simple solvent extraction for serotonin as originally described by Shellenberger and Gordon [6] gives excellent recoveries for the amines and

*To whom correspondence should be addressed.

relatively pure extracts suitable for assay. The original procedure has been modified, first to incorporate a very sensitive radioenzymatic assay for catecholamines and now a high-performance liquid chromatographic (HPLC)—fluorimetric assay by which both catecholamines and serotonin may be determined simply and with excellent sensitivity.

EXPERIMENTAL

Reagents

Norepinephrine (*d,l*-7-^{3}H, 5—15 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and S-adenosylmethionine (tritiated S-methyl, 7—15 Ci/mmole) from the Radiochemical Centre (Amersham, Great Britain). Norepinephrine bitartrate, dopamine, normetanephrine and 3-methoxytyramine hydrochlorides were purchased from Sigma (St. Louis, MO, U.S.A.). Serotonin creatinine sulfate was obtained from Fluka (Buchs, Switzerland). N-Tris(hydroxymethyl)methylglycine (Tricine) and *n*-heptanol were purchased from Hopkin and Williams (Chadwell Heath, Great Britain). Heptanol was distilled before use and after use was washed with water and redistilled for re-use. Kieselgel 60 F254 thin-layer plates, 0.25 mm thick (Merck, Darmstadt, G.F.R.), were used in chromatography. Liquifluor (New England Nuclear) and Instagel (Packard Instrument, Downers Grove, IL, U.S.A.) were used in liquid scintillation counting. Alumina, activity grade I, 70—230 mesh (Merck) was pretreated according to the method of Anton and Sayre [7]. Catechol O-methyl transferase (COMT) was prepared by a modified method of Axelrod and Tomchick [8] involving the dialysis of the 30—50% ammonium sulfate precipitated fraction against 0.001 *M* phosphate buffer pH 7.0 for 16 h. The final protein concentration was 16 to 24 mg/ml and 1-ml aliquots were stored at −20°C for up to three months before use without loss of activity. Acetonitrile, 190 nm HPLC grade was purchased from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC was redistilled from alkaline permanganate before use. All other reagents were of A.C.S. certified grade.

Chromatographic system

A 5000 series liquid chromatograph fitted with a universal loop injector (Varian, Palo Alto, CA, U.S.A.) was used in conjunction with a Micropak MCH-10 octadecylsilane reversed-phase column (300 × 4 mm I.D.). A 40-mm guard column was packed with pellicular C_{18} material (Vydac SC reversed phase, Varian). The detector was a Schoeffel FS 970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. The amines were detected by excitation at 200 nm and their fluorescence emission was selected by a Corning 7-60 glass filter with an approximate bandpass of 320—400 nm.

Brain samples

Rabbits were obtained from an inbred colony established since 1961 (Commonwealth Serum Labs., Melbourne, Australia) and were killed with an overdose of sodium pentobarbitone. The brain and spinal cord were rapidly removed and dissected over ice into cerebral hemispheres, thalamus plus limbic (thalamus, basal ganglia and hippocampus) hypothalamus, midbrain,

pons plus medulla, cerebellum, cervical cord, thoracic cord and lumbar cord. These were then frozen in liquid nitrogen, weighed and stored in liquid nitrogen for up to four weeks.

Sample preparation

The sections of brain were homogenised with a Polytron homogeniser (Kinematica, Luzern, Switzerland) at setting 5 for 10 sec in 0.4 *M* perchloric acid containing 0.1% sodium metabisulfite and 0.05% Na_2EDTA. The ratio of acid to tissue was 6:1, v/w. The homogeniser was washed with 1 ml of acid and the washings added to the homogenate. The homogenates were centrifuged at 30,000 *g* for 10 min and the supernatants collected and stored over ice. The pellets were resuspended in 2 ml of 0.4 *M* perchloric acid, centrifuged and the two supernatants pooled. The total volume was then recorded.

Extraction of catecholamines and HPLC estimation

Tritiated norepinephrine (200,000 dpm) was added to 6 ml of the supernatant and buffered to pH 7.5 to 8.0 with 5 to 6 ml of Tricine buffer (Tricine 100 m*M* and Na_2EDTA 68 m*M* in 0.525 *M* sodium hydroxide) as described by Shellenberger and Gordon [6]. Alumina (300 mg) was added and the samples shaken gently for 15—20 min. After centrifugation at 1000 *g* for 5 min, the supernatant was removed for the serotonin determination. The alumina was washed three times with 20 ml of water by gentle inversion and centrifuged for 5 min at 1000 *g*. All water was carefully removed by aspiration. The catecholamines were eluted by the addition of 0.1 *M* perchloric acid (5 ml for radioenzymatic estimation and 1 ml for HPLC) with gentle shaking for 15 min. After centrifugation the acid eluate was transferred to clean tubes and stored at 4° C. Aliquots (50 µl) of the eluates were added to 10 ml of Instagel for scintillation counting to determine the recovery of catecholamines. The eluates are stable for at least one week at 4° C.

The eluates were assayed by injection of 50—100 µl directly onto the HPLC column. The mobile phase was 0.01 *M* perchloric acid—acetonitrile (99:1) and the flow-rate was 2 ml/min. Detection was by fluorescence (see Chromatographic system). Calibration was performed by the injection of standard solutions freshly prepared from stock solutions (1 mg/ml in 0.01 *M* hydrochloric acid).

Radioenzymatic estimation of catecholamines

Norepinephrine and dopamine are estimated in the alumina eluates by enzymatic conversion to tritiated normetanephrine and 3-methoxytyramine respectively. Enzyme inhibition [8, 9] by aluminium ions was effectively overcome by 1:1 dilution of the eluates with 0.1 *M* perchloric acid.

Duplicate aliquots (50 µl) of the diluted eluates were added to 50 µl of 0.2 *M* perchloric acid in 15-ml stoppered glass tubes. Blank samples were prepared with 100 µl of 0.15 *M* perchloric acid and external standards (100, 250 and 500 pg) in 50 µl of 0.1 *M* perchloric acid.

The reaction was started by the addition of 100 µl of an enzyme mixture containing 12.5 m*M* $MgCl_2$, 600 m*M* Tris HCl pH 9.6, [^{3}H]S-adenosylmethionine (2.5 µCi) and COMT (44%, v/v), prepared in ice immediately before

use. The samples were then incubated at 37°C for 45 min. The reaction was stopped by the addition of 100 μl of 1 *M* sodium hydroxide containing 0.8 *M* boric acid, 0.08 *M* Na_2EDTA and 3 m*M* normetanephrine and methoxytyramine. The O-methylated derivatives were extracted with 4 ml of a mixture of toluene—isoamylalcohol (3:2) by shaking for 2 min and the organic phase transferred to clean tubes containing 100 μl of 0.1 *M* acetic acid. Here and in all subsequent steps phase separation was simplified by freezing the aqueous phase in dry ice—ethanol. The tritiated derivatives were extracted into the acid phase by shaking for 2 min, the phases separated by centrifugation and the acid phase was washed with a further 2 ml of toluene—isoamyl alcohol mixture. The acid extracts were dried in vacuo at 50°C and taken up in 100 μl of 0.001 *M* methanolic hydrochloric acid which was quantitatively transferred to silica gel thin-layer plates. The plates were developed in a solvent system consisting of chloroform—ethanol—ethylamine (80:15:7, water saturated). After development the plates were air dried, the bands corresponding to the derivatives located under UV illumination (254 nm), and scraped into scintillation vials. To each vial was added 1 ml of 0.05 *M* ammonia.

Vials containing normetanephrine were treated with 100 μl of 3% sodium periodate for 5 min. The reaction was stopped with 100 μl of 10% glycerol and acidified with 1 ml of 0.1 *M* acetic acid. The vanillin thus produced was extracted by brief shaking into 10 ml of toluene—Liquifluor scintillant for ^3H determination.

Methoxytyramine was extracted directly into 10 ml of toluene—isoamyl alcohol which was added to 10 ml of Instagel for ^3H determination.

The assay is linear to at least 500 pg of catecholamine. Blank values are typically 20—50 cpm for norepinephrine and 100—200 cpm for dopamine while the slopes of the standard curves are 25—35 cpm per pg of catecholamine.

Estimation of serotonin by the HPLC method

Aliquots of the buffered perchloric acid homogenates (2 ml) were taken from the alumina adsorption step, saturated with 1 g of sodium chloride and adjusted to pH 9.8 ± 0.4 with 80—120 mg of potassium carbonate. Standards were made up in 1 ml of 0.4 *M* perchloric acid and 1 ml of Tricine buffer. Then *n*-heptanol (4 ml) was added to each sample and shaken for 2 min, followed by centrifugation to separate the phases. A 3-ml aliquot of the heptanol extract is transferred to clean tubes and serotonin is back-extracted into 500 μl of 0.5 *M* NaH_2PO_4. The heptanol was carefully aspirated and the extract washed with 1 ml of *n*-heptane to remove residual heptanol.

The extract was then assayed by injection of 50 μl directly onto the HPLC column. The mobile phase was 0.01 *M* perchloric acid—acetonitrile (85:15) at a flow-rate of 2 ml/min. Calibration was by means of a curve prepared concurrently with the samples from external standards. Fluorescence detection was used (see Chromatographic system). Results with this method were compared with those obtained by using the method of Shellenberger and Gordon [6] without modification.

RESULTS AND DISCUSSION

For each sample the recovery of catecholamines was estimated by the recovery of tritiated norepinephrine. Use of this method to estimate dopamine recoveries is justified by the observation that the recoveries of several catecholamines are very similar [6, 10]. Recoveries were found to vary between 50 and 80% and were lower (50—70%) for the 1-ml eluates used in the HPLC assay. The unnatural catecholamine 3,4-dihydroxybenzylamine could be used with the HPLC assay — it elutes between epinephrine and dopamine — as an internal standard to avoid the necessity for recovery measurements, but the large variation in catecholamine levels between samples would require adjustment of the amount of internal standard for each sample.

The HPLC assay for catecholamines uses the intrinsic fluorescence of catechols upon excitation at 200 nm. In the mobile phases used for chromatography, catecholamines absorb maximally at 200, 220 and 280 nm (uncorrected) and serotonin at 205, 220, 275 and 295 nm. It was found experimentally that the signal-to-noise ratio for fluorescence detection was optimum at an excitation wavelength of 200 nm. This method is sensitive to 100 pg of norepinephrine and 300 pg of dopamine (Fig. 1), but relatively unspecific since the same conditions are used to detect serotonin. Specificity is achieved with the combination of adsorption onto alumina and reversed-phase HPLC. The chromatographic system is stable, highly reproducible and linear to at least 20 ng (the response is offscale for 60 ng of norepinephrine). Calibration is by the injection of known standards, since no difference is found between internal and external standards. The coefficient of variation of the absolute fluorescence response to the injection of 2 ng of catecholamine is less

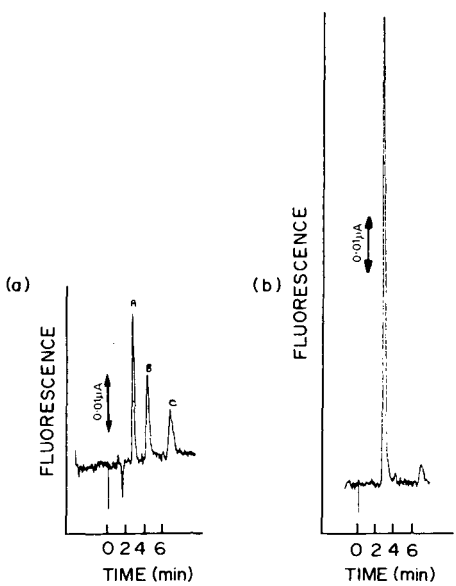

Fig. 1. Chromatograms of (a) 2 ng of norepinephrine (A), epinephrine (B) and dopamine (C) and (b) 100 μl of the eluate from a control hypothalamus weighing 40 mg.

than 10% for any one assay or for all assays over a two-month period. This is comparable with procedures using electrochemical detection [11, 12] or fluorescence after *o*-phthalaldehyde derivatisation [13]. For comparison, samples were assayed by radioenzymatic method using catechol O-methyl transferase. The procedure combines the method of Peuler and Johnson [14] and DaPrada and Zürcher [15] for the differential assay of catecholamines. The inhibitory effects on the enzyme of aluminium ions [8, 9] in the eluates are effectively overcome by dilution of the eluates so that an external standard curve may be used. However, since only 25 μl of the 5 ml of eluate are assayed, the basic sensitivity of the assay, 1—2 pg for norepinephrine and 4—8 pg for dopamine, corresponds to 200—400 pg for norepinephrine and 800—1600 pg dopamine per eluate.

The results of the comparison are shown in Fig. 2. The slopes of the regression lines are 0.961 for norepinephrine and 0.954 for dopamine or a difference of only 4%. While similar results can be obtained with either method, the speed and simplicity of the HPLC method make it preferable.

HPLC with electrochemical detection has been used to assay alumina eluates [10—12, 16] and can sometimes offer an absolute sensitivity comparable with the radioenzymatic assay [10, 12]. However, electrode life is often limited and sensitivity variable [10], while we find that fluorescence detection is very stable without any special precautions. Sasa and Blank [17, 18] have used electrochemical detection to assay butanol extracts of tissue homogenates, but not only are recoveries low and different for internal and external standards, but the chromatographic system must resolve catecholamines, serotonin and metabolites such as the metanephrines, which are also extracted. This leads to long chromatography times, which are avoided in our procedure by assaying catecholamines and serotonin independently. An HPLC assay for urinary catecholamines after alumina adsorption uses *o*-phthalaldehyde derivatisation and fluorescence detection [13], but only offers a two-fold

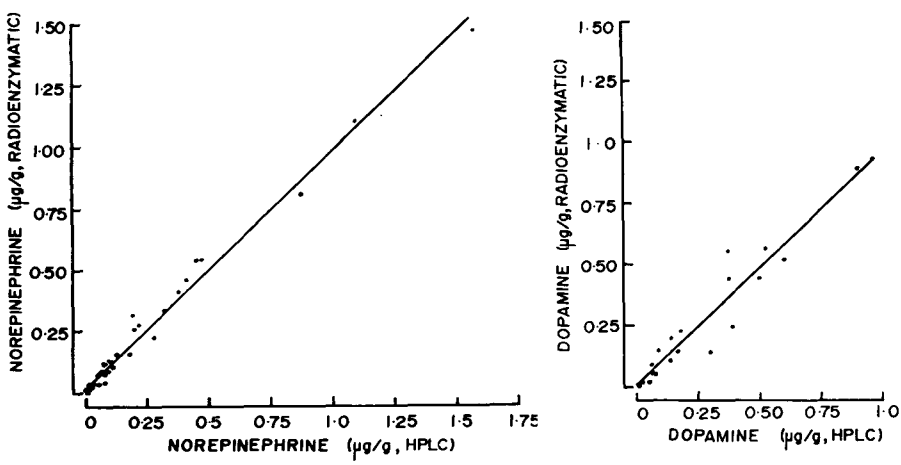

Fig. 2. Comparison between the radioenzymatic and HPLC assays for catecholamines. Samples were dilute eluates of the radioenzymatic assay. Slopes of the regression lines are 0.961 for norepinephrine and 0.954 for dopamine. Correlation coefficients are 0.993 and 0.986, respectively.

increase in sensitivity compared with our procedure which involves no derivatisation.

The extraction procedure for serotonin is taken directly from the method of Shellenberger and Gordon [6]. The high intrinsic fluorescence of serotonin when excited at low wavelengths permits the use of small samples. Some specificity is also achieved by the fact that another HPLC—fluorescence assay has shown the relative fluorescence of serotonin to be at least 40 times greater than that of its common precursors and metabolites [19]. The mild extraction procedure minimises interferences while giving recoveries of about 80%. No difference was found between internal and external standards. The calibration graph is linear within the range 0—100 ng. The reagent blank shown in Fig. 3 indicates the possibility of using much smaller samples, particularly since only 10% of the final extract is actually assayed, corresponding to about 15 mg of tissue.

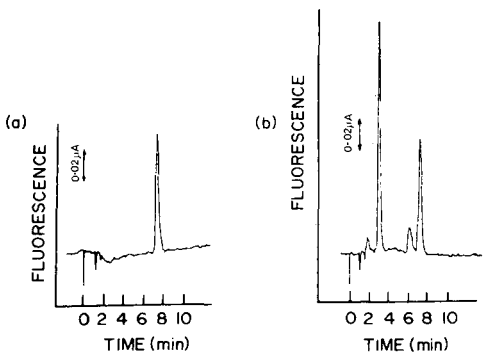

Fig. 3. Typical chromatograms for serotonin. (a) Reagent blank. The peak eluting at 8 min originates with the reagents and is present in all chromatograms. (b) Tissue sample containing serotonin which elutes at 3.2 min. The peak corresponds to approximately 3 ng of serotonin injected.

A direct comparison between the HPLC assay and the fluorimetric ninhydrin derivative assay [6] gave an excellent correlation (r = 0.999). The regression line was not significantly different from the line of identity.

Alternative serotonin assays have involved derivatisation with *o*-phthaldehyde [20] which is sensitive to about 5 ng. The ninhydrin assay is only sensitive to 30 ng but is more reliable [6]. A double enzymatic procedure has been described, but is time consuming and only sensitive to 1 ng [21]. The enzymes are unstable and must be prepared frequently. Radioimmunoassay has been found to be sensitive to 100 pg, but cross-reacts with 5-methoxytryptamine [22]. The antibody is also unstable and requires careful calibration. Our procedure involves no derivatisation, is extremely stable and can detect 20 pg of serotonin routinely.

In summary, the procedure we have described offers the advantage of simple extraction methods, rapid sensitive chromatography without derivatisation and the ability to assay catecholamines or serotonin independently if required.

REFERENCES

1 J. Axelrod, Clin. Sci. Mol. Med., 51 (1976) 415—421.
2 J.P. Chalmers, Clin. Sci. Mol. Med., 55 (1978) 45s—56s.
3 P.I. Korner, J.R. Oliver, J.A. Reynoldson, G.A. Head, V.J. Carson and M.M. Walker, Eur. J. Pharmacol., 53 (1978) 83—93.
4 J.A. Reynoldson, G.A. Head and P.I. Korner, Eur. J. Pharmacol., 55 (1979) 257—262.
5 P.I. Korner, J.A. Reynoldson, G.A. Head, J.R. Oliver and V.J. Carson, J. Cardiovascular Pharmacol., 1 (1979) 311—328.
6 M.K. Shellenberger and J.H. Gordon, Anal. Biochem., 39 (1971) 356—372.
7 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360—375.
8 J. Axelrod and R. Tomchick, J. Biol. Chem., 233 (1958) 702—705.
9 V.K. Weise and I.J. Kopin, Life Sci., 19 (1976) 625—636.
10 P. Hjemdahl, M. Daleskog and T. Kahan, Life Sci., 25 (1979) 131—138.
11 C. Refshauge, P.T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R.N. Adams, Life Sci., 14 (1974) 311—322.
12 R. Keller, A. Oke, I. Mefford and R.N. Adams, Life Sci., 19 (1976) 995—1004.
13 L.D. Mell, Jr., A.R. Dasler and A.B. Gustafson, J. Liquid Chromatogr., 1 (1978) 261—277.
14 J.D. Peuler and G.A. Johnson, Life Sci., 21 (1977) 625—636.
15 M. DaPrada and G. Zürcher, Life Sci., 19 (1976) 1161—1174.
16 H. Hashimoto and Y. Maruyama, J. Chromatogr., 152 (1978) 387—393.
17 S. Sasa and C.L. Blank, Anal. Chem., 49 (1977) 354—359.
18 S. Sasa and C.L. Blank, Anal. Chim. Acta, 104 (1979) 29—45.
19 A.M. Krstulovic and C. Matzura, J. Chromatogr., 163 (1979) 72—76.
20 R.P. Maickel, R.H. Cox, Jr., J. Saillant and F.P. Miller, Int. J. Neuropharmacol., 7 (1968) 275.
21 J.M. Saavedra, M. Brownstein and J. Axelrod, J. Pharmacol. Exp. Ther., 186 (1973) 508—515.
22 B.M. Jaffe, in B.M. Jaffe and H.R. Behrman (Editors), Methods of Hormone Radioimmunoassay, 2nd ed., Academic Press, London, 1978, pp. 527—539.

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CHROMBIO. 542

RAPID METHOD FOR DETERMINATION OF RIBOFLAVIN IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, specific, and sensitive high-performance liquid chromatographic (HPLC) method for the determination of riboflavin directly in urine samples using a fixed-wavelength spectrofluorometer is described. Centrifuged raw urine samples (50 μl) are injected onto a reversed-phase microparticulate C_{18} column. The eluent is 0.01 *M* KH_2PO_4 (pH 5.0)—methanol (65:35). This method is capable of differentiating riboflavin from riboflavin-5-phosphate, non-riboflavin fluorescing components in urine, and photo-degraded riboflavin. The method shows good reproducibility and is linear to at least 12 μg/ml. The sensitivity of this procedure, at the 95% confidence limit, determined by linear regression analysis, is estimated to be 0.05 μg/ml using peak height and 0.07 μg/ml using peak area. This HPLC method is compared to an automated fluorometric method for riboflavin. The coefficient of linear regression of this comparison is $Y = 0.858 + 0.893X$, where X is the HPLC method and Y is the fluorometric method.

INTRODUCTION

Riboflavin, vitamin B_2, is an enzyme co-factor vitamin found in most multiple vitamin or B-complex preparations. In man, riboflavin is excreted in urine apparently only as free riboflavin [1, 2]. Riboflavin excreted into the urine is often used as a measure of the relative bioavailability of vitamin formulations. The U.S.P. riboflavin assay [3] is a fluorometric method using an excitation wavelength of 440 nm and emission wavelength at 565 nm. Riboflavin is quantitated by comparing the fluorescence of the sample in the oxidized state (fluorescing form) with the reduced state (leuco or non-fluorescing form). Mellor and Maass [4] developed an automated fluorometric method for the determination of riboflavin in human urine. Their method, a modification of the U.S.P. procedure, used an excitation wavelength at 440 nm and emission wavelength at 560 nm.

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Several other assay methods have been described to determine riboflavin in biological fluids: a microbiological method [5], a protozoological assay [6] and a thin-layer chromatographic method [7].

Several high-performance liquid chromatographic (HPLC) methods to determine riboflavin in non-biological fluids using either normal-phase columns with a fluorometer [8] or reversed-phase columns with UV detection [9, 10] have been described. Williams and Slavin [11] described a HPLC method for the direct determination of riboflavin in urine using a variable-wavelength fluorometer and a 10-μl sample volume. The reproducibility of their method was not reported. They also noted the presence of detectable amounts of riboflavin phosphate in the urine from a subject on a riboflavin supplement.

The method described in this paper is a reversed-phase HPLC procedure for the direct determination of riboflavin in urine using a fixed-wavelength fluorometer, 50 μl sample volume, and phosphate buffer—methanol eluent. The concentration of riboflavin and the presence of riboflavin phosphate were determined in urine specimens from subjects who had received a multiple vitamin formulation or from control subjects on a low riboflavin diet. The reproducibility and sensitivity of this method as well as a comparison of the assay results of this HPLC method with the results from an automated fluorometric method [4] are reported.

EXPERIMENTAL

Apparatus

A Spectra-Physics Chromatronix Model 3500 high-performance liquid chromatograph (Santa Clara, CA, U.S.A.) equipped with a Valco (Houston, TX, U.S.A.) sample injection valve with a 50-μl sampling loop and a Hewlett-Packard Model 7120A strip chart recorder (10 mV input and 0.5 cm/min chart speed) was used. The detector was a fixed-wavelength spectrofluorometer (range: 4) (LDC Model 1209 FluoroMonitor, Laboratory Data Control, Riviera Beach, FL, U.S.A.). The excitation lamp was a low-pressure hot cathode mercury lamp with a phosphor coating which emitted near UV light with a range of 320—400 nm. The wavelength range for the emission filter was 400—700 nm. Peak areas were determined with a Spectra-Physics Autolab minigrator. A refrigerated centrifuge (Sorvall RC-3, Norwalk, CN, U.S.A.) was used to prepare the urine samples.

Chromatographic conditions

The column was a reversed-phase micro-particulate C_{18} (μBondapak C_{18}, particle size 10 μm, 30 cm × 4 mm, Waters Assoc., Milford, MA, U.S.A.) preceded by a C_{18} precolumn (Co:Pell ODS, 7 cm × 2.1 mm, Whatman, Trenton, NJ, U.S.A.). The eluent was 0.01 *M* KH_2PO_4 (pH 5.0)—methanol (65:35) at a flow-rate of 2.0 ml/min. The mobile phase was prepared by mixing exact volumes of methanol (distilled-in-glass, spectroscopic grade, Burdick and Jackson, Muskegon, MI, U.S.A.) and 0.01 *M* potassium monobasic phosphate solution adjusted to pH 5.0 with 1 *N* sodium hydroxide and then filtering through a 0.5-μm filter.

Standards

Riboflavin standard stock solutions were prepared to contain 100 μg/ml of U.S.P. reference standard by addition of 100 mg of riboflavin, previously dried at 105°C for 2 h, 750 ml of water and 1.2 ml of glacial acetic acid to a 1-liter flask, dissolving with heat, and diluting to volume with water. This stock solution was diluted with either urine or 0.1 *M* sodium acetate buffer (adjusted to pH 6.0 with acetic acid) to contain 1, 2, 6 and 10 μg/ml of riboflavin. All solutions were protected from light. These standards were injected onto the column via the sampling loop. The chromatogram was recorded and the peak areas or peak heights were determined.

Riboflavin-5-phosphate standard stock solution was prepared to contain 100 μg/ml in distilled water and then diluted with urine to a concentration of 10 μg/ml.

Sample analysis

Approximately 10 ml of urine were centrifuged at 1400 *g* for 10 min. A portion of the supernatant liquid was injected onto the column via the sampling loop. The chromatogram was recorded and the peak areas or peak heights were determined.

System suitability test

The resolution factor (10) for riboflavin relative to riboflavin-5-phosphate should be greater than 3. After three or more injections of a single standard, the relative standard deviation of response should be less than 2%.

RESULTS AND DISCUSSION

The retention time of riboflavin is dependent upon the methanol concentration. A ratio of 65:35 (aqueous buffer—methanol) was selected to maximize sample throughput. Total sample time was about 5 min. A typical chromatogram of riboflavin and riboflavin-5-phosphate in urine is shown in Fig. 1.

Although the excitation wavelength maximum for riboflavin is 440 nm, the excitation energy emitted by this lamp was sufficient to cause fluorescence. The output of this detector was limited, but adequate for this assay. UV absorbance could not be used in the direct determination of riboflavin in urine since large UV absorbance occurred at the solvent front and tailed into the riboflavin peak as was observed by Williams and Slavin [9].

Pooled centrifuged urine containing about 0.3 μg/ml of endogenous riboflavin, based on this HPLC assay, was spiked with riboflavin from 0.2 to 10 μg/ml. Similarly, riboflavin standards in 0.1 *M* sodium acetate (pH 6.0) from 0.4 to 10 μg/ml were prepared. The spiked urine and buffer samples were injected onto the column. The responses (peak area and peak height) of the spiked urine samples were then corrected for endogenous levels of riboflavin.

Least-square linear regression analysis was used to determine the slope, *y*-intercept, and correlation coefficient for the spiked urine samples and the standards in buffer. The results of these two analyses, given in Table I, indicate that all responses were linear with concentration to at least 10 μg/ml of riboflavin. The slopes and *y*-intercepts for corrected standards in urine and stan-

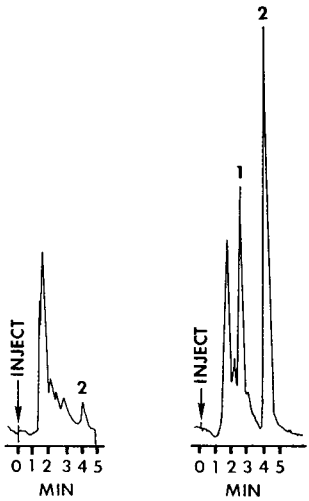

Fig. 1. Chromatograms of riboflavin and riboflavin-5-phosphate in urine. (A) Urine blank with approximately 0.1 μg/ml of endogenous riboflavin (2); (B) same urine spiked with 2.0 μg/ml each of riboflavin-5-phosphate (1) and riboflavin (2).

TABLE I

LINEARITY OF RESPONSE IN HUMAN URINE AND BUFFER SOLUTIONS

Least-square regression analysis*	Urine		Buffer (pH 6.0)	
	Peak height	Peak area	Peak height	Peak area
n	28	29	6	6
Slope	53.3	1.55×10^4	53.9	1.58×10^4
y	0.03	150	0.15	−490
r	0.999	0.999	0.999	0.999

*n = number of samples assayed; y = y-intercept; r = correlation coefficient.

dards in buffer were practically equivalent. These results indicate that standards in buffer can be used in the determination of riboflavin in urine.

The sensitivity of this method was estimated by linear regression analysis to be, at the 95% confidence limit, 0.05 μg/ml using peak height and 0.07 μg/ml using peak area. Only those concentrations less than 1.3 μg/ml were used in the calculation of sensitivity.

The precision of this method was determined by assaying samples of known concentrations of riboflavin in urine. The responses (both peak height and peak area) were corrected for endogenous riboflavin which was calculated to be 0.34 μg/ml. The results are given in Table II. The mean calculated concentration ± standard deviation and differences between actual and calculated concentration are reported. As is shown in Table II, this method showed good accuracy and reproducibility. The pooled coefficient of variation was <1% at concentrations greater than 1 μg/ml and 6.3% at concentrations less than 1 μg/ml using peak height as the response and 1.8% at concentrations greater than 1 μg/ml and

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF RIBOFLAVIN IN URINE

Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)					
	Using peak height			Using peak area		
	Concentration (mean ± standard deviation)	*n*	% Difference	Concentration (mean ± standard deviation)	*n*	% Difference
10.0	9.95 ± 0.005	4	0.5	9.95 ± 0.03	4	0.5
6.0	6.10 ± 0.006	3	1.7	6.07 ± 0.04	3	1.2
3.0	3.00 ± 0.007	2	0	3.10 ± 0.02	2	3.3
2.0	2.06 ± 0.007	2	3	2.03 ± 0.06	2	1.5
1.0	1.00 ± 0.05	3	0	1.03 ± 0.02	3	3
0.4	0.37 ± 0.005	4	7.5	0.35 ± 0.02	4	12.5
0.2	0.16 ± 0.06	3	20.0	0.15 ± 0.03	3	25.0

3.3% at concentrations less than 1 μg/ml using peak area. The average percent difference in calculated concentration relative to theoretical concentration was 1% using peak height as the response and 1.9% using peak area at concentrations greater than 0.5 μg/ml.

In bioavailability studies of multiple vitamin formulations, the subject must collect his/her urine over a 24-h period. This collected urine is usually stored at room temperature. These urine specimens are then stored at 5°C until all legs of a cross-over design are complete. Therefore, the stability of riboflavin in urine at room temperature and at 5°C was determined. Pooled urine, spiked with riboflavin (10 μg/ml), protected from light and stored at room temperature, was periodically sampled up to 24 h. Similarly, riboflavin-spiked urine was stored under refrigeration (5°C) and periodically sampled up to two weeks. Pooled urine spiked with riboflavin (10 μg/ml) was also exposed to direct natural sunlight at room temperature. These samples were then assayed according to this procedure.

For those samples protected from light and stored at room temperature or 5°C, no change in riboflavin peak response was observed. The spiked urine samples exposed to natural light showed a decrease in the peak response of riboflavin and the appearance of a second peak (retention time was about 30 sec longer than riboflavin) which also disappeared upon prolonged exposure to light as is shown in Fig. 2. These results indicate that this HPLC procedure can differentiate between photodegraded and undegraded riboflavin. Riboflavin in urine, when protected from light, appeared to be stable in urine for at least 24 h at room temperature and at least 2 weeks at 5°C.

This HPLC method was compared to an automated fluorometric method for the determination of riboflavin in urine. Urine samples from human subjects receiving a 10-mg riboflavin tablet or a multivitamin tablet containing 10 mg of riboflavin as well as urine samples from the same subjects on a low riboflavin diet and no riboflavin supplement were assayed by this HPLC—fluorometric method and by an automated fluorometric method [2]. It should be noted that no detectable levels of riboflavin phosphate were observed in these urine specimens by the HPLC method. In the fluorometric method, the apparent riboflavin content of urine was calculated from the difference in fluorescent intensity before and after reduction with sodium hydrosulfite, by comparison

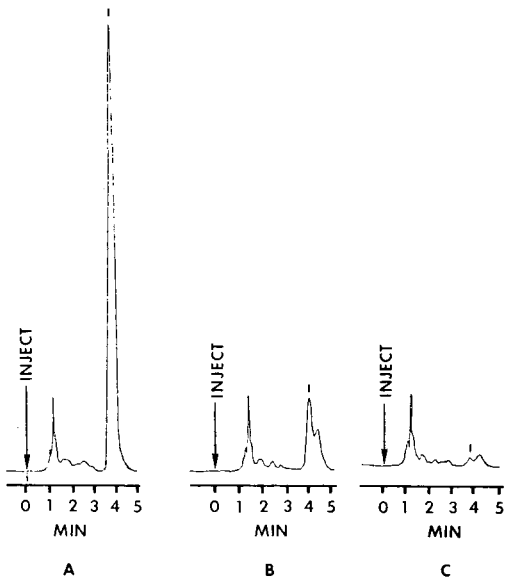

Fig. 2. Stability of riboflavin in urine in natural light. Riboflavin (10 μg/ml) (1) under following experimental conditions: A = initial, B = 1 h in natural sunlight, C = 4 h in natural sunlight.

with the intensity of known concentrations of riboflavin assayed simultaneously.

The data from these two determinations were compared using linear regression analysis and paired *t*-test. A graphical representation of this comparison is given in Fig. 3. The coefficients of the linear regression analysis are as follows: $Y = 0.865 + (0.888 \pm 0.050)\ X$ ($p = 0.05$) where X = HPLC data and Y = fluorescence data; $r = 0.991$. Both coefficients were significantly greater than

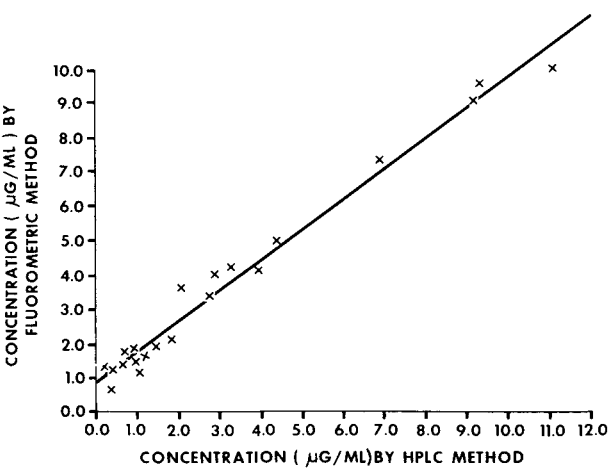

Fig. 3. Comparison of the HPLC method with a fluorometric method for the determination of riboflavin concentration in human urine.

zero. The paired t-test indicated that the fluorometric and HPLC results were significantly different [$p = 0.01$ ($t_{value} = 5.303$, $df = 23$)].

In the comparison of the HPLC and fluorometric method a few points at higher concentrations heavily weighed the estimation of the overall slope. Linear regression analysis of those riboflavin concentrations less than 5 μg/ml were determined. The regression equation is as follows: $Y = 7.3 + (0.98 \pm 0.13) X$ ($p = 0.05$, $n = 20$, $r = 0.97$) where X = determinations by HPLC, Y = determinations by fluorometric method. For concentrations less than 5 μg/ml, the slope was not significantly different from one ($p = 0.05$, $n = 20$). The y-intercept was still significantly greater than zero.

These analyses indicated that the assay results from this HPLC method and an automated fluorometric differed, but were highly correlated. The HPLC method has been demonstrated to be specific. As was shown in Figs. 1 and 2, this method differentiated riboflavin, riboflavin phosphate, and photodegradation products of riboflavin. The fluorometric method may not have the same degree of specificity, and may be quantitating a residual fluorescing component of urine which is also reduced to a non-fluorescing component.

In conclusion, this HPLC method, which uses a fixed-wavelength spectrofluorometer for the determination of riboflavin directly in urine, is a simple, reproducible and sensitive procedure. Assay results of human urine specimens by this HPLC method and by an automated fluorometric method were different, but highly correlated.

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REFERENCES

1 W.J. Jusko and G. Levy, J. Pharm. Sci., 56 (1967) 58.
2 B. Stripp, Acta Pharmacol. Toxicol., 22 (1965) 353.
3 United States Pharmacopeia, 19th rev., Mack Publishing Co., Easton, PA, 1975, p. 627.
4 N.P. Mellor and A.R. Maass, Advances in Automated Analysis, Vol. 9, Mediad, Tarrytown, NY, 1973, p. 67.
5 M. Telegdy-Kouats and M. Heqedus, Elelmez. Ipar, 24 (1970) 358.
6 H. Baker, O. Frank, S. Feingold, R. Gellene, C. Leevy and S.H. Hutner, Amer. J. Clin. Nutr., 19 (1966) 17.
7 C. Haworth, R. Oliver and R. Swaile, Analyst (London), 96 (1971) 432.
8 D. Willmer and W.G. Haney, Jr., J. Pharm. Sci., 64 (1974) 588.
9 R.L. Kirchmeier and R.P. Upton, J. Pharm. Sci., 67 (1978) 1444.
10 R.B.H. Wills, C.G. Shaw and W.R. Day, J. Chromatogr. Sci., 15 (1977) 262.
11 A.R. Williams and W. Slavin, Chromatogr. Newsletter, 5 (1977) 9.
12 United States Pharmcopeia, 19th rev., Mack Publishing Co., Easton, PA, 1975, p. 640.

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CHROMBIO. 536

MEASUREMENT OF FAECAL BILE ACID SULPHATES

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SUMMARY

A method is described for the measurement, by difference, of the sulphate fractions of faecal bile acids. A solvolysis step (for the deliberate hydrolysis of the bile acid sulphates) was added to the procedure of sample homogenisation, extraction, enzymatic hydrolysis and thin-layer chromatography. The bile acids were quantitated by gas—liquid chromatography of their methyl ester and trifluoroacetate methyl ester derivatives on 3% QF-1 columns. The total bile acid excretion in 15 control subjects was 603 ± 71 mg/24 h ($\bar{x}$ ± S.E.M.). The major bile acid peaks (mg/24 h) were: lithocholic acid, without solvolysis 118 ± 26 and including solvolysis 175 ± 30; deoxycholic acid 60 ± 8 and 90 ± 18 and chenodeoxycholic acid 13 ± 7 and 15 ± 7. It was concluded that bile acid sulphates may form a considerable proportion of the total bile acids excreted in man.

INTRODUCTION

For several substances hepatic sulphation provides an effective detoxifying mechanism. Many potentially toxic drugs, for example, are rendered harmless by sulphation in the liver which ensures their secretion in the bile and faeces. Although the major primary and secondary bile acids are largely conserved within the entero-hepatic circulation by intestinal and hepatic transport mechanisms, they are also partially sulphated in the human liver [1,2]. First reported by Palmer [3] in 1967, sulphation has been shown to enhance the excretion of bile acids, particularly lithocholic acid [4,5]. This hepatic sulphation of bile acids probably influences the physiology of the major bile acids in health but it becomes particularly important in cholestasis and during chenodeoxycholic acid treatment of gallstones. In cholestasis the urinary excretion of bile acids becomes the major route for their elimination from the body [4]. During chenodeoxycholic acid therapy, increased amounts of its principal bacterial metabolite, lithocholic acid, form in and are absorbed from the intestine. In man, sulphation of lithocholic acid prevents its accumulation within

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the liver and hence the tissue damage which might well ensue; in animals where hepatic sulphation is less well developed, lithocholate accumulation does occur and is associated with changes in liver structure and function [6].

Despite this, surprisingly little is known about the excretion of faecal bile acid sulphates in man [7,8] and to date no methods for measuring faecal bile acid sulphates have been published. We have modified an established procedure [9] for faecal bile acid determination based on a technique for the analysis of urinary bile acid sulphates [10], to enable us to quantitate the proportion of faecal bile acids excreted as sulphate esters.

METHOD

The method used was modified from technique of Grundy et al. [9] for measuring faecal bile acids. The procedure is illustrated schematically in Fig. 1. Details of the steps involved were as follows.

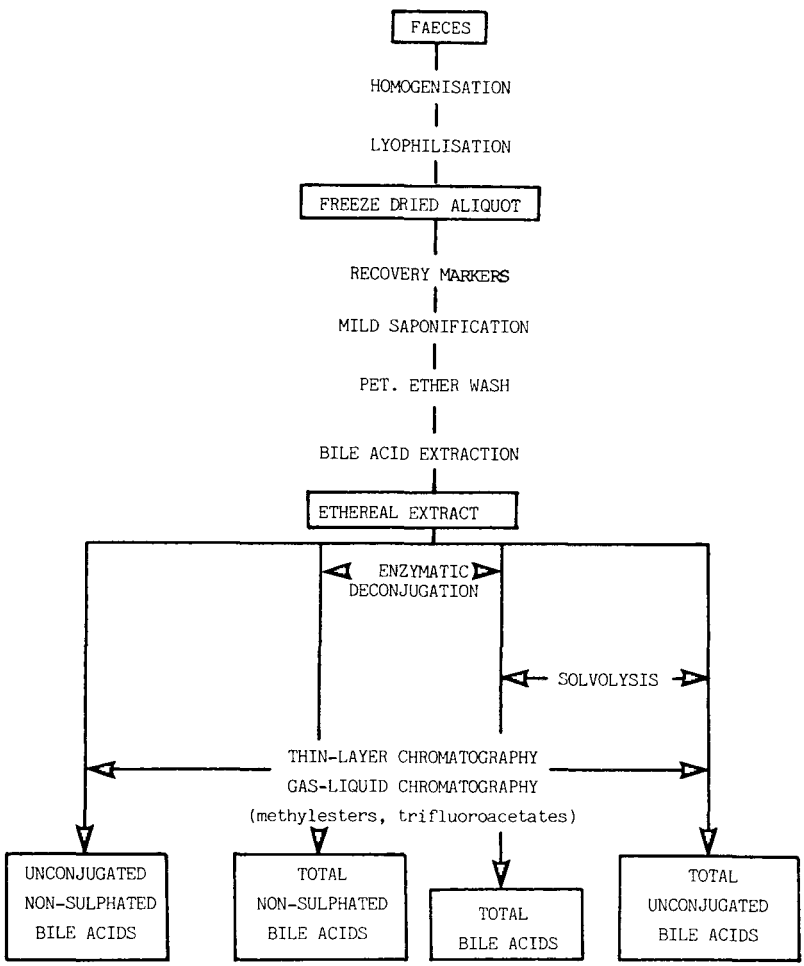

Fig. 1. Scheme of the steps involved in the separation procedure.

Homogenisation

The faecal samples (usually 72-h collections) were homogenised at 4°C in distilled water, the final weights of homogenate being adjusted to 2500 g. Aliquots (25 ml) of the homogenate were then freeze-dried and weighed.

Mild saponification

To approximately 800 mg freeze-dried material [2,4-^{14}C] chenodeoxycholic acid (10^4 dpm) and [^{3}H] cholesterol (10^4 dpm) were added as recovery markers of acidic and neutral sterols together with 80 ml 1 *M* sodium hydroxide (in 90% ethanol). The mixture was refluxed with continuous stirring for 2 h and then centrifuged at 3500 *g* for 10 min. The resulting pellet was washed with 20 ml 1 *M* sodium hydroxide (in 50% ethanol) and the washings pooled with the original supernatant.

Petroleum ether wash

The pooled supernatant was transferred to a separating flask and 20 ml water with 150 ml petroleum ether (b.p. 00—00°C) added. Following vigorous shaking by hand during 1 min the lower phase, which contained the bile acids was kept, and the upper phase washed with 20 ml 1 *M* sodium hydroxide (in 50% ethanol). The ethanolic washings were pooled. The upper phase, which contained the [^{3}H] cholesterol was sampled for scintillation counting.

Bile acid extraction

The pooled aqueous ethanol fraction was evaporated to approximately 50 ml and the mixture then acidified to pH < 1 with 11.5 *M* hydrochloric acid. This acidic phase was then promptly extracted with diethyl ether (3 × 150 ml). The ethereal washings were pooled, washed with water and divided into 8 equal volumes, each of which was evaporated to dryness under air in a fume cupboard, the test tubes being placed in a water bath at 50°C to hasten the process. The samples were then subjected to one of the four pathways illustrated in Fig. 1, each being carried out in duplicate.

Enzymatic deconjugation

The method of deconjugation used was essentially that of Nair et al. [11]. The ethereal residue was dissolved in 0.6 ml methanol and 0.066 *M* phosphate buffer (5 ml) was added. The pH of the mixture was adjusted to 5.6 and the test tubes placed in a water bath at 37°C for 10 min. *Clostridium welchii* solution (Sigma, St. Louis, MO, U.S.A.; 0.5 ml) was then added and the incubation period continued for 4 h. A standard aqueous 2 m*M* taurocholate solution was carried through with each batch of analyses to ensure completeness of the enzymatic conjugation. The pH was lowered to < 1 with 11.5 *M* hydrochloric acid and the bile acids rapidly extracted with 3 × 15 ml diethyl ether. The ether extracts were washed with water and then evaporated to dryness.

Solvolysis

The method used for solvolysis was essentially that of Burstein and Lieberman [12]. The ethereal residue was dissolved in 1 ml absolute ethanol and 0.5 ml 2 *M* hydrochloric acid added. The samples were subjected to three 10-sec

bursts of ultrasonic agitation. Acetone (9 ml) was then added and the solutions incubated at 37°C for 2 days. Following evaporation to dryness, the residue was dissolved in 4 ml of 5% methanolic potassium hydroxide and refluxed for 1.5 h. The solution was evaporated to dryness under vacuum. The residue was dissolved in 1 ml water, the solution acidified to pH < 1 and the bile acids extracted into diethyl ether (3 × 10 ml).

Thin-layer chromatography

All fractions were subjected to thin-layer chromatography (TLC) on 0.5-mm silica gel G. The plates were first developed in hexane—ethyl acetate (9:1) and then in isooctane—ethyl acetate—acetic acid (10:10:2) [13]. Following location with iodine, the unconjugated bile acid areas of each plate were scraped into a column fitted with a sintered glass disc and the bile acids were then eluted with 40 ml chloroform—methanol (2:1).

Gas—liquid chromatography

The bile acid methyl esters were prepared with diazomethane [14]. The apparatus used was a Pye GCV gas—liquid chromatograph fitted with an autoinjection system (Pye Unicam, Cambridge, Great Britain). A preliminary chromatogram was obtained from each sample in order to select the most appropriate internal standard (such as 7-ketocholanoic acid, hyodeoxycholic acid, or hyocholic acid). The solution of bile acid methyl esters was evaporated to dryness and the internal standard added (200 μg in 200 μl methanol). A further 200 μl methanol was added and the solution Vortex mixed. An aliquot (1 μl) was then injected onto a 3% QF-1 column, temperatures: injection 250°C; column 240°C and detector 260°C. The remaining solution was evaporated to dryness and the trifluoroacetate derivatives of the bile acid methyl esters prepared [15]. Automatic injection was made onto the same column, temperatures: injection 230°C; column 230°C and detector 250°C. Identification was thus made using the peak shift technique [14].

RESULTS

Recovery experiments

(i) Recoveries of ^{14}C-labelled bile acids added as aqueous solutions (2 m*M*) to faecal samples before homogenisation and subjected to the whole procedure were (mean ± S.D., $n = 6$ in each case): lithocholic acid 102 ± 2.9%; deoxycholic acid 99 ± 2.1%; chenodeoxycholic acid 99 ± 4.0%; cholic acid 97.5 ± 2.5%; glycocholic acid 84.2 ± 6.8%; glycochenodeoxycholic acid 84.2 ± 6.7%; taurocholic acid 68.9 ± 10.3%.

The poor recovery of taurocholic acid is probably explained by the fact that although its enzymatic deconjugation was maximum at 4 h, it was only 75.2 ± 5.6% complete. The degree of deconjugation obtained with glycocholic acid was 92.1 ± 2.5% and with glycochenodeoxycholic 89.1 ± 4.1%. Conversion of conjugated bile acid to unconjugated bile acid because of inadvertent hydrolysis during reflux was approximately 12% for glycochenodeoxycholic and glycocholic acids but only 5% for taurocholic acid.

(ii) Recovery of [3-$^{35}SO_4$]-lithocholic acid [16] added in aqueous solution to the faecal samples before homogenisation was 85.0 ± 5.2%. The solvolysis procedure was applied to standard preparations of the 3α-sulphates of lithocholic, chenodeoxycholic, deoxycholic, cholic and taurolithocholic acids and to the 3,7-disulphate derivative of chenodeoxycholic acid. After solvolysis and using the TLC system of Cass et al. [17] only the parent acid of each preparation was detected.

(iii) Completeness of extraction of bile acids was estimated by comparing the amount of radioactivity recovered from a faecal sample (containing ^{14}C-bile acids) in a patient who had ingested [^{14}C]chenodeoxycholic acid (whilst on a normal solid diet), using the above procedure with that obtained by direct oxidation in an Intertechnique sample oxidiser. The recovery after 1 h reflux was 91 ± 0.4% ($\bar{x}$ ± S.D.; n = 3) and after 2 h reflux 99 ± 0.1% of that obtained by sample oxidation.

Reproducibility studies

(i) The results obtained by replication (n = 6) of the analysis of a sample, each replicate being the mean of duplicate determinations are given in Table I.

(ii) The recovery of [^{14}C]chenodeoxycholic acid carried through with each analysis was 77 ± 6% ($\bar{x}$ ± S.E.M.; n = 124).

TABLE I

BILE ACID FRACTIONS OF ONE SAMPLE ANALYSED IN DUPLICATE SIX TIMES

Figures denote $\bar{x}$ ± S.D. (mg/24 h) values.

	Total	Total non-sulphated	Unconjugated non-sulphated	Total unconjugated
Lithocholic	41.4 ± 1.9	37.9 ± 3.0	14.1 ± 1.3	16.9 ± 1.6
Deoxycholic	61.2 ± 3.0	49.3 ± 1.1	38.0 ± 3.2	48.3 ± 3.0
Chenodeoxycholic	66.8 ± 1.4	65.1 ± 2.9	47.1 ± 3.1	47.8 ± 2.8
Ursodeoxycholic	38.1 ± 1.1	35.5 ± 2.7	25.0 ± 1.7	27.2 ± 2.2
Cholic	31.0 ± 1.6	23.2 ± 2.0	18.7 ± 1.3	22.4 ± 1.6

Qualitative demonstration of the presence of bile acid sulphates in human faeces

To date, no means of measuring bile acid sulphates directly in biological samples has been described and all the techniques used require solvolysis, i.e. hydrolysis of the sulphate ester followed by estimation of the parent acid. Although the approach used in the present study was similar to that previously used for bile [18], serum [19] and urine [10] in that the sulphate contribution was determined by difference, it was considered necessary to provide some direct demonstration of the presence of bile acid sulphates. This was done as follows:

(i) Six ethereal extracts (Fig. 1) prepared from faecal samples as described above were, before solvolysis, subjected to TLC using hexane—ethyl acetate (9:1) and isooctane—ethyl acetate—acetic acid (10:10:2) [13]. The unconjugated bile acids were visualised in iodine. The chromatogram was further developed in chloroform—methanol—acetic acid—water (65:24:10:5) [20] when the unconjugated bile acids moved towards the solvent front and several

additional bands were seen, which corresponded in R_F to the published values for bile acid sulphates. Lithocholic acid sulphate was identified by repeating the third development after including taurolithocholic, lithocholic and lithocholic acid sulphate as standards. The presence of lithocholic acid sulphate was confirmed by the use of two-dimensional TLC. The solvent system chloroform—methanol—acetic acid—water (65:24:15:9) [17], was used in the first direction and ethyl acetate—butanol—acetic acid—water (8:6:3:3) [21] at right angles to the first direction. Lithocholic acid sulphate was included as standard for the second development. Lithocholic acid sulphate was clearly demonstrated.

(ii) Four of the ethereal extracts were subjected to Sephadex LH-20 column chromatography as described by Stiehl et al. [22]. Four fractions were eluted with chloroform—methanol (1:1) and four with methanol. Each fraction was subjected to TLC examination for unconjugated bile acids and sulphated bile acids using the methods described above. Sulphated bile acids were found only in the last two fractions eluted and solvolysis followed by gas–liquid chromatography confirmed the presence of lithocholic, deoxycholic and chenodeoxycholic sulphates.

Normal values

The mean total faecal bile acid excretion in 15 control subjects (8 males, 7 females, mean age 41 ± S.E.M. 5.8 years, range 17—84) was 603 (± S.E.M. 71) mg/day. The amounts of the major bile acid fractions are shown in Table II.

TABLE II

CONCENTRATIONS OF MAJOR BILE ACID FRACTIONS FOUND IN CONTROL SUBJECTS

n = 15; bile acid fractions (mg/24 h, $\bar{x}$ ± S.E.M.).

Bile acid	Total	Total non-sulphated	Total unconjugated	Unconjugated non-sulphated
Lithocholic	175 ± 30	118 ± 26	122 ± 29	97 ± 27
Deoxycholic	90 ± 18	60 ± 8	59 ± 10	47 ± 8
Chenodeoxycholic	15 ± 7	13 ± 7	6 ± 3	5 ± 2

DISCUSSION

None of the methods previously described for faecal bile acid measurement in man have deliberately included a solvolysis step. Our results and those of other workers [8] indicate that bile acid sulphates may form a considerable proportion of the total bile acids excreted in man and should be either knowingly included or excluded in such determinations.

Without the aid of mass spectrometry it is questionable whether one can confidently analyse the complex mixture of bile acids found in human faeces [23]. Studies using gas chromatography with mass spectrometry [23,24] have shown that whilst deoxycholic and lithocholic acids are quantitatively the most important bile acids there are a large number of minor components which together make a significant contribution to the total amount of faecal bile acids. In normal subjects these minor bile acids have been reported to account for as

much as 40% total faecal bile acids [25]. In the present study the problem of accurate quantitation without the use of mass spectrometry is further complicated by the inclusion of bile acid sulphate determination.

The retention times of the different bile acids were established with standards; where standards were not available the values given in the literature were used for comparison.

The difficulties of accurate sulphate determination are considerable and it must be borne in mind that:

(1) in vitro solvolysis by faecal bacteria may occur during homogenisation [26],

(2) acid catalysed solvolysis [27] may occur in the presence of most organic solvents including diethyl ether [28] and

(3) low pH (pH < 1) is essential for quantitative extraction of sulphates from aqueous solutions. Thus any step for extraction of bile acid sulphates must be performed at low pH and with speed if inadvertent solvolysis is to be avoided. It is probable that methods which use prolonged acid extraction procedures [29] include measurement of bile acid sulphates as their parent acids.

(4) Although enzymatic hydrolysis of conjugated bile acid sulphates has been described by other workers [21,22] to-date no detailed studies have been made of either the degree of sulphation on the rate of hydrolysis or of the nature of products.

There is considerable variation in the reported normal ranges for faecal bile acids in man. One important factor in this variation must be methodological differences. For example, results for the synthesis of bile acids (input into pool) obtained using isotopic methods are usually higher than the results obtained by chemical analysis of faecal bile acids (output from pool [30]). The findings in the present study are similar to the results obtained using isotopic techniques. If, however, the sulphate contribution is omitted then the results are similar to those found by many workers using chemical analyses.

The above procedure, therefore, whilst it provides no information on the type of bile acid sulphate present, does allow one by means of a simple addition to a well established procedure, to measure all the fractions which constitute faecal bile acid excretion in man.

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REFERENCES

1 A. Stiehl, P. Czygan, W. Fröhling, M. Liersch and B. Kommerel, in L. Bianchi, W. Gerok and K. Sickinger (Editors), Liver and Bile, MTP Press, Lancaster, 1977, pp. 129—138.
2 R.H. Palmer, in W. Taylor (Editor), The Hepatobiliary System, Plenum Press, New York, London, 1976, pp. 227—243.
3 R.H. Palmer, Proc. Nat. Acad. Sci. U.S., 58 (1967) 1047—1050.
4 A. Stiehl, R. Raedsch and B. Kommerell, Digestion, 12 (1975) 105—110.
5 A.E. Cowan, M.G. Korman, A.F. Hofmann, O.W. Cass and S.B. Coffin, Gastroenterology, 69 (1975) 67—76.

6 T.R. Gadacz, R.N. Allan, E. Mark and A.F. Hofmann, Gastroenterology, 70 (1976) 1125—1129.
7 M.T. Podesta, G.M. Murphy, G.E. Sladen and R.H. Dowling, Clin. Sci. Mol. Med., 54 (1978) 32 Abstract.
8 G. Salvioli, R. Salati, M. Pastorello, A. Gibertini and B.M. Sala, in Hepatology, Rapid Literature Review IX (1978), Falk Foundation, Freiburg, p. 187, Abstract 207, Eur. Ass. Study of the Liver.
9 S.M. Grundy, E.M. Ahrens, Jr., and T.A. Miettinen, J. Lipid Res., 6 (1965) 397—410.
10 G.P. van Berge Henegouwen, K.H. Brandt, H. Eyssen and G. Parmentier, Gut, 17 (1976) 861—869.
11 P. Nair, M. Gordon and T. Reback, J. Biol. Chem., 242 (1967) 7—11.
12 S. Burstein and S. Liebermann, J. Biol. Chem., 233 (1958) 331—335.
13 P. Eneroth, J. Lipid Res., 4 (1963) 11—16.
14 P. Eneroth and J. Sjovall, in P.P. Nair and D. Krichevsky (Editors), The Bile Acids, Vol. I, Plenum Press, New York, London, 1971, pp. 121—171.
15 W.J.A. VandenHeuvel, J. Sjovall and E.C. Horning, Biochim. Biophys. Acta, 48 (1961) 596—599.
16 R.O. Mumma, Lipids, 1 (1966) 221—223.
17 O.W. Cass, A.E. Cowan, A.F. Hofmann and S.B. Coffin, J. Lipid Res., 16 (1975) 159—160.
18 R.C. Danzinger, A.F. Hofmann, J.L. Thistle and L.J. Schoenfield, J. Clin. Invest., 52 (1973) 2809—2821.
19 R. Galeazzi, E. Kok and N.B. Javitt, in G. Paumgartner and A. Stiehl (Editors), Bile Acids in Health and Disease, MTP Press, Lancaster, 1977, pp. 71—74.
20 S. Barnes, J.L. Gollan and B.H. Billing, Biochem. J., 166 (1977) 65—73.
21 G. Parmentier and H. Eyssen, J. Chromatogr., 152 (1978) 285—289.
22 A. Stiehl, D.L. Earnest and W.H. Admirand, Gastroenterology, 68 (1975) 534—544.
23 P. Eneroth, B. Gordon, R. Ryhage and J. Sjovall, J. Lipid Res., 7 (1966) 511—523.
24 P. Eneroth, B. Gordon and J. Sjovall, J. Lipid Res., 7 (1966) 524—531.
25 S.J. Gordon, L.J. Miller, L.J. Haeffner, M.D. Kinsey and O.D. Kowlessar, Gut, 17 (1976) 58—67.
26 T.J. Imperato, C.E. Way, L.J. Chen and R.J. Bolt, J. Bacteriol., 130 (1977) 545—547.
27 M.B. Goren and G. Jansen, J. Lipid Res., 9 (1968) 226—236.
28 G.P. van Berge Henegouwen, R.N. Allan, A.F. Hofmann and P.Y.S. Yu, J. Lipid Res., 18 (1977) 118—122.
29 E. Evrard and G. Jansen, J. Lipid Res., 9 (1968) 226—236.
30 M.T.R. Subbiah, N.E. Tyler, M.D. Buscaglia and L. Marai, J. Lipid Res., 17 (1976) 78—84.

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LIPID CONTENT OF HUMAN PLATELETS QUANTITATED BY THIN-LAYER CHROMATOGRAPHY IN COMBINATION WITH FLAME IONIZATION DETECTION

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SUMMARY

The lipid contents of human platelets from twenty-one healthy adults were analysed using thin-layer chromatography in combination with flame ionization detection.

The weight per cent of neutral lipids in human platelets was 14.6%, which consisted mainly of free cholesterol, that of phosphatidylethanolamine 24.9%, phosphatidylserine plus phosphatidylinositol 6.8%, phosphatidylcholine 35.2% and sphingomyelin 18.6%. Free cholesterol in 10^8 platelets was estimated as 7 μg and phospholipids as 46 μg from calibration standards. The reproducibility was satisfactory and the procedure could be performed quickly and simply.

INTRODUCTION

Dysfunctions of platelets play important roles in the pathogenesis of atherosclerosis [1—3]. Various properties of platelets including adhesiveness, aggregation, coagulation factors, prostaglandins, anti-platelet antibody and membrane glycoprotein, can now be measured in many clinical laboratories. In addition to these examinations, lipid analysis of platelets is required to investigate the role of lipids in the various functions of platelets. However, only a few papers concerning lipid analysis of human platelets have appeared, with technical difficulties demanding massive platelets beyond clinical applications.

This paper presents a simple and rapid method for the quantitative analysis of platelet lipids as a clinical and practical improvement, using a combination of thin-layer chromatography (TLC) and flame ionization detection (FID).

MATERIALS AND METHODS

Subjects

Platelets were obtained from 21 healthy adults (8 females and 13 males). The mean age, serum cholesterol and serum phospholipid of the subjects were 25 years, 162 mg/dl and 169 mg/dl, respectively. All of them were confirmed as having normolipidemia with normal platelet functions as regards aggregation, adhesiveness, volume, and malondialdehyde (MDA) release induced by arachidonic acid.

Preparation of platelets

Five millilitres of venous blood were taken from the subjects after overnight fasting using a siliconized tube containing 3.8% citrate. The blood was centrifuged at 100 *g* for 10 min at 23°C. The supernatant (platelet-rich plasma) was centrifuged for 10 min at 23°C. The platelets in the pellet were resuspended in 1.5 m*M* Tris·HCl buffer (pH 7.4) containing 0.15 m*M* NaCl, 5 m*M* KCl and 1.5 m*M* EDTA. After being rinsed twice with the same buffer, the platelets in the pellet were counted using a Hemalog 8 (Technicon Co., Tokyo, Japan). The number of platlets obtained from 5 ml of blood was approximately $1-8 \times 10^8$.

Analytical method for neutral lipids

Platelet lipids were extracted with chloroform—methanol (2:1, v/v) containing cholesterol acetate (MW 428, 10 mg/dl) as an internal standard. After the solvents were rinsed twice with saline, the top layer was discarded and the lower and intermediate layers were filtered through filter paper (Type 5A, Toyo Co., Tokyo, Japan). The solution of platelet lipids was evaporated in a water-bath at 40°C under reduced pressure. The dried samples were then dissolved in 200 μl of Folch's solution [4], and 3 μl of this solution were applied to a Chromarod for TLC. Development was in methanol for 10 mm from the point of application; the rod was then air-dried. Neutral lipids (cholesterol ester, cholesterol acetate, triglyceride, free cholesterol and phospholipid) were developed in *n*-hexane—diethyl ether (9:1, v/v) for 40 min at 20°C. The Chromarod was passed through a flame ionization detector after drying for 3 min at 110°C.

Thin-layer chromatography in combination with flame ionization detection

TLC was performed on a "Chromarod" (Iatron, Tokyo, Japan), a quartz rod 0.9 mm in diameter and 152 mm long, coated with silica gel. The rod was passed through a flame ionization detector (Iatron) after chromatographic development under the following conditions: hydrogen pressure 1.4 kg/cm^2, air flow-rate 2000—2500 ml/min, scanning speed gear 40 T, chart speed 240 mm/min, and recorder range 0.1 V. Each fraction, calibrated by flame ionization detector, was identified by using the purified standard such as cholesterol ester, triglyceride, free fatty acid and phosphatidylcholine (Sigma, St. Louis, MO, U.S.A.), and the values were quantitated by measuring the step height of the integrating signal in triplicate samples.

The height ratio of individual neutral lipid to internal standard (cholesterol acetate) was divided by the sum of the ratio and demonstrated as weight per cent.

For free cholesterol and phosphatidylcholine, calibration curves were obtained by adding various amounts of standard free cholesterol and phosphatidylcholine; the values of free cholesterol and phosphatidylcholine in platelets were expressed as μg per 10^8 platelets.

Analysis of phospholipids

Phospholipid in platelets was extracted by Folch's solution. The following procedures were the same as the procedures described above. Phospholipid was subfractionated in chloroform—methanol—water (60:30:3.5, v/v), containing 500 mg/dl of 2,3-di-*tert.*-butyl-1,4-methylphenol (BHT). Each fraction was identified using a standard mixture of phosphatidylcholine, sphingomyelin (Sigma), phosphatidylethanolamine (purified from *Escherichia coli*), phosphatidylinositol (purified from yeast), and phosphatidylserine (purified from bovine brain) (by courtesy of Prof. S. Nozima, School of Pharmacy, Tokyo University, Tokyo, Japan). Each fraction was expressed as a percentage by measuring the step height of the integrating curve of each fraction and calculating the weight per cent compared with that of total phospholipid.

RESULTS

Patterns of standard mixture of neutral lipids and of phospholipids

A standard mixture of neutral lipids was developed by TLC—FID (Fig. 1). Six peaks were separated clearly including cholesterol ester, triglyceride, free fatty acid, free cholesterol, phospholipid, and cholesterol acetate as internal standard. *n*-Eicosane, *n*-dodecylbenzene and lithocolic acid could also be de-

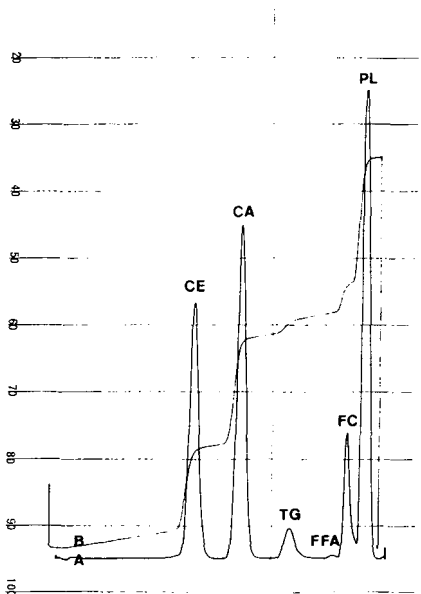


Fig. 1. Pattern of standard mixture of neutral lipids. CE, cholesterol ester; CA, cholesterol acetate; TG, triglyceride; FFA, free fatty acid; FC, free cholesterol; PL, phospholipid. B, integrating curve; A, differential curve.

tected by TLC—FID although cholesterol acetate was superior as internal standard in this system from the point of view of sensitivity and overlapping. A standard mixture of phospholipids could be clearly separated into five fractions which were identified as phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin (Fig. 2).

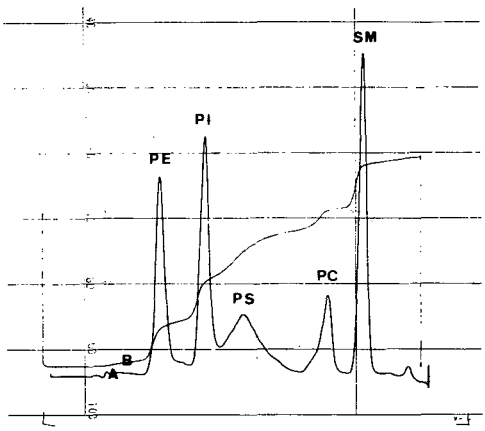


Fig. 2. Pattern of standard mixture of phospholipids. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

Patterns of platelet lipids

Neutral platelet lipids were determined by TLC—FID as cholesterol ester, triglyceride, free fatty acid, free cholesterol, and phospholipid (Fig. 3).

The peaks of free cholesterol and phospholipid were sharp and high, al-

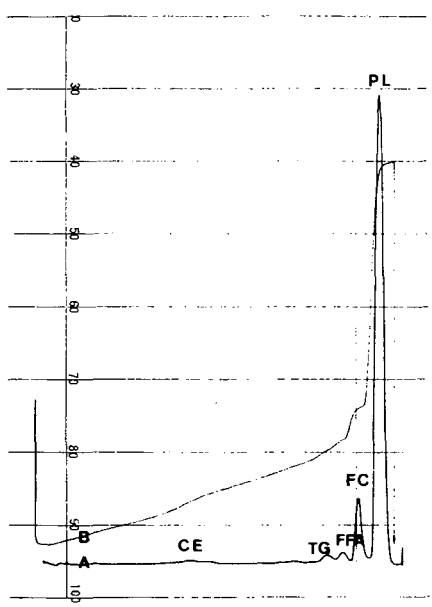
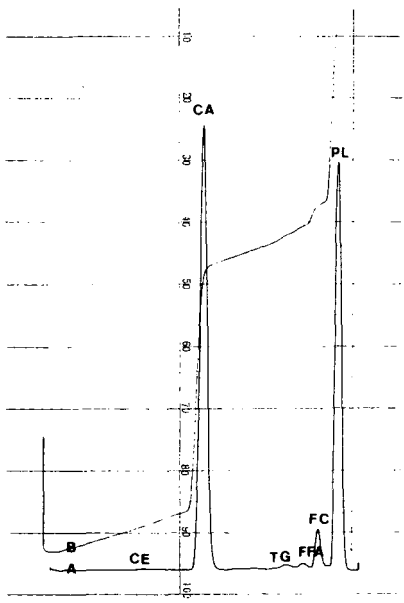


Fig. 3. Pattern of neutral lipids of platelets. Abbreviations as in Fig. 1.



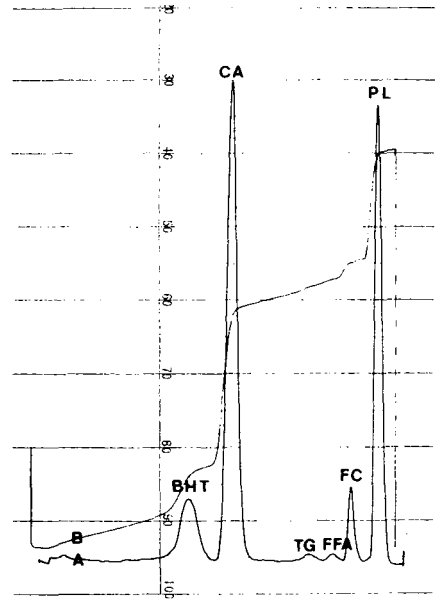


Fig. 4. Pattern of neutral lipids of platelets containing cholesterol acetate (CA) as internal standard. Abbreviations as in Fig. 1.

Fig. 5. Patterns of neutral lipids of platelets containing cholesterol acetate (CA) as internal standard and BHT as an antioxidant. Abbreviations as in Fig. 1.

though the peaks of cholesterol ester, free fatty acid, and triglyceride were lower and in many cases they were unrecognizable. The pattern of neutral lipids containing cholesterol acetate as an internal standard was no different from that without cholesterol acetate (Fig. 4). Neutral lipids of platelets with BHT added as an antioxidant and cholesterol acetate as an internal standard also separated into clear fractions without interference from one other (Fig. 5).

Platelet phospholipids were subfractionated into four peaks corresponding to phosphatidylethanolamine, phosphatidylinositol plus phosphatidylserine, phosphatidylcholine, and sphingomyelin (Fig. 6). Phosphatidylcholine and phosphatidylserine could not be separated clearly into two peaks by TLC—FID.

Reproducibility of the TLC—FID method

The reproducibility of the TLC—FID method was examined by analysing one sample of platelets ten times. The mean value and standard deviation of the weight per cent was calculated and the variation coefficients were 8.4% for free cholesterol, 37.0% for phosphatidylethanolamine, 45.0% for phosphatidylinositol plus phosphatidylserine, 11.1% for phosphatidylcholine and 3.5% for sphingomyelin.

Standard curves of free cholesterol and phosphatidylcholine

The standard curves of free cholesterol and phosphatidylcholine showed good linearity. The factor of each standard curve was $Y = 250.80\ X + 18$ for free cholesterol and $Y = 431.00\ X$ for phosphatidylcholine.

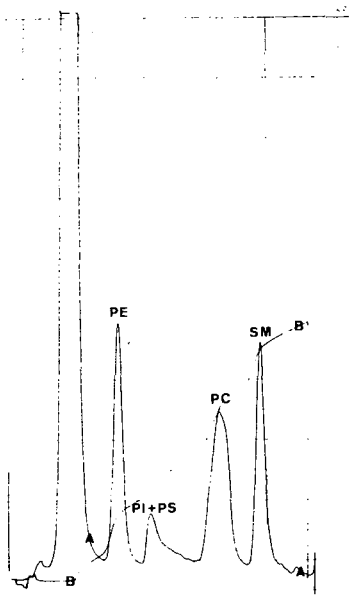


Fig. 6. Patterns of phospholipids of platelets. Abbreviations as in Fig. 2.

TABLE I

PERCENTAGE OF TOTAL LIPIDS IN WHOLE PLATELETS

	This paper (n=21)	Marcus et al. [5]
Neutral lipids	14.6±3.6	21.0
Phosphatidylethanolamine	24.9±4.5	20.0
Phosphatidylserine	6.8±3.4	8.0
Phosphatidylinositol		3.4
Phosphatidylcholine	35.2±3.1	31.4
Sphingomyelin	18.6±2.6	14.5
Lysolecithin	–	0.6
Cardiolipin	–	0.4
Gangliosides	–	0.5

TABLE II

ABSOLUTE AMOUNTS OF FREE CHOLESTEROL AND PHOSPHOLIPID AND PERCENTAGES OF PHOSPHOLIPID SUBFRACTIONS

	FC*	PL*	PE**	PI+PS**	PC**	SM**
This study	7.32±1.10	46.06±8.36	29.6±4.9	7.8±3.9	41.1±3.9	22.1±2.8
Shattil and Cooper [7]	11.22	34.44				
Nördoy and Rödset [10]		23.62	31.6	10.1	44.7	13.5
Marcus et al. [5]			27.0	15.1	38.4	17.0

*μg per 10^8 platelets.

**Percentage of each subfraction of phospholipids.

Lipid content of platelets

The values of platelet lipids were determined as weight percentages (Table I). Neutral lipids were about 15% and their major part consisted of free cholesterol. Phospholipids were approximately 80% and were subdivided into approximately 35% of phosphatidylcholine and 20% of phosphatidylethanolamine and sphingomyelin.

The values (μg) per 10^8 platelets calculated from the standard curve indicated also that 46 μg of phospholipid was major as compared with 7 μg of free cholesterol (Table II). Lysolecithin, cardiolipin and gangliosides could not be detected by TLC—FID.

DISCUSSION

It has been suggested that the lipid contents of platelets are important factors in inducing atherosclerosis through aggregation of platelets under hyperlipoproteinemic conditions [6, 7]. Vandamme and Vankerckhoven studied plasma lipid analysis by TLC—FID, and reported that the method correlated well with the conventional method of quantitating lipids in plasma [8]. Platelet lipid analysis in conjunction with plasma lipid analysis might provide useful information for evaluating the hyperaggregability of platelets in patients with hyperlipidemia.

A rapid and simple method to quantitate lipids in platelets is desirable for examining the possible role of platelet lipids in atherogenesis. The purpose of this study was to examine the validity of the TLC—FID method to quantitate platelet lipids, and to determine the absolute concentrations of the individual lipids in platelets from healthy adults.

As an internal standard for neutral lipids, *n*-eicosane, *n*-dodecylbenzene, lithocholic acid and cholesterol acetate were examined; cholesterol acetate was found to be most suitable since it did not overlap or interfere with coexisting peaks.

BHT added in the solvent as an antioxidant for neutral lipids was found to overlap completely the peak of cholesterol ester so that BHT was used only for the separation of phospholipids.

The TLC—FID patterns of human platelets demonstrated cholesterol ester, triglyceride, free fatty acid and free cholesterol as neutral lipids, and phospholipids containing phosphatidylethanolamine, phosphatidylinositol plus phosphatidylserine, phosphatidylcholine and sphingomyelin, but not lysolecithin, cardiolipin or gangliosides. The patterns of TLC—FID of these lipids in human platelets was compatible with that reported by Vandamme and Vankerckhoven [6].

The minimum amount detected by this TLC—FID method as a peak under the most sensitive conditions was 0.05 μg using cholesterol acetate. The peaks of the minor neutral lipids were usually low and were not necessarily detected as reliable peaks from 10^8 platelets. This would contribute to our finding of a lower percentage of neutral lipids compared to Marcus et al. [5].

Free cholesterol was quantitated most satisfactorily and reliably from the standard curve in this method. The lower amounts of free cholesterol found in our platelets compared to the data of Shattil and Cooper [9] obtained by gas—liquid chromatography were presumably due to the difference in the

methods, because there was no difference in the levels of plasma cholesterol and phospholipid between the two groups.

With regard to the phospholipid, the highest values were obtained in our study compared to others [7, 10]. We used phosphatidylcholine alone purified from egg yolk for the standard curve. There is a difference in the composition of the free fatty acids in phosphatidylcholine from egg yolk and that of platelets. The free fatty acid in phosphatidylcholine of egg yolk examined in our laboratory was C16:0, 38.4%; C18:0, 16.8%; C18:1, 18.4%; C18:2, 16.4%; whereas that of platelets reported by Marcus et al. [5] was C16:0, 34.1%; C18:0, 14.1%; C18:1, 27.0%; C18:2, 6.9%; C20:4, 12.0%; indicating an excess of C20:4 in platelets.

In addition, phosphatidylcholine is a major component of platelet phospholipids. These factors were considered to be a cause of the higher values for phospholipid found in our study.

As the subfraction of platelet phospholipids, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin were completely separated while phosphatidylinositol and phosphatidylserine were hardly separated from each other. This evidence may contribute to the lower amount of phosphatidylinositol plus phosphatidylserine and the larger amount of sphingomyelin found in our study compared to reported data [5, 10].

Trace amounts of lysolecithin, cardiolipin and gangliosides are known to exist in platelets, but they could not be detected by our method [5].

There are many advantages in employing the TLC—FID method to quantitate the lipid content of platelets. Firstly, concentration of different lipids can be determined in a single procedure. Since the molar ratio of free cholesterol to phospholipid has a significant role concerning the fluidity of platelet plasma membranes [7, 9], the values of the cholesterol/phospholipid molar ratio determined by this method are more reliable than those measured by conventional techniques which quantitate cholesterol and phospholipid separately.

Quantitation of the subfractions of phospholipids is feasible using the TLC—FID method. The Chromarod can be utilized repeatedly after it has passed through the flame ionization detector. Finally, it is very beneficial that the amount of sample required for chromatography of both neutral lipid and phospholipids is only 6 μl, since the remainder of the sample (194 μl) can be used for further lipid analysis such as fatty acids.

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REFERENCES

1 L.A. Harker, R. Ross and J.A. Glomset, in G. de Gaetano and S. Garattini (Editors), Platelets: A multidisciplinary Approach, Raven Press, New York, 1978, p. 89.
2 W.G. Van Aken, in W.D. Reitsma (Editor), Atherosclerosis, Excerpta Medica, Amsterdam, 1979, p. 17.
3 M.M. Bern, Diabetes, 27 (1978) 342.

4 J.P. Folch, M. Lees and S. Sloane, J. Biol. Chem., 226 (1957) 497.
5 A.J. Marcus, L.B. Safier and H.L. Ullman, in G.J. Nelson (Editor), Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism, Wiley-Interscience, New York, 1972, p. 417.
6 A.G.A. Carvalho and R.W. Colman, N. Engl. J. Med., 290 (1974) 434.
7 S.J. Shattil and R.A. Cooper, J. Lab. Clin. Med., 89 (1977) 231.
8 D. Vandamme and G. Vankerckhoven, Clin. Chim. Acta, 89 (1978) 231.
9 S.J. Shattil and R.W. Cooper, J. Clin. Invest., 55 (1975) 636.
10 A. Nördoy and J.M. Rödset, Diabetes, 19 (1970) 698.

Journal of Chromatography, 182 (1980) 311—316
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MINIATURE TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC SEPARATION OF LECITHIN AND SPHINGOMYELIN*

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SUMMARY

A miniature two-dimensional thin-layer chromatographic procedure employing silica gel impregnated glass-microfiber chromatography sheets (commercial product, ITLC-type SG sheets) has been developed for the separation of lecithin (L) and sphingomyelin (S) from a standard lipid mixture containing L, S, lysolecithin, phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine, phosphatidyl glycerol, and diphosphatidyl glycerol. The newly developed procedure eliminates possible interference from PI and PS. Complete separation of L and S was easily achieved with chromatographic solvent migration times of approximately 3 and 2 min for the first and second dimensions, respectively. The lipids were visualized by charring and fluorescent staining techniques. The procedure has been adapted for the separation of L and S from amniotic fluid samples.

INTRODUCTION

Since Gluck et al. [1] described the thin-layer chromatographic (TLC) separation of amniotic fluid lecithin (L) and sphingomyelin (S) in 1971, the L/S ratio procedure has become the most popular chemical method for the estimation of fetal pulmonary maturity [2].

Phosphatidyl inositol (PI) and phosphatidyl serine (PS) have been shown to interfere in some TLC separation procedures for L and S [3—5]. Giudicelli et al. [6] have demonstrated that the acetone precipitation procedure does not remove PI and PS. However, they have removed these interferences by a two-di-

*Editorial remark. According to Editorial opinion the technique described herein, employing silica gel impregnated glass-microfiber chromatography sheets, may more appropriately be classified as a paper chromatography procedure.

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mensional TLC separation [6, 7]. Other researchers have removed these interferences by adsorption onto diethylaminoethyl cellulose [4, 5].

To significantly reduce the TLC solvent migration time, researchers have employed silica gel impregnated glass-microfiber sheets (commercial product, ITLC^{TM}-type SG sheets) [3, 5, 8—13]. The separation of lecithin and sphingomyelin has been reported to require less than 5 min [8]. A commercial kit employing ITLC-type SG sheets for the determination of L/S ratios in amniotic fluid has been marketed by Gelman (Ann Arbor, MI, U.S.A.) [14]. Phospholipid separation is achieved in approximately 3 min.

In this paper, lecithin and sphingomyelin have been separated from a phospholipid mixture by two-dimensional TLC on ITLC-type SG chromatography sheets. This new procedure has combined the separation advantages of the two-dimensional technique with the rapid separation achieved on ITLC sheets. The phospholipids were visualized by charring and fluorescent staining techniques [1, 5, 15].

MATERIALS AND METHODS

Each of the following phospholipids were obtained in duplicate from Sigma (St. Louis, MO, U.S.A.) and from Supelco (Bellefonte, PA, U.S.A.): phosphatidyl glycerol (PG, egg yolk, from Sigma; PG, bacterial, from Supelco), diphosphatidyl glycerol (DPG, bovine), phosphatidyl inositol (plant), and phosphatidyl serine (bovine). Commercially prepared 1:1 L/S ratio standards, lecithin (Type III E, egg yolk), sphingomyelin (bovine brain), and phosphatidyl ethanolamine (PE, Type I, bovine brain; Type III, egg yolk) were purchased from Sigma. Synthetic lysolecithin (LL) was obtained from Calbiochem (San Diego, CA, U.S.A.).

The following Certified ACS grade chemicals were purchased from Fisher Scientific (Winnipeg, Canada): ammonium sulfate, chloroform, isopropanol, methanol and methylene chloride. Absolute ethanol and methanol were from the Standard Chemical Company (St. Boniface, Canada). Concentrated sulfuric acid and ammonium hydroxide (28—30% NH_3) were purchased from Canadian Industries (St. Boniface, Canada). The 2′,7′-dichlorofluorescein (DCF) was from Eastman Kodak (Rochester, NY, U.S.A.).

A 20 × 20 cm ITLC-type SG chromatography sheet was cut to size, 6.5 × 10.0 cm, to fit a Seprachrom chromatography chamber (Gelman, Ann Arbor, MI, U.S.A.). One hole was punched, 2 cm from the bottom and 2 cm from the right edge, with a Gem handpunch containing a 3-mm circular die (McGill, Marengo, IL, U.S.A.). Prior to use, the miniature sheets were heated in an oven at 100°C for 30 min and stored in a desiccator above calcium sulfate.

Chromatography solvent system I consisted of methylene chloride—ethanol—water (100:25:3, v/v). Solvent system II was composed of chloroform—methanol—ammonium hydroxide (170:20:3, v/v). Both solvent systems were prepared fresh weekly and stored in tightly-stoppered brown bottles at room temperature. Both solvent systems were shaken vigorously for one full minute just prior to use.

The ammonium sulfate—sulfuric acid charring reagent [15] was prepared by adding 20 g of ammonium sulfate and 4 ml of concentrated sulfuric acid to a

100-ml volumetric flask which was filled to volume with distilled water. The fluorescent visualization stain [5] was prepared by adding 1.5 mg of 2′,7′-dichlorofluorescein to a 100-ml volumetric flask which was filled to volume with isopropanol.

Phospholipid standard No. 1 was prepared to contain LL, L, S and PG each at 3.0 mg/ml of chloroform—methanol (9:1, v/v). Standards Nos. 2—5, were each similarly prepared to contain LL, L, S and one of the following: DPG, PI, PS or PE, respectively. Standard No. 6 was similarly prepared to contain LL, L, S, DPG, PG, PE, PI and PS.

The standard application technique of Popowicz [16] was employed, as previously described [5]. One microliter of phospholipid mixture No. 1 was applied to a blank chromatography disc. The disc was air dried and inserted into the prepunched hole of a miniature chromatography sheet. Solvent system I was mixed vigorously for one full minute. Three milliliters were pipetted into a Seprachrom chromatography trough. The chromatography chamber was assembled and the solvent was allowed to migrate 60 mm above the origin. This required approximately 3 min. The chromatogram was air dried for 5 min. To permit chromatographic separation in the second dimension, the sheet was cut to size (6.5 × 6.5 cm). The second solvent system was allowed to migrate 40 mm above the origin. This required approximately 2 min. Following a 5-min air drying, the phospholipids were visualized by a DCF staining technique as previously described [5] or by spraying with 50% sulfuric acid and charring above a hot plate. Chromatographic separation and visualization of standards Nos. 1—6 were repeatedly performed as described above. R_F values were calculated for each of the phospholipids tested.

A standard chromatography disc was prepared to contain LL, L, and S, each at 3 μg, and DPG, PG, PE, PI and PS, each at a concentration of 5 μg. Two-dimensional thin-layer chromatographic separation was performed as described above. Following a 5-min air drying, the chromatography sheet was immersed for 3 min in the ammonium sulfate—sulfuric acid charring reagent. The sheet was removed and placed in an upright position for approximately 5 min, to allow excess reagent to drain. The chromatogram was placed on a 350°C hot plate for 2 min. The charred phospholipids appeared as grey to black spots on a white background. The spots were observed most clearly when the chromatogram was illuminated from behind. The chromatogram was photographed with Kodak high-contrast copy film (HC710) rated at ASA 25, and developed for 4 min in diluted Dektol [stock Dektol—water (1:1, v/v), Kodak Canada, Toronto, Canada]. Photography was by Audio Visual Services, University of Regina, Regina, Canada.

The miniature two-dimensional glass-microfiber TLC separation technique was evaluated for the isolation of amniotic fluid L and S. Amniotic fluid sample preparation was as previously described [8, 9]. Chromatographic separation, visualization, and subsequent photography were performed as described above.

RESULTS AND DISCUSSION

Solvent system I was evaluated for its ability to separate L and S from a mixture containing eight phospholipids. Similar to the observations of previous researchers [3—5], PI and PS were observed to interfere with the accurate determination of the L/S ratio. Both PI and PS formed elongated spots under the conditions employed in this study. At times, PI and PS were distributed through both the L and S spots on the chromatogram.

The separation of L and S from PI and PS was achieved in the second dimension with solvent system II. Minimal migration of PI and PS was observed to occur in the second dimension (Fig. 1). The R_F values have been calculated for the first and second dimensions for each of the phospholipids studied (Table I).

TABLE I

THE R_F VALUES OF PHOSPHOLIPIDS COMMON TO AMNIOTIC FLUID

Each value reported represents an average of at least ten test results.

Phospholipid	First dimension R_F	Second dimension R_F
Lysolecithin	0.23*	0.28*
Sphingomyelin	0.37*	0.38*
Lecithin	0.55*	0.53*
Phosphatidyl serine	0.71**	0.20
Phosphatidyl inositol	0.57**	0.17
Phosphatidyl glycerol	0.77*	0.61*
Phosphatidyl ethanolamine	0.81	0.59
Diphosphatidyl glycerol	0.95	0.79

*Each value reported represents an average of at least twenty-five results.
**Approximate R_F results were calculated from diffusely distributed PI and PS spots (see Fig. 1). Refer to text for complete details.

It should be noted that the PI and PS values were inadvertently transposed in a preliminary abstract presentation [17]. Although standard mixtures of PI and PS were found to form elongated spots in the first dimension, more discrete and compact spots were observed when amniotic fluid samples were analyzed (Fig. 2). Ageing and decomposition of PI and PS in the standard mixtures may account for the observed variation. Another variation, labelled X in Fig. 1, was found to be either a contaminant or a decomposition product within a commercial PG preparation.

Previous publications [5, 8, 9] and the marketing of a commercial kit [14] attest to the fact that a rapid and reliable separation is achieved between L and S on ITLC-type SG chromatography sheets with solvent system I. The incorporation of solvent system II in the second dimension enhances the separation distance and eliminates interference from PI and PS.

The R_F values for each of the phospholipids analysed have been observed to vary under the influence of a wide range of adverse experimental conditions, e.g. seasonal fluctuations in laboratory temperature, different lot numbers of ITLC sheets, and inter-laboratory differences. The latter is primarily attributed to variations in reagents, glassware, and laboratory personnel. However, in all

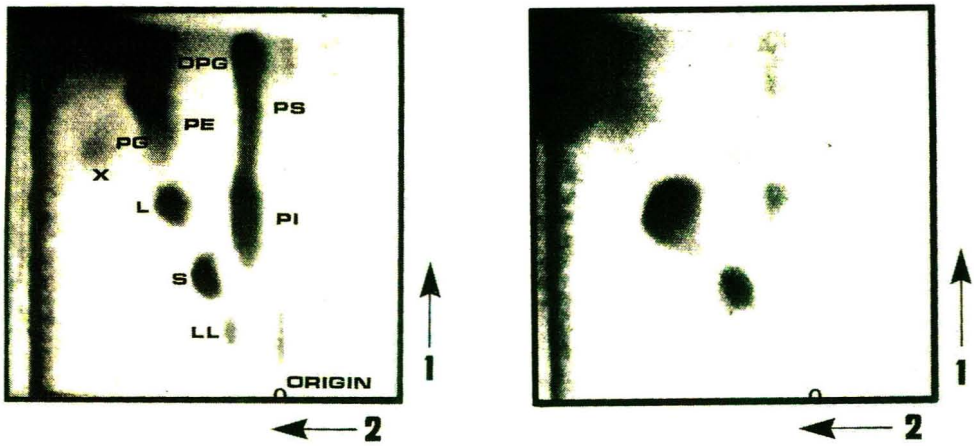

Fig. 1. Miniature two-dimensional thin-layer chromatogram depicting the separation of lecithin (L) and sphingomyelin (S) from a standard lipid mixture containing L, S, lysolecithin (LL), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG). Refer to text for complete details.

Fig. 2. Miniature two-dimensional thin-layer chromatogram depicting the separation of lecithin and sphingomyelin from other lipids contained in an amniotic fluid sample taken after fetal lung maturity. Refer to text for complete details.

cases it was observed that the relative positions of the phospholipids remained constant, and that complete separation of L and S was always achieved for both standards and patient samples. Representative chromatographic separations of L and S from amniotic fluid specimens collected before and after fetal lung maturation are depicted in Figs. 3 and 2, respectively. Maturity or immaturity was verified by comparison to another laboratory procedure [18] and by examination of the case histories.

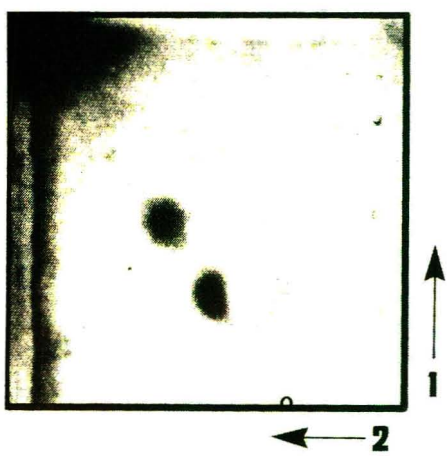

Fig. 3. Miniature two-dimensional thin-layer chromatogram depicting the separation of lecithin and sphingomyelin from other lipids contained in an amniotic fluid sample taken prior to fetal lung maturity. Refer to text for complete details.

Preliminary investigation employed a 50% sulfuric acid spray and charring above a hot plate for visualization of the phospholipids. Subsequent visualization with an ammonium sulfate—sulfuric acid charring reagent [15] was more convenient, and improved the charring consistency (see Figs. 1—3).

Solvent system I was observed to be stable for at least six weeks when stored in a tightly stoppered amber bottle. Reproducible results were achieved as long as the solvent was shaken vigorously for one minute before use. Solvent system II has been stable for up to 7 days, when stored in a tightly stoppered bottle.

The present two-dimensional chromatographic separation requires approximately 3 min for solvent migration in the first dimension and 2 min for solvent migration in the second dimension. The drying times are also rapid for the ITLC chromatograms, as the solvent evaporates quickly from the glass-microfiber sheets. Economy is achieved with the small size of the ITLC sheet and the minimal volumes of solvents required.

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REFERENCES

1 L. Gluck, M.V. Kulovich, R.C. Borer, P.H. Brenner, G.G. Anderson and W.N. Spellacy, Amer. J. Obstet. Gynecol., 109 (1971) 440.
2 C.E. Wilde and R.E. Oakley, Ann. Clin. Biochem., 12 (1975) 83.
3 C.F. Cusick, Ann. Clin. Biochem., 13 (1976) 379.
4 L. Gosselin and J.M. Foidart, Clin. Chim. Acta, 81 (1977) 209.
5 D.S. Ng and K.G. Blass, J. Chromatogr., 163 (1979) 37.
6 Y. Giudicelli, R. Pecquery, M. Lacasa, M. Boscameric and R. Nordmann, Clin. Chim. Acta, 71 (1976) 445.
7 Y. Giudicelli, R. Pecquery, C. Magyar, M. Lacasa and R. Nordmann, Clin. Chim. Acta, 60 (1975) 335.
8 K.G. Blass, R.J. Thibert and T.F. Draisey, J. Chromatogr., 89 (1974) 197.
9 K.G. Blass, R.J. Thibert and T.F. Draisey, Clin. Chem., 19 (1973) 1394.
10 E.H. Coch, G. Kessler and J.S. Meyer, Clin. Chem., 20 (1974) 1368.
11 E. Coch, J.S. Meyer, G. Goldman and G. Kessler, Clin. Chem., 19 (1973) 967.
12 E.H. Coch and G. Kessler, Clin. Chem., 18 (1972) 490.
13 N.L. Sass, R. Alvarado and J.P. Martin, Biochem. Med., 15 (1976) 217.
14 Gelman Seprachrom™ L/S Ratio System, Procedure Manual, Gelman Instrument Company, Ann Arbor, MI, Technical Bulletin 31, 1974.
15 T. Ziminski and E. Borowski, J. Chromatogr., 23 (1966) 480.
16 J. Popowicz, J. Chromatogr., 7 (1962) 271.
17 R.L. Briand, D.S. Ng and K.G. Blass, Clin. Biochem., 11 (1978) P-2.
18 L. Gluck, M.V. Kulovich and R.C. Borer, Clinics in Perinatology, 1 (1974) 125.

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DETERMINATION OF URIC ACID IN SERUM USING ISOTACHOPHORESIS

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SUMMARY

An operational system is described for the isotachophoretic determination of uric acid in serum, making use of column coupling. The method has been compared with a standard enzymatic procedure. With the present technique small amounts of serum (ca. 3 μl) can be applied without any pretreatment. Urate recovery was 99.0—100.5%. Under the non-physiological measuring conditions used, 12—28% of control serum uric acid was bound to macromolecules of molecular weight exceeding 25,000. The day-to-day variations of the isotachophoretic procedure were smaller than those of the enzymatic method, whereas standard deviations were comparable. The isotachophoretic procedure is less influenced by certain metabolites.

INTRODUCTION

Uric acid is the end-product of purine catabolism in man. An abnormal concentration of uric acid in body fluids may be indicative of a number of disturbances. An increased level of uric acid in serum (hyperuricemia) is seen in primary gout: although there is a normal excretion rate, uric acid is overproduced or it is underexcreted [1].

In secondary gout, hyperuricemia might result either from increased nucleic acid turn-over (for example, hematologic disorders, leukemia), or from decreased renal excretion of uric acid induced by drugs or dietary factors. Also in a number of genetic disorders, such as glycogen storage disease, Down's syn-

drome and psoriasis, is hyperuricemia a common feature [1, 2].

The methods currently used to assay uric acid in serum and urine are based on either chemical or enzymatic oxidation to allantoin. The enzymatic method seems to be the method of choice because of its sensitivity, accuracy and specificity [3, 4]. Analytical techniques such as high-performance liquid chromatography (HPLC) and isotachophoresis can be applied for determination in biological fluids of a series of metabolites, including uric acid. Methods using HPLC have been reported for serum urate [5], blood and urine [6]. Isotachophoresis has been employed in the analysis of nucleotides in muscle extracts [7] and urinary purines and pyrimidines, including uric acid [8]. Until now isotachophoresis has not been used for the rapid determination of serum uric acid.

Isotachophoresis is an electrophoretic separation method, taking advantage of the non-diluting phenomenon of the sample zone in the steady-state [9]. In this paper an isotachophoretic procedure is described for the quantification of uric acid in serum.

Unlike other available methods, such as the colorimetric [10] and the enzymatic [3, 4] methods, the determination of uric acid by HPLC and isotachophoresis is much less hampered by interfering substances, such as drugs and biological metabolites. In contrast to the HPLC procedure, where pretreatment of biological samples is often necessary (e.g. removal of proteins), the sample can mostly be applied directly in isotachophoresis. Moreover, the ratio of free to protein-bound urate can be determined conveniently using a simple ultrafiltration step.

MATERIALS AND METHODS

Materials

Uric acid (sodium salt), HCl (Titrisol^{R}), tris(hydroxymethyl)aminomethane (Tris), ϵ-aminocaproic acid (EACA) and morpholino-ethanesulfonic acid (MES), all analytical grade, were purchased from Merck (Darmstadt, G.F.R.). Hydroxyethylcellulose (HEC) was obtained from Polysciences (Warrington, PA, U.S.A.; Cat. No. 5568); a 0.5% (w/v) stock solution was purified by ion exchange. Uricase was purchased from Løvens Kemiska Fabrik (Ballerud, Denmark). Serum was prepared from venous blood after clotting (2 h at room temperature) and centrifugation for 10 min at 1000 *g* (4°C). The samples were stored at −20°C. Ultrafiltration CF 25 centriflow filters (mol. wt. cut-off 25,000) were purchased from Amicon (Oosterhout, The Netherlands).

Methodology

In the present study isotachophoretic equipment was used in which two Teflon capillaries with different internal diameters were mounted [11, 12]. This set-up is of special interest for the analysis of biological samples. In such samples constituents are often present at a low concentration and the concentration of various compounds can differ by an order of magnitude. This is the case in serum, for example, where the chloride concentration can exceed the concentration of uric acid by a factor of up to 500. In such mixtures the analysis time required to obtain sufficient information on a given compound in-

creases when its concentration decreases. The present column coupling system [11, 12] alleviates this problem. Basically the equipment consists of two tubes with inside diameters of 0.8 mm (pre-separation tube) and 0.2 mm (separation tube). In the pre-separation tube a high electric current is permitted. At a well-defined distance from a "tell-tale" detector (conductivity type), mounted in the pre-separation compartment, a special construction (bifurcation) allows the separation tube to branch off from the pre-separation tube. The zones of interest can be easily selected via the "tell-tale" detector and separated from the "sample-train", migrating isotachophoretically in the pre-separation tube. In fact, the very efficient separation characteristic of isotachophoresis is applied for both sample pre-separation and final separation. A high sample load is permitted without a significant increase in analysis time. High ratios of concentrations between sample constituents are tolerated and different operational systems can be applied in one analysis. Moreover different electrophoretic principles (for example, isotachophoresis and zone electrophoresis) can be applied [9].

The enzymatic determination of serum uric acid was performed in the laboratory of the Department of Neurology (University Hospital, Nijmegen) with an ABA 100 bichromatic analyser (Abbott). The determination of uric acid is based on the successive action of three purified enzymes which are added to the reaction mixture: uricase, catalase and aldehyde dehydrogenase [4]. The formation of NADPH from $NADP^+$ in the latter reaction (measured at both 340 and 380 nm) is used for the quantification of uric acid. Sera containing known concentrations of uric acid were used as standards.

RESULTS

The operational conditions for the isotachophoretic determination of uric acid are listed in Table I. The leading electrolyte (pH 5.0) contains Cl^- as the leading ion; the terminating electrolyte (pH 6.5) consists of morpholino-ethanesulfonic acid and Tris. The analysis time with the column coupling system is 12 min.

Physiological uric acid concentrations normally range between 0.15 and 0.40 m*M*. In Fig. 1 the calibration curve for uric acid in this range is shown. As can be expected in isotachophoresis there is a linear relationship between the uric acid zone length and the amount of uric acid injected.

TABLE I

OPERATIONAL SYSTEM FOR THE ISOTACHOPHORETIC DETERMINATION OF URIC ACID

	Electrolyte	
	Leading	Terminating
Anion	Chloride	MES
Concentration (*M*)	0.01	0.005
Counter-ion	EACA	Tris
pH	5.00	6.5
Additive	0.25% HEC	
Driving current (A)	20×10^{-6}	

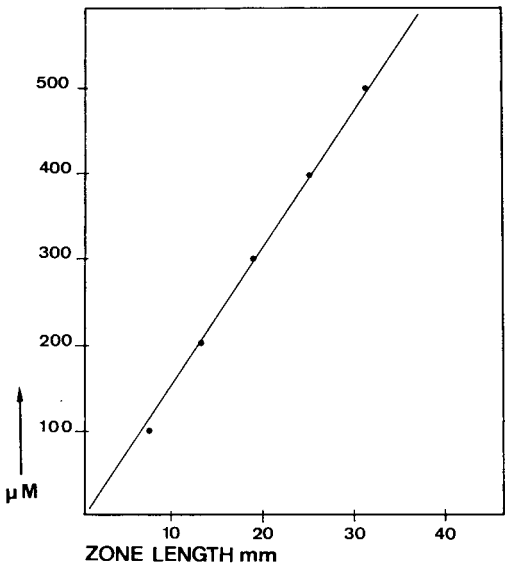

Fig. 1. Calibration curve for uric acid. Operational conditions, see Table I. Injected volume: 3 μl.

When standard amounts of uric acid were added to serum, extensively dialysed against 0.9% NaCl, the isotachophoretic analyses yielded recoveries of 99.0—100.5% (data not shown). The uric acid zone was eliminated after treatment of the serum with purified uricase. In addition, over-spiking confirmed the identity of the uric acid zone.

In order to estimate the amount of uric acid bound to serum proteins under our experimental conditions, the recovery from ultrafiltered and non-ultrafiltered samples was compared. When undialysed pooled serum from several healthy controls was passed through an Amicon CF 25 filter (mol. wt. cut-off 25,000), 85.1% of the total serum uric acid was recovered in the ultrafiltrate, indicating that in this sample approximately 15% was bound to proteins with a molecular weight exceeding 25,000. The lower amount of uric acid in the ultrafiltrate as compared to non-filtered samples was not due to the CF 25 filter; when a standard solution of uric acid (474 μM in water) was passed through it, the recovery was 99.4%. Also the effect of high pH on the binding of urate to serum proteins was studied. The pH of normal serum samples (pH 7.2—7.4) was adjusted with NaOH to pH 10.0 and after ultrafiltration the urate binding turned out to decrease to approximately 7%.

Some serum samples showed turbidity, as judged from visual inspection. Those samples were rapidly passed through a Millipore filter (Millex^{R}, 0.22 μm). This did not affect the recoveries.

Serum samples from six healthy controls were assayed for uric acid using the isotachophoretic method and the enzymatic method. The samples were used either directly or after ultrafiltration. The serum uric acid values obtained with both methods showed an acceptable correlation (Table II): correlation coefficient 0.98. Twelve to twenty-eight per cent of the uric acid seemed to be associated with macromolecules of molecular weight exceeding 25,000. The day-to-

TABLE II

ISOTACHOPHORETIC AND ENZYMATIC DETERMINATION OF URIC ACID IN SERA FROM SIX HEALTHY CONTROLS

No	Isotachophoresis			Enzymatic method		
	NF* (μM)	UF** (μM)	Bound*** (%)	NF* (μM)	UF** (μM)	Bound*** (%)
1	374	329	12	390	303	22
2	392	282	28	383	283	26
3	294	224	24	292	233	20
4	483	415	14	483	400	17
5	361	298	17	375	317	15
6	463	385	17	498	365	27

*NF = not ultrafiltered.
**UF = ultrafiltered (CF 25).
***Bound = the percentage of uric acid removed by ultrafiltration.

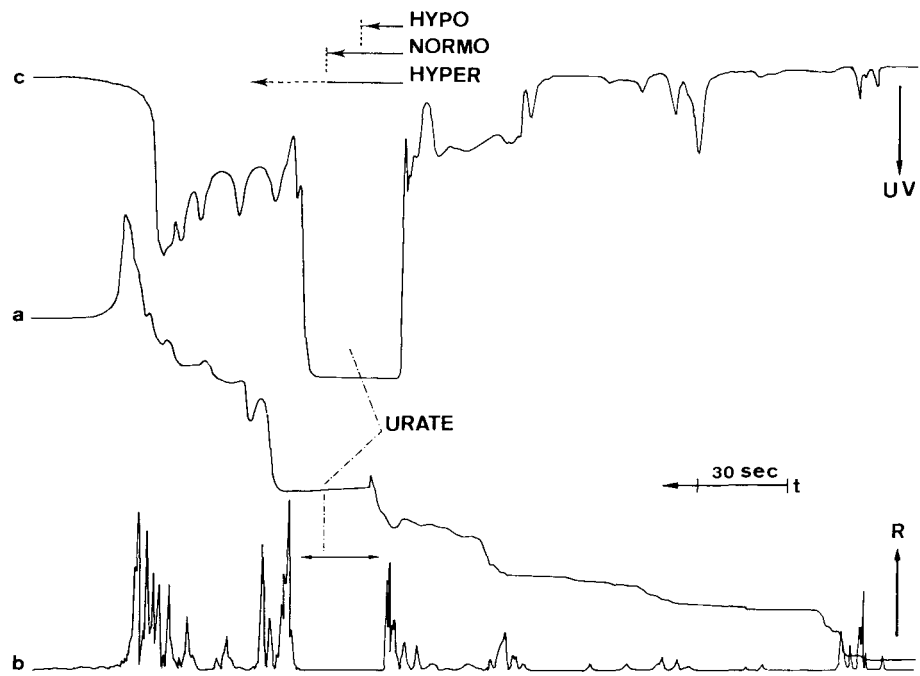

Fig. 2. Isotachophoretic separation of a hyperuricemic serum. The horizontal arrows give the uric acid zone lengths of hypo-, normo- and hyperuricemic sera under standardised operational conditions (Table I). a: conductivity signal, R = increasing resistance. b: differential signal of the conductivity signal. c: UV signal, transmission at 280 nm.

day variation for a repeatedly tested sample was ca. 2% with the isotachophoretic method and ca. 10% with the enzymatic method.

The uric acid zone length is directly representative of the serum concentration under the present operational conditions. In hypo-uricemic sera the zone length will be less than 13.5 mm. Normo- and hyperuricemic sera will give zones of up to 25 mm and longer than 25 mm, respectively (Figs. 1 and 2).

TABLE III

THE EFFECT OF HOMOGENTISIC ACID ON URIC ACID DETERMINATION BY ISOTACHOPHORESIS AND THE ENZYMATIC METHOD

Addition	Isotachophoresis (μM)	Enzymatic method (μM)
None	348	348
0.5 g/l homogentisic acid	348	364
5.0 g/l homogentisic acid	346	676

TABLE IV

SERUM URIC ACID CONCENTRATIONS IN HYPERURICEMIC PATIENTS RECEIVING VARIOUS DRUGS

Diagnosis	Medication	Uric acid (μM)	
		Enzymatic method	Isotachophoresis
Gout (male, age 63)	Zyloric	286	299
Rheumatoid arthritis with hyperuricemia (female, age 68)	Hygroton, Selokene, Penicillinamide, Indocid Seresta	620	647
Rheumatoid arthritis with hyperuricemia (female, age 44)	Baktrimel, Primnison, Torecan	356	366

Several metabolites and drugs can interfere with the enzymatic determination of uric acid. An example is homogentisic acid, a compound which occurs in increased quantities in urine of patients with alkaptonuria, an inborn error of amino acid metabolism [13, 14]. When unphysiologically high amounts of homogentisic acid were added to samples, no effect on the isotachophoretic determinations was seen. However, with the enzymatic method higher values than those actually present were recorded (Table III).

Serum uric acid values in three initially hyperuricemic rheumatologic patients, who were treated with a number of drugs, were in close agreement when determined by both procedures (Table IV). None of the drugs seemed to interfere with the uric acid determination according to both methods.

DISCUSSION

The present isotachophoretic method for the determination of uric acid levels in serum is quantitative, reliable and reproducible (Fig. 1). In contrast to the general practice in HPLC [6] there is no need for deproteinisation: the samples can be applied directly. However, also a HPLC system without deproteinisation has been described [5]. It should be pointed out here, that in HPLC it is sometimes difficult to predict the retention behaviour of a given compound. In isotachophoresis this is more predictable on the basis of the pK_a values and mobilities of the compounds under study. An additional advantage of isotachophoresis over HPLC is the flexibility of the system: no column packing and equilibration is necessary if rapid switching from one electrolyte

system to another is needed when different conditions have to be tested. Once an electrolyte system has been chosen the isotachophoretic analyses can be done with very low day-to-day variations (< 2%). This value is lower than that obtained with the enzymatic method with a common day-to-day variation of 5—6% [15]. At the present time the isotachophoretic serum uric acid determination is more accurate than the enzymatic method, although the latter is faster when automated.

The physiological significance of the binding of urate to plasma proteins is still disputed. Reversible interactions between urate and serum albumin, low-density-β-lipoprotein, β_2-macroglobulins and α_1,α_2-globulin have been reported [16, 17].

Percentages of 20—40% of bound urate have been described under different conditions of temperature, ionic strength, buffers, etc. [16, 18, 19]. Our values agree with these data (Table II). Reduced binding capacity of plasma proteins might lead to higher levels of free uric acid; in patients with gout such a decreased binding capacity has been reported [16, 18]. It has also been shown that several drugs, such as salicylates, phenylbutazone and probenecid, reduce urate binding in vitro [20]. It should be stressed, however, that all of these studies, including our own, were done under non-physiological conditions. Therefore, no conclusion can be drawn regarding the physiological significance, especially because in vivo measurements have shown that at 37°C the percentage of urate bound is small [21].

Homogentisic acid is a metabolite which occurs in increased quantities in the urine of alkaptonuric patients. It interferes with the enzymatic uric acid determination at 340 nm by causing lower values than those actually present to be recorded [4]. No effect was seen with isotachophoresis in the presence of homogentisic acid, whereas increased levels were read with the enzymatic procedure carried out with the bichromatic (380 and 340 nm) analyser ABA-100 (Table III). No attempts were made to elucidate this experimentally, but the differences might be attributed to the use of a bichromatic analyser in the present study, in contrast to a monochromatic determination at 340 nm only [4].

A number of drugs used in the treatment of three initially hyperuricemic patients did not interfere with any of the methods employed: the uric acid values obtained were comparable (Table IV).

The usefulness of isotachophoresis in screening for inborn errors of purine and pyrimidine metabolism by analysing urinary bases and nucleosides has already been demonstrated [8]. We have recently developed operational systems for the analysis of purine and pyrimidine nucleosides and bases in serum [22]. For both experimental and clinical purposes an alternative analytical approach is opened up, such as for the pharmacokinetic analysis of drug metabolism, or in tracing the consequences of metabolic disturbances using body fluids and cell lysates.

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REFERENCES

1 J.B. Wyngaarden and W.N. Kelley, in J.B. Stanburry, J.B. Wyngaarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Disease, McGraw Hill, New York, 4th ed., 1978, p. 916.
2 D.S. Newcombe, Inherited Biochemical Disorders and Uric Acid Metabolism, University Park Press, Baltimore, MD, 1975.
3 L. Liddle, J.E. Seegmiller and L. Laster, J. Lab. Clin. Med., 54 (1959) 903.
4 R. Haeckel, J. Clin. Chem. Clin. Biochem., 14 (1976) 101.
5 W.D. Slaunwhite, L.A. Pachlan, D.C. Wenke and P.T. Kissinger, Clin. Chem., 21 (1975) 1427.
6 J.A. Milner and E.G. Perkins, Anal. Biochem., 88 (1979) 560.
7 D.C. Gower and R.C. Woledge, Sci. Tools, 24 (1977) 17.
8 A. Sahota, H.A. Simmonds and R.H. Payne, J. Pharm. Methods, 2 (1979) 303.
9 F.M. Everaerts, J.L. Beckers and Th.P.E.M. Verheggen, Isotachophoresis: Theory, Instrumentation and Applications, Elsevier, Amsterdam, Oxford, New York, 1976.
10 N. Kageyama, Clin. Chim. Acta, 31 (1971) 421.
11 F.M. Everaerts, Th.P.E.M. Verheggen and F.E.P. Mikkers, J. Chromatogr., 169 (1979) 21.
12 Th.P.E.M. Verheggen, F.E.P. Mikkers and F.M. Everaerts, Protides Biol. Fluids, 27 (1980) 723.
13 A.E. Garrod, Inborn Errors of Metabolism, Frowde, Hodder and Stoughton, London, 1909.
14 B.N. la Du, V.G. Zannoni, L. Laster and J.E. Seegmiller, J. Biol. Chem., 230 (1958) 251.
15 K. Lamers, Department of Neurology, University Hospital, Nijmegen, personal communication.
16 J.O. Alvsaker, Scand. J. Clin. Lab. Invest., 17 (1965) 467.
17 J.O. Alvsaker, J. Clin. Lab. Invest., 18 (1966) 227.
18 J.R. Klinenberg and I. Kippen, J. Lab. Clin. Med., 75 (1970) 503.
19 P.C. Farrell, R.D. Popovich and A.L. Babb, Biochim. Biophys. Acta, 243 (1971) 49.
20 R. Bluestone, I. Kippen, J.R. Klinenberg and M.W. Whitehouse, J. Lab. Clin. Med., 76 (1970) 85.
21 A.E. Postlethwaite, R.A. Gutman and W.N. Kelley, Metabolism, 23 (1974) 771.
22 F. Oerlemans, Th. Verheggen, F. Mikkers, F. Everaerts and C. de Bruyn, Adv. Exp. Med. Biol., 122 (1980) in press.

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QUANTITATIVE DETERMINATION OF HEMOGLOBIN A_1c BY THIN-LAYER ISOELECTRIC FOCUSING

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SUMMARY

A method for the quantitation of hemoglobin A_1c using isoelectric focusing is reported. Hemolysates were prepared and stabilised with carbon monoxide and with potassium cyanide before the quantitation. The two preparations gave identical results. The potassium cyanide method is simple and adequate for routine purposes, but the cyanohemiglobin compound remains stable for one week only. The carbon monoxide method is more laborious, but the carboxyhemoglobin derivative remains stable for up to one year.

Quantitations of the separated fractions emerging after isoelectric focusing were made with spectrophotometry or with densitometry. No significant difference in the results could be shown. Reproducibility tests were improved by introducing transferrin as an internal standard.

The specificity of the method was checked by the in vitro addition of oral hypoglycemic drugs and of insulin.

INTRODUCTION

A hitherto unknown hemoglobin component in hemolysates prepared from the blood of diabetic subjects was first noted by Rahbar [1] and later identified as hemoglobin A_1c (HbA_1c). Koenig et al. [2] demonstrated that a high A_1c value was an indicator of poor diabetic regulation. Consequently, the development of adequate methods for the routine quantitative determination of HbA_1c is important.

Previously described methods for the determination of HbA_1c revealed different disadvantages. Trivelli et al. [3] developed a rather laborious ion-exchange chromatographic method with a low capacity and with no separation between the hemoglobin fractions F and A_1c. Modifications of the method of Trivelli et al. [3] have been described [4,5] but they all revealed lack of specificity, as no separation between HbA_1a, HbA_1b and HbA_1c, nor between the HbF and the HbA_1c fractions could be obtained.

This paper describes a rapid and specific method for quantitation of HbA_1c, adequate for clinical purposes in the monitoring of metabolic regulation in diabetic patients.

MATERIALS AND METHODS

Chemicals

The polyacrylamide gels (1 mm thick) specially designed for HbA_1c determinations (LKB Products, Bromma, Sweden) had a specific narrow-range pH gradient. The anolyte (0.05 *M* L-glutamic acid), the catholyte (0.1 *N* sodium hydroxide), potassium cyanide, tetrachloromethane and ammonium hydrogen carbonate were all of analytical grade (Merck, Darmstadt, G.F.R.). Human-transferrin was obtained from Behringwerke (Marburg-Lahn, G.F.R.). Chromatographically purified HbA_1c was obtained from Isolab. (Akron, OH, U.S.A.). Carbon monoxide was of specially purified grade. The staining solution consisted of 1 g Coomassie Brilliant Blue R-250 (Merck) dissolved in 1 l of an aqueous solution containing ethanol (25%) and acetic acid (8%). This solution should be freshly prepared every day. Fixing solution: 57.5 g trichloroacetic acid and 17.25 g sulphosalicylic acid were added to 500 ml distilled water. Destaining solution: 500 ml ethanol and 160 ml acetic acid were mixed and diluted to 2 l with distilled water. Preserving solution: 40 ml of glycerol were added to 400 ml of the destaining solution.

Equipment

The isoelectric focusing was carried out on a LKB Multiphor System 2117 equipped with a constant power supply LKB 2103. To maintain a constant temperature (15°C) a LKB multitemp 2209 was used. Sample application pieces were LKB Paratex (10 × 20 mm) and the electrode focusing strips were LKB 2118-106. The measurements were carried out on a spectrophotometer LKB 2074 and a soft laser scanner SL-504 from Biomedical Instruments (Chicago, IL, U.S.A.).

Preparation of hemolysates

A 2-ml volume of venous whole blood was collected in a heparin tube. A 160-μl volume of capillary blood was drawn into heparin coated microhematocrit tubes (Beckmann, Fullerton, CA, U.S.A.). Erythrocytes that had been washed three times with five volumes of saline were hemolysed with eight volumes of distilled water. The cell debris was extracted with half a volume of tetrachloromethane. After centrifugation for 30 min at 2000 *g* the upper layer was separated and equilibrated with carbon monoxide for 5 min at 37°C. This resulted in a concentration of hemoglobin equal to 2—2.5 mmol/l. The carboxyhemoglobin was stored at —70°C until analysis. Stored in this way the carboxyhemoglobin is stable for one year [6].

The cyanohemiglobin method was as described for the carboxyhemoglobin method except that 0.03 *M* potassium cyanide was used to hemolyse the erythrocytes.

Preparation of hemolysates, densitometric method

The preparation of the hemolysates was as described under the spectrophotometric method except for the addition of transferrin as the internal standard (1.5 mg) for venous, and (0.4 mg) for capillary blood samples. Furthermore the concentration of total hemoglobin in the hemolysates was diminished 30 times by dilution with distilled water. This resulted in a hemoglobin concentration equal to 60—100 μmol/l before application on the gel slab.

Isoelectric focusing

The gel was placed on a glass cooling plate in an electrophoresis box and kept at 15°C during the separation procedure.

The gel was pre-run at 20 W for 30 min. Next, 30 sample application pieces were placed at a distance of 5 mm from the cathodic electrode focusing strip. Each sample application piece was covered with 15 μl of hemolysate and the gel was supplied with a constant effect of 20 W for 2 h. The hemolysate had been quantitatively removed from the sample application pieces within the first 30 min of electrophoresis. At that time the application pieces were removed to avoid tailing phenomena. To ensure maximum separation the gel was supplied with an effect of 30 W for the last 10 min.

Evaluation of hemoglobin A_1c concentration

Spectrophotometric method. The hemoglobin A_1c bands were cut off and transferred to plastic tubes containing 1 ml of a 1:1 mixture of 0.05 *M* ammonium hydrogen carbonate and 0.015 *M* potassium cyanide buffered to pH 8.4, and eluted in a rotary mixer (20 rpm) for 4 h. The carboxyhemoglobin samples were eluted in ammonium hydrogen carbonate only.

The total concentration of hemoglobin (C_{Hb}) in the hemolysate was determined by dilution of 15 μl of the hemolysate in 10 ml of ammonium hydrogen carbonate.

The extinctions of carboxyhemoglobin and cyanohemiglobin were measured spectrophotometrically at 417 nm. The concentrations of the components were determined from the molar extinction coefficients (see below).

$$\frac{\text{Ext. (total, COHb)} \times \text{dilution (5341)} \times 10^3}{201{,}000} = C_{COHb}$$

The HbA_1c concentration was calculated in percent of total hemoglobin (measured as COHb):

$$\frac{\text{Ext. } (HbA_1c) \times 100}{\text{Ext. (total, COHb)} \times \text{dilution (10)}} = P_{HbA_1c}$$

Consequently, the concentration of HbA_1c expressed in mmol/l may be calculated from the formula:

$$C_{HbA_1c} = P_{HbA_1c} \times C_{COHb}$$

To improve the spectrophotometric measurements a carbon monoxide stabilised hemolysate with known HbA_1c concentration was analysed every day. The hemolysate was kept at —70°C.

Densitometric method. On completion of the isoelectric focusing procedure the gel was stained according to the method described by Jeppson et al. [6]. The HbA_1c concentrations were read from standard curves made from densitometer traces of samples containing varying but known concentrations of HbA_1c as evaluated by the spectrophotometric method (cf. Fig. 3).

RESULTS

Measurements of dilutions (1:70 to 1:63,000) were carried out for venous as well as for capillary blood samples, using both the cyanohemiglobin as well as the carboxyhemoglobin methods (Table I). Proportionality between extinction and concentration was obtained for hemoglobin concentrations below 2.7 μmol/l. The extinction coefficients of the two hemoglobin derivatives used in the calculations of the HbA_1c are 201,000 mol^{-1} cm^{-1} l and 222,000 mol^{-1} cm^{-1} l for carboxyhemoglobin and cyanohemiglobin, respectively. The values were calculated by using the formula: $E \times$ dilution $= \epsilon \times c \times 1$ where E is the measured extinction value, c is the hemoglobin concentration and ϵ the extinction coefficient. The mean (n=10) of the extinction values measured on hemolysates diluted 5341 times was used as the E value. These measurements were done at room temperature on carbon monoxide stabilised as well as potassium cyanide stabilised hemolysates. The concentration of total hemoglobin was measured spectrophotometrically (n=10) and the mean used as the c value (Van Kampen's modification of Drabkin's method [7]). The corresponding ϵ values were calculated from the formula.

TABLE I

EVALUATION OF THE RANGE FOR TOTAL HEMOGLOBIN IN THE HEMOLYSATES GIVING DIRECT PROPORTIONALITY BETWEEN THE CONCENTRATIONS AND THE OBTAINED EXTINCTION VALUES

Measurements done using carbon monoxide stabilised hemolysates.

Dilution of hemolysate	Extinction value (cm^{-1})	Calculated conc. of hemoglobin in the hemolysates (μmol/l)*
70	2.545	12.661
700	1.278	6.358
1400	1.270	6.318
2100	0.917	4.562
2800	0.713	3.547
3500	0.548	2.726
7000	0.260	1.297
14,000	0.129	0.642
21,000	0.081	0.403
28,000	0.061	0.303
49,000	0.038	0.189
63,000	0.022	0.109

$$^{*}C_{Hb} = \frac{\text{Ext.} \times 10^{6}}{201{,}000}$$

To determine the optimal concentration of hemoglobin giving the best separation, samples containing different concentrations of total hemoglobin were isoelectrically focused (Fig. 1). Day-to-day variations revealed the smallest deviations using hemolysates containing total hemoglobin in the concentration range 2.0—2.5 mmol/l. The coefficient of variation (C.V.) was never above 4% measuring the same hemolysates five times within nine days. The carboxyhemoglobin and the cyanohemiglobin methods were shown to give identical results. Nine samples were analysed using both methods. Mean ± S.D. for the two methods were 0.78 ± 0.02 and 0.78 ± 0.03 mmol/l, respectively.

Comparison between the spectrophotometric and a new densitometric quantitation was carried out. A condition for obtaining reliable results is that the range for the total hemoglobin concentration in the hemolysates is of an order giving direct proportionality between the transmission of the monochromatic laser light through the HbA_1c band and the concentration of the hemoglobin fraction. Measurements were carried out using different dilutions of the same hemolysate. The peak area of the HbA_1c peak in the densitometer trace (Fig. 2) was compared to the corresponding concentration (Table II). As demonstrated a total hemoglobin concentration up to 248 μmol/l revealed direct proportionality between the HbA_1c concentration present and the corresponding peak area.

The calibration of the densitometer was carried out by analysing the HbA_1c concentration in hemolysates from non-diabetic as well as from diabetic subjects. Each concentration was spectrophotometrically determined (carbon monoxide method) as the mean of five determinations. Hemolysates from the

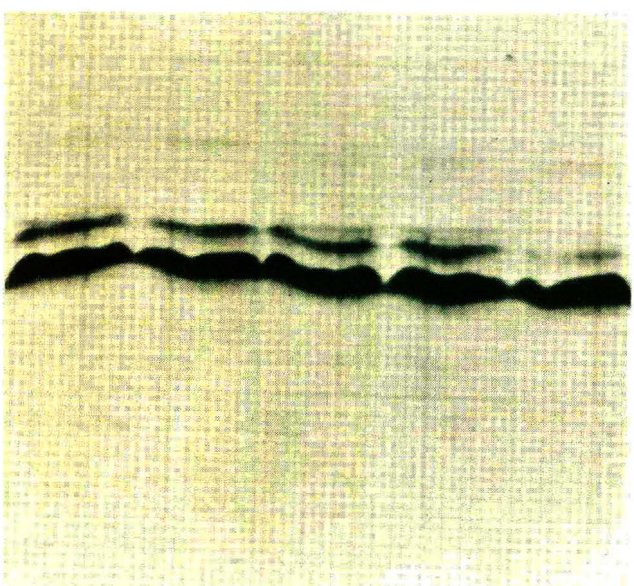

Fig. 1. Typical hemoglobin isoelectric focusing patterns from different patients. The HbA_1c fraction (upper band) is demonstrated to be clearly separated from the HbA_1 fraction (lower band). The four left-hand tracks are from diabetics, and the right-hand track is a smaller HbA_1c fraction from a non-diabetic.

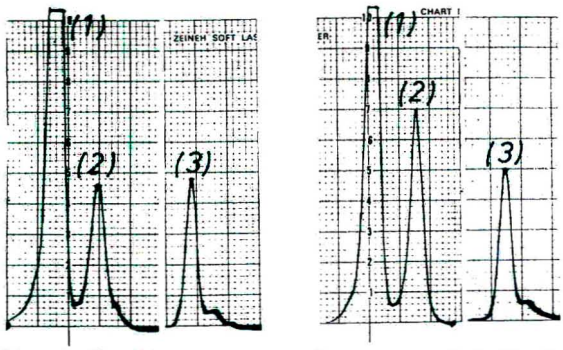

Fig. 2. Densitometer traces from a non-diabetic (left) and from a diabetic subject (right). The HbA_1c peak (2) is substantially higher in the right chromatogram correlated to the transferrin peak (3). The HbA_1 fraction is represented by peak (1). The *y*-axis gives the relative intensity, and the *x*-axis the paper speed.

TABLE II

EVALUATION OF THE RANGE FOR TOTAL HEMOGLOBIN CONCENTRATIONS IN THE HEMOLYSATES GIVING DIRECT PROPORTIONALITY BETWEEN THE CONTENT OF HbA_1c AND THE OBTAINED HbA_1c PEAK AREAS, ACHIEVED BY THE DENSITOMETRIC QUANTITATION

Concentration of total hemoglobin in the hemolysate (carbon monoxide stabilised) (μmol/l)	Peak area (arbitrary units) by densitometric measurements of HbA_1c
62	9.0
93	14.0
124	23.0
155	26.0
186	30.0
248	40.0
310	68.5
434	82.0
620	95.0

same subjects were then densitometrically quantitated using the peak area of HbA_1c. The method was improved substantially by adding the same amount of transferrin as an internal standard to each application piece before the isoelectric focusing procedure. The peak area of HbA_1c and the ratio (R) between the peak areas of HbA_1c and transferrin were used as a measure for the HbA_1c concentration (Fig. 3).

The comparability of the spectrophotometric and the densitometric method was investigated by analysing ten different samples using both methods. No significant difference between the methods was found using the paired *t*-test.

The reproducibility of the densitometric method was carried out by analysing the same sample ten times within the same day (mean ± S.D. = 0.73 ± 0.03 mmol/l).

The proof of the identity of the HbA_1c band on the gel was carried out by comparing the isoelectric focusing parameters of the chromatographically purified HbA_1c.

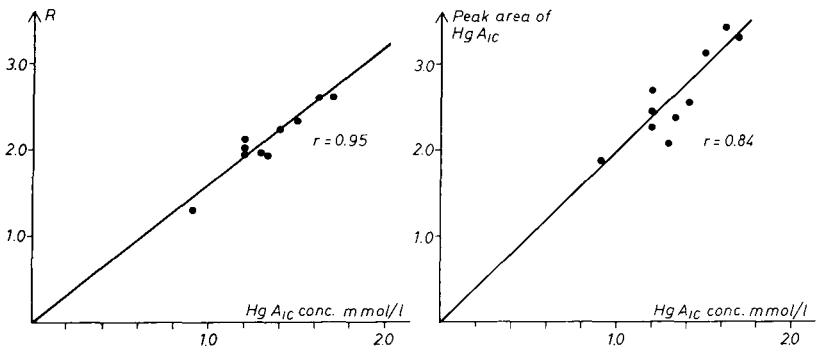

Fig. 3. Calibration curves constructed on the basis of known HbA_1c concentrations plotted against peak area of HbA_1c (right) and the ratio of peak areas of HbA_1c and the internal standard (left).

The specificity of the methods was examined by measuring the HbA_1c concentration in hemolysates with and without the addition of drugs commonly used by diabetics. Two drugs, metformine and tolbutamide, were added in amounts giving a concentration equal to 1 g/l hemolysate. Regular insulin (monocomponent) was added in amounts of 1200 IU/l. In no cases, either using spectrophotometry or densitometry, did the addition of these hypoglycemic drugs influence the HbA_1c concentrations.

DISCUSSION

The absorption maxima of carboxyhemoglobin and cyanohemiglobin have both been reported to be 421 nm [8]. However, our experiments using distilled water for dilution revealed an absorption maximum at 417 nm (25°C). To ensure that the maximum absorption wavelengths of the compounds were independent of the concentrations, spectral analyses were carried out on carboxyhemoglobin and cyanohemiglobin solutions in different concentrations. No influence on the absorption maximum of the concentration was found within the usual range of concentrations (0.6—2.7 μmol/l).

Our experiments revealed that whole blood when kept in a refrigerator (4—6°C) is stable for five days in the absence of stabilising agent. Hemolysates stabilised with potassium cyanide are stable for a period of up to nine days. This could be due to the formation of a hemoglobin A_3 fraction giving a reduction of the HbA_1c value [6]. Spicer et al. [9] found that after the addition of glycerol to the cyanohemiglobin it was possible to store the derivative at —12°C for a longer period of time. Our investigations did not confirm this finding. Deep freezing of the hemolysate should be avoided under all circumstances, due to flocculations in the hemolysate.

Experiments indicated that spectrophotometric determinations gave identical results independent of the stabilising agent (carbon monoxide or potassium cyanide). Furthermore the concentration of HbA_1c is shown to be equal in capillary as well as in venous blood samples. Identical results are obtained for total hemoglobin concentrations in the hemolysates with concentrations between 2 and 2.5 mmol/l. Reproducibility tests display very small deviations

with a coefficient of variation not greater than 4%. Furthermore the day-to-day variations as measured five times within nine days revealed only small fluctuations sufficient to claim a steady level in the clinic.

A very important parameter when using isoelectric focusing is the application of an optimal amount of total hemoglobin on the application pieces. In order to quantitate the concentration of HbA_1c by spectrophotometry relatively large amounts of hemoglobin (30—40 nmol) have to be isoelectrically focused. However, too much hemoglobin causes poor separation. Therefore, optimal conditions must be determined experimentally. Table I shows direct proportionality between the extinction values obtained and the degree of dilution for concentrations of total hemoglobin in the hemolysates ranging from 0.6 to 2.7 μmol/l. This concentration range ensures an adequate HbA_1c amount on the separated gel band. Isoelectric focusing followed by densitometric quantitation of HbA_1c requires an amount of 1—2 nmol total hemoglobin to ensure sufficient sensitivity. As demonstrated in Table II no amounts of total hemoglobin larger than 2 nmol should be applied (15 μl of 155 μmol/l) if proportionality between concentrations and the HbA_1c peak areas is to be ensured. Fig. 2 illustrates the densitometer trace of a HbA_1c determination. The separation between HbA_1 and HbA_1c is not complete, giving an additional area to the HbA_1c peak. However, this additional area has no significance and as demonstrated in Fig. 3 a straight calibration line is achieved when plotting the ratio of the peak areas against the concentration of HbA_1c. In conclusion, spectrophotometric and densitometric quantitations of HbA_1c have been demonstrated to give equal sensitivity and reproducibility. This means that results obtained by different groups using either one of the two methods can be immediately compared. This is of the utmost importance for utilization of these measurements routinely.

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REFERENCES

1 S. Rahbar, Clin. Chim. Acta, 22 (1968) 296.
2 R.J. Koenig, C.M. Peterson, R.C. Jones, C. Saudek, M. Lehrman and A. Cerami, N. Engl. J. Med., 295 (1976) 417.
3 L.A. Trivelli, H.M. Ranney and H. Tien Lai, N. Engl. J. Med., 284 (1971) 353.
4 P.A. Kynoch and H. Lehman, Lancet, ii (1977) 16.

5 E.C. Abraham, T.A. Huff, N.D. Cope, J.B. Wilson, E.D. Bransome and T.H.J. Huisman, Diabetes, 27 (1978) 931.
6 J.O. Jeppson, B. Franzen and K.O. Nilsson, Sci. Tools, 25 (1978) 69.
7 E.J. van Kampen and W.G. Zijlstra, Clin. Chim. Acta, 6 (1961) 538.
8 A. Makarem, in R.J. Henry, D.C. Cannon and J.W. Winkelman (Editors), Clinical Chemistry, Principles and Technics, Harper and Row, Hagerstown, 1974, p. 1111.
9 K.M. Spicer, R.C. Allen and M.G. Buse, Diabetes, 27 (1978) 384.

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Biomedical Applications

CHROMBIO. 541

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ASSAY FOR LOW LEVELS OF RETINOIC ACID IN HUMAN BLOOD

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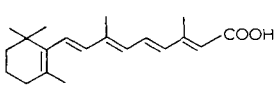
SUMMARY

A rapid and sensitive method of analysis for retinoic acid in human blood has been developed using gas chromatography—mass spectrometry for separation and detection. The retinoic acid is isolated by solvent extraction into petroleum ether, and converted to methyl retinoate by reacting with dimethylformamide dimethylacetal. The method has been applied to the study of retinoic acid in human blood after subtotal inunction, total inunction and intravenous injection of retinoic acid. The sensitivity limit of 1 ng/ml blood is realized with a 10-ml blood sample.

INTRODUCTION

Retinoic acid is an active compound of Tretinoin (Johnson & Johnson, New Brunswick, NJ, U.S.A.), a topical application acne drug. The ability to monitor low levels of retinoic acid in human blood in a relatively short time would be of considerable value in studying the metabolism of retinoic acid.



retinoic acid

Retinoic acid concentrations in plasma following oral administration have been measured in humans by Jurkowitz [1] and in pigs by Nelson et al. [2] using a colorimetric method. The limit of detection reported by these authors was in the order of submicrogram quantities of retinoic acid per ml of plasma. Liquid–gel partition chromatography has also been used by Ita et al. [3] in separating retinoic acid from its analogs in plasma samples from rats. Recently, high-performance liquid chromatography has become a popular method for

separation and identification of natural retinoids [4–7]. The limits of detection claimed by these authors varied from 1 ng [4] to 6.7 ng [5] of retinoic acid in standard solutions; however, no limit of detection was reported from actual experimental samples using nonradioactive retinoic acid.

A gas chromatographic—mass spectrometric (GC—MS) method was developed for detecting as little as 2 ng of nonradioactive retinoic acid per ml of human plasma. The purpose of this study was to develop a method for detection and quantitation of retinoic acid in human plasma. This method requires a simple clean-up and derivatization prior to GC—MS analysis; thus it allows the analysis of a large number of clinical samples, with the limit of detection down to 1 ng retinoic acid per ml of blood.

EXPERIMENTAL

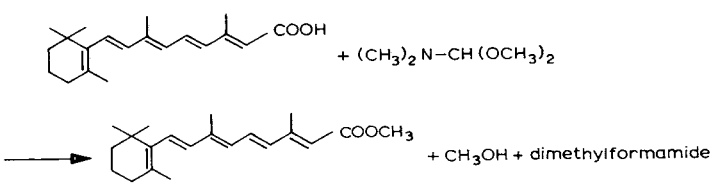
Extraction

Blood samples were drawn from patients into J-Vac (Jelco Lab.) tubes containing anticoagulant ethylenediaminetetraacetic acid and antimycotic agent potassium sorbate. Blood of ^{14}C-labeled (at the carboxylic group) retinoic acid (Amersham Corp., Amersham, Great Britain) (3.8 ng/ml) was added to the blood samples as an internal standard when internal standard was used. The Amersham material was a mixture of 38% of ^{14}C-labeled retinoic acid and 62% of nonlabeled retinoic acid as determined by our mass spectrometer. This ratio was used to calculate the amount of nonradioactive retinoic acid originally in the samples by correcting for contribution from the 62% nonlabeled retinoic acid in the added internal standard.

Plasma samples were obtained by centrifuging approximately 30 ml of blood at 2600 *g* for 25 min. To each 10 ml of plasma in a glass-stoppered centrifuge tube, 1 ml of 2 *N* HCl, 10 ml of ethanol and 25 ml of petroleum ether (b.p. 39—56°C) were added. After shaking on a mechanical shaker for 10 min, the tube was cooled and centrifuged at 2600 *g* for 5 min. The petroleum ether layer was evaporated to dryness in a 2-ml amber vial with a stream of nitrogen gas, and the vial was immediately capped with a PTFE-lined rubber seal. All steps were performed in the absence of direct light due to the lability of retinoic acid.

GC—MS method

Fifty microliters of dimethylformamide dimethylacetal (Aldrich, Milwaukee, WI, U.S.A.) were added to the vial to convert retinoic acid to methyl retinoate [8]. The conversion of retinoic acid to the volatile methyl retinoate derivative enables separation on a glass GC column (6 ft. × 2 mm I.D.) packed with 3% SE-30 on 80—100 mesh Chromosorb W HP (Supelco, Bellefonte, PA,



U.S.A.) at a column temperature of 230°C and injector temperature of 250°C. Three microliters of the dimethylformamide dimethylacetal solution were used for each GC—MS injection. The molecular ions of methyl retinoate at *m/z* 314 [9] and methyl ^{14}C-labeled retinoate at *m/z* 316 were monitored by a Varian MAT 311A mass spectrometer equipped with a two-stage Watson-Biemann separator, and operating at 70 eV in the electron-impact mode. The separator temperature was 250°C and the helium flow-rate was 50 ml/min. Signal output from the electron multiplier was recorded on a Varian A-25 potentiometer strip chart recorder.

A typical selected ion monitoring trace of *m/z* 314 from a sample containing all-*trans*-retinoic acid is shown in Fig. 1A. For samples which also contained 13-*cis*-retinoic acid the methyl 13-*cis*-retinoate was clearly separated from the

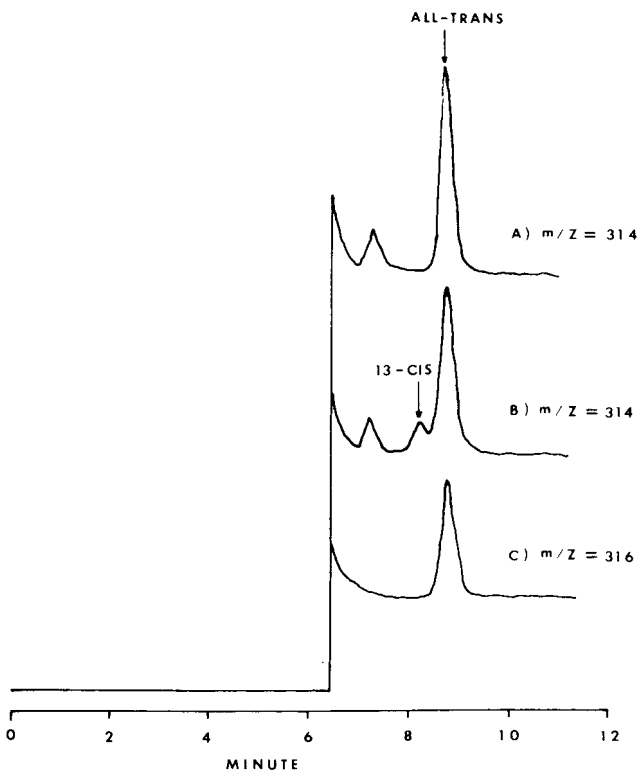


Fig. 1. Selected ion monitoring traces of methyl retinoate from samples: (A) *m/z* = 314 trace of sample with all-*trans*-retinoic acid; (B) *m/z* = 314 trace of sample with mixture of all-*trans*- and 13-*cis*-retinoic acids; (C) *m/z* = 316 trace of sample with ^{14}C-labeled (at the carboxylic group) all-*trans*-retinoic acid as an internal standard. Column: glass column (6 ft. × 2 mm I.D.) packed with 3% SE-30 on 80—100 mesh Chromosorb W HP at 230°C.

methyl all-*trans*-retinoate (Fig. 1B). A typical *m/z* 316 trace of ^{14}C-labeled (at the carboxylic group) retinoic acid used as an internal standard is shown in Fig. 1C.

In order to determine the degree of esterification of retinoic acid a known amount of retinoic acid was dissolved in dimethylformamide dimethylacetal.

Peak intensity of methyl retinoate from this solution was compared with a standard solution made by dissolving a known amount of pure methyl retinoate* in dimethylformamide dimethylacetal. It was found that the esterification of retinoic acid with dimethylformamide dimethylacetal was complete without heating. This made it possible to work with small amounts of retinoic acid in biological systems because the procedure does not require heating, extensive extraction or separation. These operations usually cause the loss of small amounts of labile retinoic acid in the samples.

RESULTS AND DISCUSSION

Percent recovery from spiked blood samples

In order to determine the efficiency of extracting retinoic acid from blood, blood samples were spiked with known amounts of nonradioactive retinoic acid (Table I), and extracted according to the procedure described under

TABLE I

RETINOIC ACID CONCENTRATION MEASUREMENTS FROM BLOOD SAMPLES SPIKED WITH VARIOUS LEVELS OF RETINOIC ACID

Level of spiking (ng retinoic acid per ml plasma)	Concentration measured (ng retinoic acid per ml plasma)*	Percent recovery**
49	28.9	59
20	11.2	56
10	6.0	60
6.9	3.0	43
3.4	1.6	47
1.7	0.8	47
1.6	0.7	44

*Average result from two measurements.
**S.D. = 6.6%.

Experimental without using internal standard. Percent recovery of the spiked retinoic acid measured by the GC—MS method described above was found to be between 40 and 60% with standard deviation of 6.6%. The percent recovery of retinoic acid from spiked plasma samples was also found to be between 40 and 60%, thus indicating no significant difference between the blood and plasma samples in recovery.

Clinical samples

Subtotal inunction. Ten female human subjects were applied with 0.025% Tretinoin Cream (a Johnson & Johnson product containing 0.025% retinoic acid) to the entire arms, legs and back two times a day, at least 8 h apart, for 28 consecutive days. Blood specimens were collected for analysis before application started (day 0), 14 days and 28 days after application started (day

*Obtained from R.J. Gander, Organic and Polymer Chemistry Dept., Johnson & Johnson Research Center.

14 and day 28, respectively). All subjects fasted for 12 h before blood specimens were collected. The specimens were analyzed according to the procedure described under Experimental, first with internal standard and then without internal standard. The results show that no retinoic acid at a limit of detection of 2 ng retinoic acid per ml of plasma was found from any of these specimens.

Total inunction. A male human volunteer was whole body treated with 1% retinoic acid cream, and blood specimens were collected for analysis during the treatment. No retinoic acid was detected in the blood samples of this rather extensive and drastic topical application of retinoic acid (see Table II).

TABLE II

RETINOIC ACID IN BLOOD FOLLOWING TOTAL INUNCTION AND INTRAVENOUS INJECTION

Sample No.*	ng retinoic acid per ml plasma**
1	not detected***
2	14.6
3	5.4

*No. 1: 5.9 ml of plasma from blood taken from patient being treated topically (whole body) with 1% retinoic acid cream. No. 2: 11.8 ml of plasma from blood taken 5 min after intravenous injection of 0.5 mg of retinoic acid into human volunteer. No. 3: 3.5 ml of plasma from blood taken 1 h after injection described in No. 2.
**Average result from two measurements.
***The limit of detection was 2 ng retinoic acid per ml plasma.

Intravenous injection of retinoic acid solution. A male human volunteer was intravenously injected with a total of 0.5 mg of retinoic acid dissolved in solution. Blood specimens were collected for analysis 5 min and 1 h after the injection. The results show a level of 14.6 ng and 5.4 ng retinoic acid per ml of plasma, respectively (see Table II).

CONCLUSIONS

A relatively rapid, sensitive and specific method for analysis of retinoic acid in human blood at a limit of detection of 2 ng per ml of plasma was developed. Results indicated that no detectable amount of retinoic acid was found in normal human blood or after topical application of retinoic acid. Low levels of retinoic acid were detected in blood following 0.5 mg intravenous injection. The non-detectable level of retinoic acid in blood following topical applications suggests low level of absorption and/or rapid metabolism of retinoic acid.

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REFERENCES

1 L. Jurkowitz, Arch. Biochem. Biophys., 98 (1962) 337.
2 E.C. Nelson, B.A. Dehority and H.S. Teague, Anal. Biochem., 11 (1965) 418.
3 Y.L. Ita, M. Zile, H. Ahrens and H.F. DeLuca, J. Lipid Res., 15 (1974) 517.
4 A.M. McCormick, J.L. Napoli and H.F. DeLuca, Anal. Biochem., 86 (1978) 25.
5 C.A. Frolik, T.E. Tavela and M.B. Sporn, J. Lipid Res., 19 (1978) 32.
6 R.M. McKenzie, D.M. Hellwege, M.L. McGregor, N.L. Rockley, P.J. Piquetti and E.C. Nelson, J. Chromatogr., 155 (1978) 379.
7 R. Hanni and F. Bigler, Helv. Chim. Acta, 60 (1977) 881.
8 J.-P. Thenot, E.C. Horning, M. Stafford and M.G. Horning, Anal. Lett., 5 (1972) 217.
9 R.L. Lin, G.R. Waller, E.D. Mitchell, K.S. Yang and E.C. Nelson, Anal. Biochem., 35 (1970) 435.

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Biomedical Applications

CHROMBIO. 555

ELECTRON-CAPTURE—GAS CHROMATOGRAPHIC DETERMINATION OF ATENOLOL IN PLASMA AND URINE, USING A SIMPLIFIED PROCEDURE WITH IMPROVED SELECTIVITY

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(Received December 10th, 1979)

SUMMARY

A sensitive gas chromatographic method for the quantitative analysis of atenolol in human plasma and urine is described. Atenolol is extracted with dichloromethane containing heptafluorobutanol to improve the extraction ability. Derivatization with trifluoroacetic anhydride in diethyl ether gives a bistrifluoroacetyl derivative which is more selectively detected by an electron-capture detector than is the corresponding heptafluorobutyryl derivative. The method allows determination down to 20 nmol/l (5 ng/ml) in 1 ml of sample with a relative standard deviation below 10%.

INTRODUCTION

Atenolol is a selective β_1-adrenergic receptor antagonist with an acetamide substituent. This hydrophilic group contributes to a low distribution ratio for atenolol between an aqueous phase and organic solvents, and extraction procedures using 45—70% of 1-butanol in the organic phase have been suggested [1—4]. Purification by pre-extraction followed by extraction with ethyl acetate has also been used [5]. However, such extraction solvents will dissolve a considerable amount of water and the co-extraction of interfering substances will be extensive. Assay methods for atenolol in biological fluids have been based on either gas chromatography (GC) with electron-capture detection [1, 2, 5] or liquid chromatography with fluorometric detection [3, 4, 6].

In the GC methods for atenolol either heptafluorobutyric anhydride [1, 2] or pentafluoropropionic anhydride [5] is used in the derivatization procedure. Without special precautions, such as back-extraction, these reagents will give high background signals, resulting in difficulties in obtaining accuracy and reproducibility at low atenolol levels. The method described includes a single efficient batch extraction with heptafluorobutanol in dichloromethane and a more selective acylation reaction with trifluoroacetic anhydride.

EXPERIMENTAL

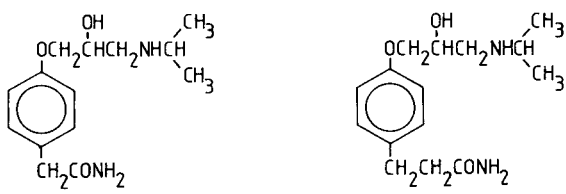
Apparatus

A Hewlett-Packard 5700 gas chromatograph equipped with a pulse-modulated ^{63}Ni electron-capture detector and an 1-mV recorder was used for the analysis. The glass column (2 m × 2 mm I.D.) was filled with 3% OV-1 on Gas-Chrom Q (100—120 mesh) (Applied Science Labs., State College, PA, U.S.A.) and operated at 180°C. An injector temperature of 200°C and a detector temperature of 300°C were chosen. The flow-rate of the carrier gas (argon with 5% methane) was 30 ml/min.

A Varian MAT 112 gas chromatograph—mass spectrometer was used to characterize the derivatives of atenolol and of the internal standard.

Reagents and chemicals

Dichloromethane, diethyl ether and toluene, obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) were purified by distillation. 1H,1H-Heptafluorobutanol was purchased from Bristol Organics (Bristol, Great Britain). Trifluoroacetic anhydride (Fluka, Buchs, Switzerland) was purified by distillation and stored at −20°C. Sodium chloride (pro analysi from Merck, Darmstadt, G.F.R.) was baked at 500°C for 8 h. Atenolol and the internal standard, H 155/87 (Fig. 1), were supplied by the Department of Organic Chemistry, AB Hässle. Standard solutions of atenolol and the internal standard were prepared in dilute hydrochloric acid (0.01 mol/l) to produce working standard solutions with concentrations of 6 μmol/l.



ATENOLOL H 155/87

Fig. 1. Molecular structures of atenolol and the internal standard, H 155/87.

Glassware

All tubes, pipettes and other glassware were washed in a laboratory dishwasher with detergent at pH 12, rinsed with phosphoric acid solution (pH 2) and with deionized water and finally dried at 60°C.

Determination of distribution ratio

Distribution ratios (D) for atenolol between aqueous buffer solutions (pH 11—12, I = 0.10) and dichloromethane were determined by shaking in centrifuge tubes for 30 min at 25°C. The distribution constant was found to be 0.26. Solid sodium chloride was added to the aqueous phase in some of the studies and varying percentages of heptafluorobutanol to the dichloromethane phase

in others. The concentration of atenolol was determined photometrically in the aqueous phase before and after equilibration, the concentration of atenolol in the organic phase being calculated from the difference. Correction was made for the increase in volume of the aqueous phase after addition of sodium chloride. The results are shown in Figs. 2 and 3.

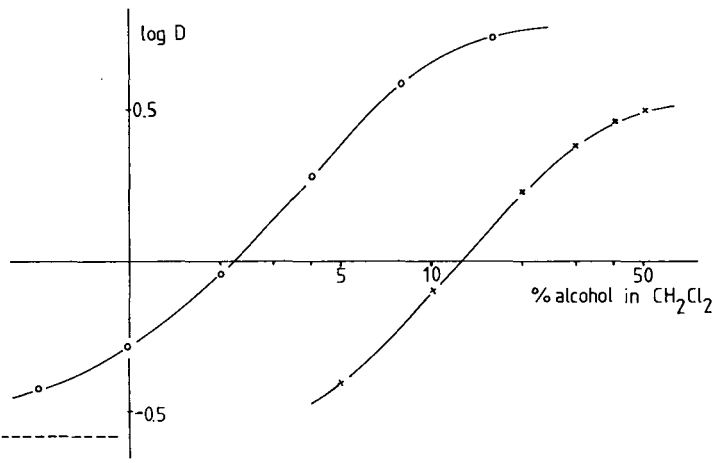


Fig. 2. Distribution ratio (D) of atenolol. Organic phase: dichloromethane containing varying amounts of 1-butanol (×) or heptafluorobutanol (○). Aqueous phase: carbonate buffer solution, pH = 11, I = 0.10. The dotted line represents the distribution ratio in the absence of added alcohol.

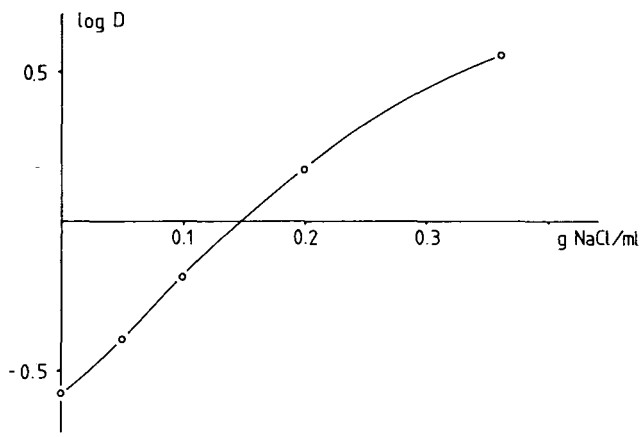


Fig. 3. Distribution ratio (D) of atenolol between dichloromethane and an aqueous phase (sodium hydroxide 0.1 mol/l) containing sodium chloride.

Analytical procedure

Urine or plasma (0.1—1.0 ml) was transferred to a 15-ml centrifuge tube (fitted with a PTFE-lined screw cap) containing 100 µl (0.6 nmol) of the internal standard solution and 0.5 g of sodium chloride. Sample volumes of less

than 1.0 ml were corrected by adding water. The aqueous phase was made alkaline by adding 50 μl of a sodium hydroxide solution (2 mol/l) followed by 50 μl of phosphate buffer (pH = 12, I = 2) and extracted with 10 ml of dichloromethane containing 3% (v/v) of heptafluorobutanol. After shaking for 10 min and centrifuging, the organic layer was transferred to a second screw-capped tube and evaporated to dryness at 40°C under a gentle stream of dry nitrogen. The residue was dissolved in 500 μl of diethyl ether and 40 μl of trifluoroacetic anhydride was added. The reaction mixture was allowed to stand for 15 min at room temperature and then evaporated to dryness under a gentle stream of dry nitrogen at room temperature. The residue was dissolved in 300 μl of toluene and 2 μl were injected into the gas chromatograph.

Quantitation

Three reference samples were prepared by adding 100 μl of the atenolol working standard solution (6 μmol/l) to 1 ml of blank plasma or urine. These samples were analysed as described above. The peak height ratio of the atenolol derivative to the internal standard derivative was calculated for each chromatogram. The average of the peak height ratios for the reference samples was used for the quantitative evaluation of the authentic samples.

RESULTS AND DISCUSSION

Extraction

As an alternative to l-butanol, Hartvig et al. [7] have suggested the addition of heptafluorobutanol to the extraction solvent. Heptafluorobutanol added to dichloromethane improved the distribution ratio of atenolol significantly, as shown in Fig. 2. Heptafluorobutanol, 6% (v/v), in dichloromethane gave about the same distribution ratio as 50% l-butanol. Using our method, 3% heptafluorobutanol in dichloromethane (D = 1.35) will give an extraction degree of about 92% with a phase volume ratio (V_{org}/V_{aq}) of 8.

The extraction of atenolol into dichloromethane can also be improved by the addition of sodium chloride to the aqueous phase (Fig. 3). By saturation of the aqueous phase with sodium chloride (0.36 g/ml) and with a phase volume ratio of 8, 96.5% of atenolol will be extracted into the organic phase.

Combining the addition of sodium chloride with the presence of heptafluorobutanol in the dichloromethane phase as proposed in the method will give a quantitative extraction of atenolol (> 99%). The shaking procedure should be performed with care as the presence of heptafluorobutanol in the organic phase in combination with sodium chloride in the aqueous phase may otherwise cause formation of an emulsion that makes extraction more difficult. Since the internal standard H 155/87 contains one methylene group more than atenolol (Fig. 1), the extraction of this substance will always be better.

Derivatization

Amines extracted from the biological sample will undergo acylation when treated with perfluorated anhydrides. The electron-capture response of such derivatives is very dependent on the type of anhydride used. Mono-derivatives of trifluoroacetic anhydride are between 100 and 1000 times less sensitive to

electron-capture detection than derivatives of heptafluorobutyric anhydride, as reported by Ervik et al. [8]. However, di-derivatives of the same type as acylated atenolol are of almost equal sensitivity irrespective of the character of the perfluoroanhydride used (Walle and Ehrsson [9]). The use of trifluoroacetic anhydride as acylating agent will thus increase the selectivity of the method for atenolol and related compounds compared to most co-extracted interfering amines.

Gas chromatograms from analysed plasma samples (Fig. 4) demonstrate the advantage of trifluoroacetic anhydride over heptafluorobutyric anhydride with regard to the extent of interfering peaks. The derivatization reaction is performed according to the method by Scales and Copsey [1]. No increase in the relative formation of atenolol derivative was achieved by using another solvent or by adding a catalyst such as triethylamine. In the studies of the reaction conditions, a known amount of 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)ethane

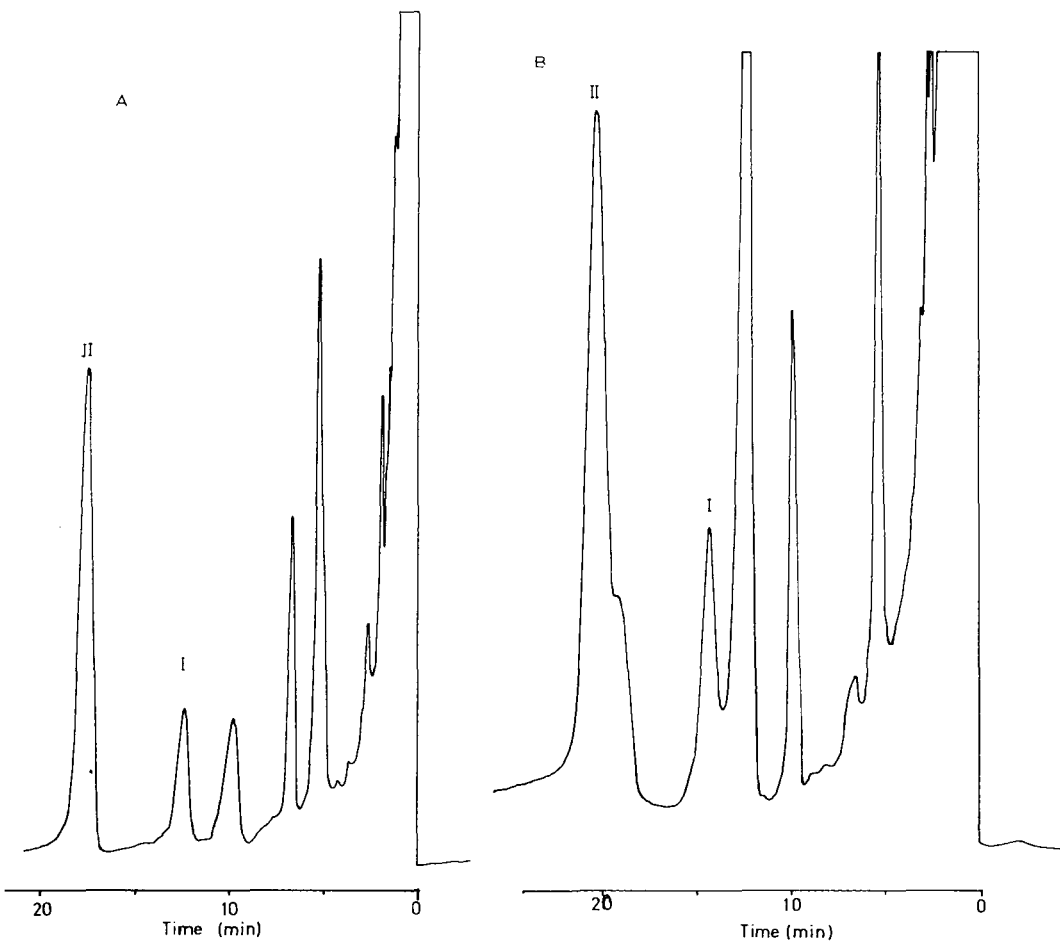


Fig. 4. Gas chromatograms obtained by analysing the same plasma sample by using (A) trifluoroacetic anhydride and (B) heptafluorobutyric anhydride as the derivatizing reagent. Peaks: I = atenolol (0.3 μmol/l) and II = internal standard.

(DDT) was added to the solution before injection as an internal marker. In instances where the catalyst was present, the resulting reaction mixture was purified according to the method of Walle and Ehrsson [10]. The influence of the anhydride concentration in diethyl ether was also examined and the results are shown in Fig. 5. The highest relative recovery is obtained when the concentration of the anhydride is about 8% (v/v). The formation of the derivative is completed within 15 min at room temperature. The derivative is stable in toluene for several days. Acylation of the amide group results in the formation of a nitrile, as shown by Scales and Copsey [1]. The structure of the derivative was confirmed by mass spectrometry (Fig. 6).

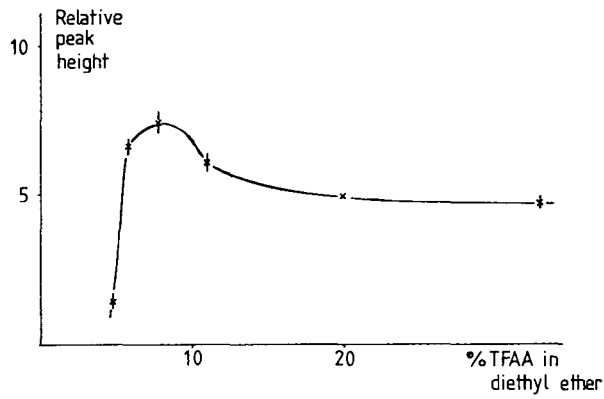


Fig. 5. Influence of trifluoroacetic anhydride (TFAA) concentration on the formation of the trifluoroacetyl derivative of atenolol. Reaction conditions: time, 15 min and room temperature.

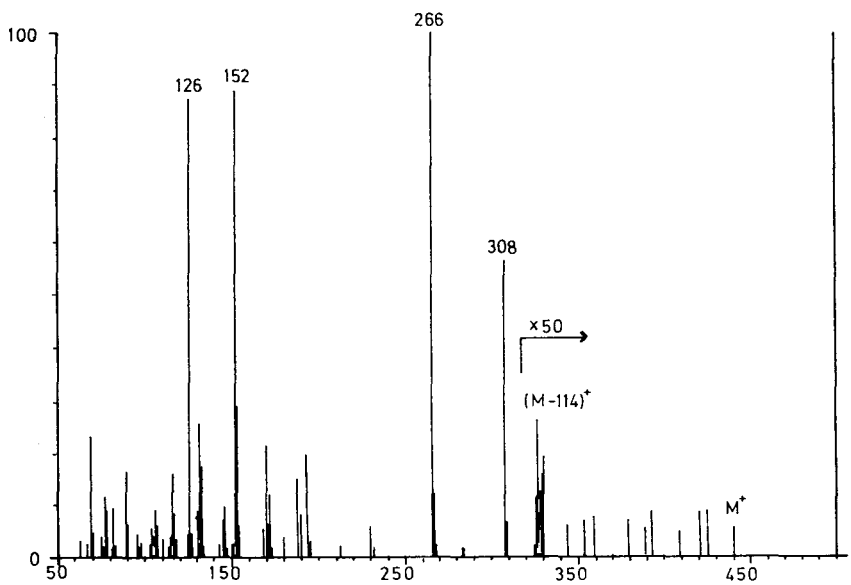


Fig. 6. Mass spectrum of the trifluoroacetyl derivative of atenolol. Varian MAT 112, GC inlet, electron impact, 60 eV.

Quantitative evaluation

Standard curves were constructed by analysing plasma and urine samples, to which known amounts of atenolol had been added. The curves were straight and passed through the origin, indicating no losses or interferences.

The precision of the method was studied within the concentration range of 1.3 μmol/l to zero. The relative standard deviation was below 10% down to a concentration of 20 nmol/l of sample, and this level was defined as the minimum determinable concentration when using 1 ml of sample.

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REFERENCES

1 B. Scales and P.B. Copsey, J. Pharm. Pharmacol., 27 (1975) 430.
2 J.O. Malbica and K.R. Monson, J. Pharm. Sci., 64 (1975) 1992.
3 O.H. Weddle, E.N. Amick and W.D. Mason, J. Pharm. Sci., 67 (1978) 1033.
4 Y.-G. Yee, P. Rubin and T.F. Blaschke, J. Chromatogr., 171 (1979) 357.
5 S.H. Wan, R.F. Maronde and S.B. Matin, J. Pharm. Sci., 67 (1978) 1340.
6 S. Decourt and B. Flouvat, J. Chromatogr., 174 (1979) 258.
7 P. Hartvig, N.-O. Ahnfelt, M. Hammarlund and J. Vessman, J. Chromatogr., 173 (1979) 127.
8 M. Ervik, T. Walle and H. Ehrsson, Acta Pharm. Suecica, 7 (1970) 625.
9 T. Walle and H. Ehrsson, Acta Pharm. Suecica, 7 (1970) 389.
10 T. Walle and H. Ehrsson, Acta Pharm. Suecica, 8 (1971) 27.

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CHROMBIO. 531

ANALYSIS OF TRICYCLIC ANTIDEPRESSANT DRUGS BY GAS CHROMATOGRAPHY USING NITROGEN-SELECTIVE DETECTION WITH PACKED AND CAPILLARY COLUMNS

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SUMMARY

A gas chromatographic method using either a conventional packed column (3% SP-2250) or a capillary column (SE-30) for the measurement of therapeutic plasma concentrations of tricyclic antidepressant drugs and their demethylated metabolites is described. The technique is based on a simple hexane extraction of drug from alkalinized plasma followed by derivatization with heptafluorobutyric anhydride for the measurement of demethylated compounds. Subsequently, parent drugs and derivatives are chromatographed and detected using a nitrogen-selective detector. A comparison of the results using both types of chromatographic systems is discussed.

INTRODUCTION

Tricyclic antidepressants continue to be one of the most widely used classes of psychotropic drugs employed in clinical practice on a long-term basis. Most of the available evidence [1—4] indicates that monitoring of plasma levels in individual patients during chronic administration is useful for optimizing the therapeutic response and avoiding toxic side-effects; however, there is no consensus on this matter at the present time [5, 6].

Among various analytical techniques used to measure tricyclic antidepressant plasma levels in recent years, gas chromatography (GC) employing a nitrogen-sensitive detector appears to be the most suitable for routine laboratory needs [7—10]. While this method is being successfully applied to monitoring steady-state levels of tricyclic antidepressants and their secondary amino derivatives, it does not appear to be sufficiently sensitive to measure reliably small concentrations of the latter compounds in single-dose pharmacokinetic studies or

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when low steady-state plasma levels are present. Derivatization of the secondary amine formed in vivo during treatment with the parent compound has occasionally been used to measure each amine separately in the same plasma sample [8, 11—13].

We now describe our results using a simple derivatization technique for the routine measurement of small quantities of secondary amine tricyclic antidepressants (desipramine, nortriptyline, demethylclomipramine, and maprotiline) along with their respective parent compounds (tertiary amines). Derivatization with heptafluorobutyric anhydride considerably increases the sensitivity of detection and allows the accurate measurement of plasma tricyclic antidepressant concentrations down to 5 ng/ml.

MATERIAL AND METHODS

Maprotiline (MAPRO), imipramine (IMI), desipramine (DMI), amitriptyline (AMI), nortriptyline (NT) and cyclobenzaprine (CBP) were checked by GC—MS analysis and kindly supplied by Dr. G. Belvedere (Istituto Mario Negri, Milan, Italy).

All the reagents [*n*-hexane—isoamyl alcohol (98.5:1.5, v/v), ethyl acetate and toluene] were of analytical grade (Merck, Darmstadt, G.F.R.). Heptafluorobutyric anhydride (HFBA), a 25% (v/v) solution in ethyl acetate, to derivatize the secondary amines was obtained from Pierce (Rockford, IL, U.S.A.). A solution of NaOH, 0.5 *N* (Merck) was also used.

Ethanolic solutions of the antidepressant drugs in the form of hydrochloride salts, containing 1 and 10 ng μl^{-1}, and CBP, containing 10 ng μl^{-1}, all calculated as free bases, were used. These solutions were stored at 4°C and they were found to be stable for up to three months.

All the glassware used was silanized using a solution (10%, v/v) of dimethylchlorosilane (DMCS) in toluene (Pierce); 2 ml of the DMCS solution were added to each tube. After agitation for 20—30 min the reagent was discarded and the remaining DMCS was evaporated with a nitrogen stream; the pipettes were rinsed with the DMCS solution and subsequently dried. This was done to avoid the absorption of tricyclic antidepressants, especially of the secondary amines, on to the glass surface of the tubes during extraction.

Extraction procedure

The internal standard marker (IS) used to measure MAPRO, CLOMI and DECLOMI was CBP (20 μl of a 10 ng μl^{-1} solution) for both packed and capillary columns. The IS for IMI and DMI, as well as for AMI and NT, was CLOMI (20 μl and 10 μl of a 10 ng μl^{-1} solution, respectively) for the packed column. With the capillary column, for IMI and DMI, the IS was NT (20 μl of a 10 ng μl^{-1} solution); while the IS for AMI and NT was CLOMI (10 μl of a 10 ng μl^{-1} solution). An ethanolic solution of IS was added to a conical tapered tube containing 1 ml of NaOH and 2 ml of plasma sample (to give pH $\approx$12). The samples were extracted with *n*-hexane—isoamyl alcohol (7 ml) for 20 min on a rock 'n roll shaker, centrifuged (5 min at 1000 *g* and 4°C), and then 6.5 ml of the organic phase were transferred to a clean tube. The hexane phase was evaporated to dryness in a water bath at 60°C under a gentle stream of nitrogen

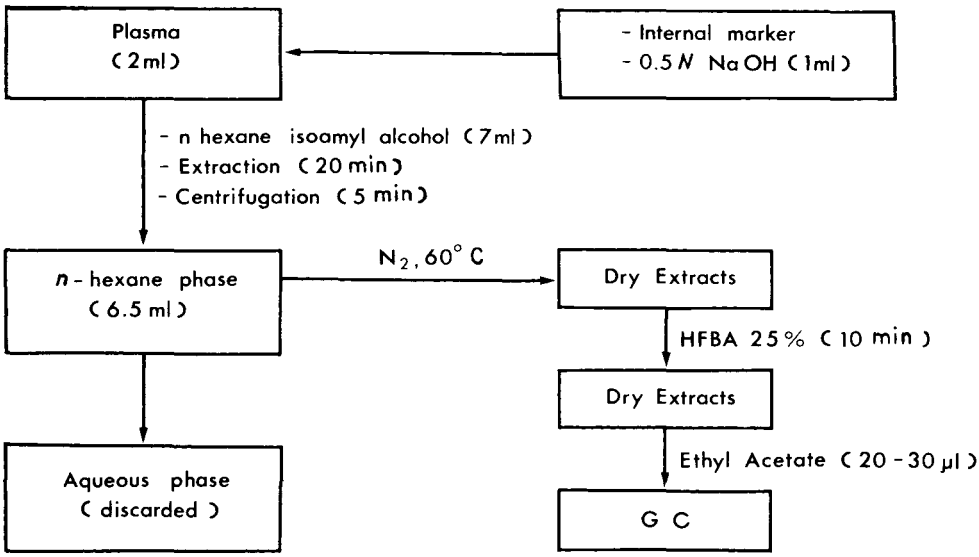

Fig. 1. Extraction scheme used for the analysis of tricyclic antidepressants and their metabolites.

and reacted with a solution of HFBA (200 µl) for 10 min at 60°C. The excess reagent was evaporated off (as described for hexane) and the dry residue was dissolved in 20 µl of ethyl acetate and 3—4 µl were injected into the gas chromatograph. The extraction scheme is shown in Fig. 1.

Gas chromatographic conditions

Two Perkin Elmer 910 gas chromatographs, dual column, equipped with nitrogen-selective detectors were used.

In the first chromatograph, two columns, 2 m × 4 mm I.D., were packed with 3% SP-2250 coated on Supelcoport 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.). The columns were conditioned as follows: a temperature program (5°C/min) from 80 to 300°C with a helium flow-rate of 50 ml/min, then 4 h at 300°C without a gas flow, and finally 48 h at 300°C with a helium flow-rate of 50 ml/min. The oven temperature was 260°C, the injector port and detector temperatures were 300°C; the carrier gas (helium) flow-rate was 50 ml/min. The detector was heated at about 400—450°C (helipot at 650) with a hydrogen flow-rate of 2 ml/min and an air flow-rate of 100 ml/min.

An LKB capillary column, type 2101-502, 25 m × 0.37 mm I.D., was used in the second chromatograph. The open-tubular column was coated with SE-30. The GC conditions were the same as used for the packed columns for MAPRO, IMI, DMI, CLOMI and DECLOMI (see above). For AMI and NT the column temperature was 250°C. The carrier gas was helium at a flow-rate of 1.5 ml/min; helium was also used as make-up gas with a flow-rate of 40 ml/min. A solid injector of the type described by Ros [14] and supplied by Spiral (Dijon, France) was used for the application of samples.

Mass spectrometry

A VG 7070 mass spectrometer, equipped with a Hewlett-Packard 5710A gas chromatograph was used to determine the identity of the peaks obtained by the GC analysis. The GC column was packed with a 3% SP-2250 coated on Supelcoport 80—100 mesh (Supelco), the helium (carrier gas) flow-rate was ca. 15 ml/min, the oven temperature was 260°C, the molecular separator temperature was 250°C. The mass spectra were obtained using the electron-impact mode. The parameters were: electron-beam energy 70 eV, trap current 200 μA, acceleration energy 4 kV, ion-source temperature 250°C, the resolution was set at $\approx$1000.

RESULTS

Typical chromatograms with packed and capillary columns, obtained from extracts of spiked plasma samples of MAPRO, IMI, DMI, CLOMI, DECLOMI, AMI and NT are presented in Figs. 2 and 3; the retention times and other GC parameters obtained with both techniques are listed in Table I. Comparison with the chromatograms obtained from a blank plasma sample shows that

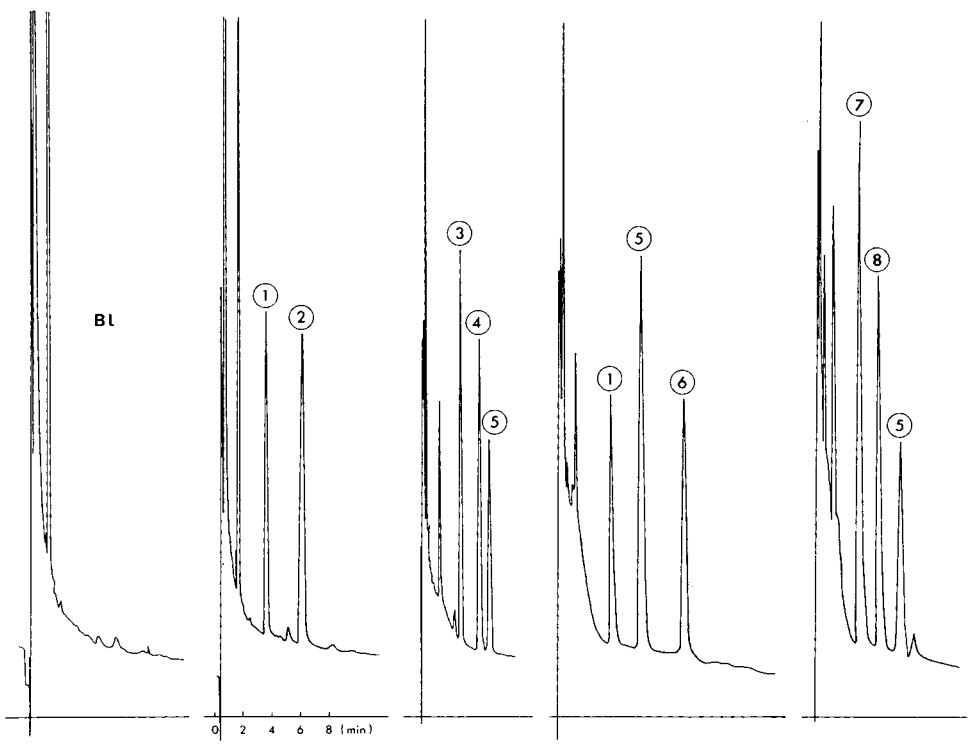

Fig. 2. Gas chromatograms of plasma sample extracts obtained with a packed column from a blank (Bl) and from spiked samples containing: 100 ng ml^{-1} of maprotiline (2), its internal standard cyclobenzaprine (1); 100 ng ml^{-1} of imipramine (3) and desipramine (4) with the internal standard clomipramine (5); 100 ng ml^{-1} of clomipramine and desmethylclomipramine (6), the internal standard being cyclobenzaprine; 200 ng ml^{-1} of amitriptyline (7) and nortriptyline (8), with the internal standard clomipramine (5).

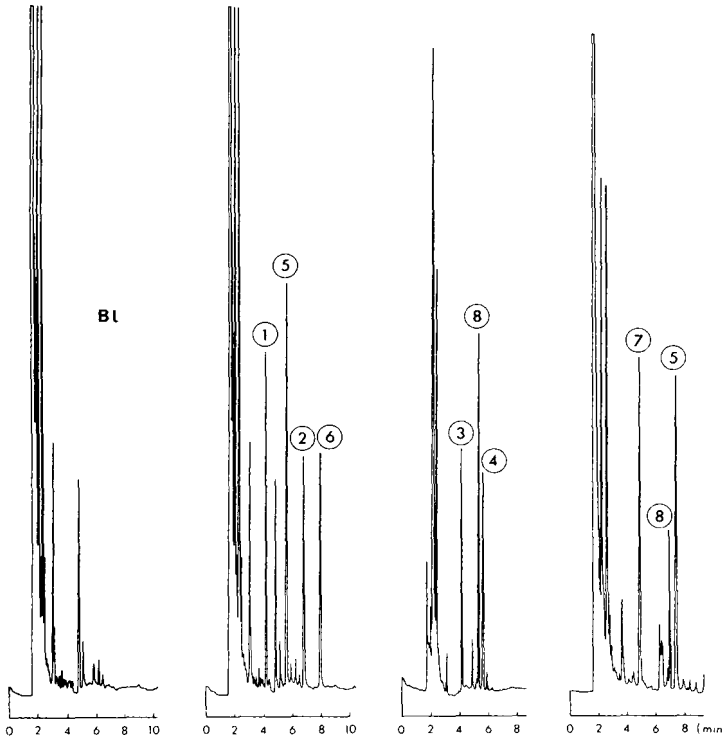

Fig. 3. Gas chromatograms of plasma sample extracts obtained with a capillary column from a blank (Bl) and from spiked samples containing: 100 ng ml^{-1} of maprotiline (2), 100 ng ml^{-1} of clomipramine (5) and desmethylclomipramine (6), the internal standard being cyclobenzaprine (1); 50 ng ml^{-1} of imipramine (3) and desipramine (4), with the internal standard nortriptyline (8); 100 ng ml^{-1} of amitriptyline (7) and nortriptyline (8), with the internal standard clomipramine (5).

endogenous compounds extracted from plasma did not interfere with drug measurement (Figs. 2 and 3).

The calibration curves prepared with tricyclic antidepressant plasma concentrations between 10 and 200 ng ml^{-1} were linear and the coefficient of variation (C.V.) of the day-to-day results at low concentrations did not exceed 15% (Fig. 4). Table II illustrates the accuracy of the analysis at low and high plasma concentrations with both packed and capillary columns.

All the secondary amines such as MAPRO and the demethylated metabolites DMI, DECLOMI and NT were derivatized on the secondary amino group with HFBA to the corresponding heptafluorobutyric amides. The GC—mass spectrometric (MS) analysis confirmed the formation of the amide derivatives of MAPRO (molecular ion at *m/e* 473), DECLOMI (*m/e* 496), DMI (*m/e* 462) and NT (*m/e* 459). The ethyl acetate solutions of the derivatives were stable even at room temperature for at least four days. The tertiary amines (IMI, CLOMI, AMI and CBP) did not appear to react with HFBA; this was evidenced by the same GC retention time and confirmed by GC—MS: IMI (molecular ion at *m/e* 280), CLOMI (*m/e* 314), AMI (*m/e* 277) and CBP (*m/e* 275).

TABLE I

RETENTION TIMES (t_R), NUMBER OF THEORETICAL PLATES (n) AND SYMMETRY FACTORS (Sym) OF TRICYCLIC ANTIDEPRESSANTS OBTAINED WITH PACKED AND CAPILLARY COLUMNS, BEFORE AND AFTER DERIVATIZATION OF THE SECONDARY AMINES WITH HFBA

	3% SP-2250 packed column*			SE-30 capillary column*		
	t_R (min)	n	Sym	t_R (min)	n	Sym
MAPRO	5.5	2300	0.75	7.2	27200	0.58
MAPRO-HFBA	7.5	3000	0.90	10.4	47300	0.86
IMI	3.5	2500	0.86	5.7	41900	0.69
DMI	4.3	2300	0.76	5.9	26300	0.58
DMI-HFBA	5.3	2800	0.96	8.6	50300	0.93
AMI	3.1	2500	0.88	5.4	45100	0.81
NT	3.9	2400	0.77	5.5	25700	0.58
NT-HFBA	4.6	2700	0.87	7.9	48200	0.88
CLOMI	6.1	2800	0.90	8.5	39900	0.73
DECLOMI	7.5	2500	0.79	9.0	33200	0.59
DECLOMI-HFBA	9.9	3100	1.00	11.8	48200	0.89
CBP	4.3	2600	0.91	5.8	36400	0.72

*Oven temperature = 250°C.

TABLE II

MEAN VALUES AND ACCURACY OF THE TRICYCLIC ANTIDEPRESSANT ANALYSES AT LOW- AND HIGH-SPIKED PLASMA CONCENTRATIONS WITH PACKED (3% SP-2250) AND CAPILLARY (SE-30) COLUMNS

	MAPRO	IMI	DMI	CLOMI	DE-CLOMI	AMI	NT
3% SP-2250 column							
Low conc. (20 ng ml^{-1})	20.2	18.9	20.4	18	21.4	20.2	20.8
± C.V. (n = 8)	14	8	14	3	4	4	6
High conc. (200 ng ml^{-1})	200.0	199.4	200.4	201.8	201.7	199.4	200.8
± C.V. (n = 8)	15	14	12	5	7	4	7
SE-30 capillary column							
Low conc. (10 ng ml^{-1})	9.9	9.5	9.9	10.3	9.6	9.8	10.3
± C.V. (n = 8)	11	8	8	9	4	11	11
High conc. (200 ng ml^{-1})	200.6	198.7	199.3	201.8	200.1	199.	199.5
± C.V. (n = 8)	10	8	8	5	5	11	12

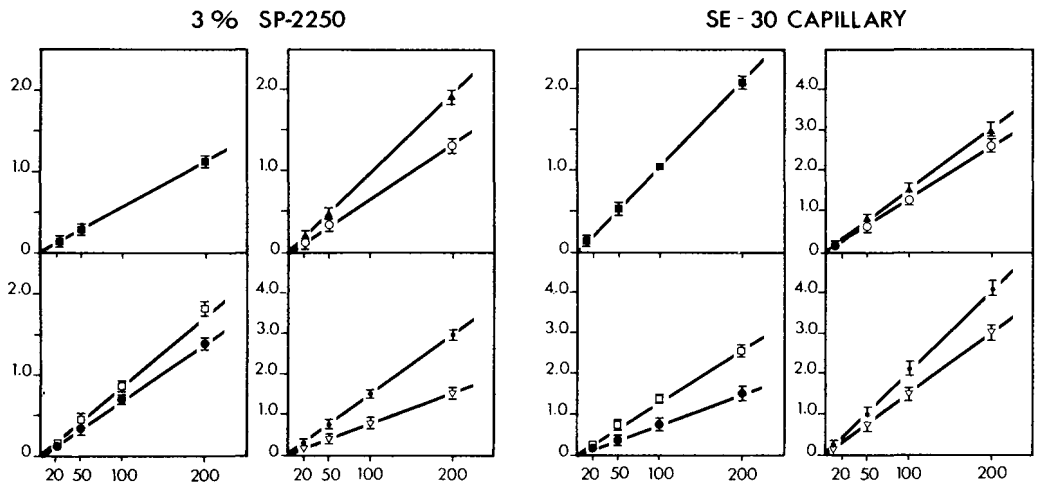

Fig. 4. Calibration curves of TCA plasma sample extracts obtained by the peak height ratio method (peak height tricyclic antidepressant/peak height internal standard) with a packed column (3% SP-2250) and a capillary column (SE-30). ■ = MAPRO, ▲ = IMI, ○ = DMI-HFBA, □ = CLOMI, ● = DECLOMI-HFBA, • = AMI, ▽ = NT-HFBA.

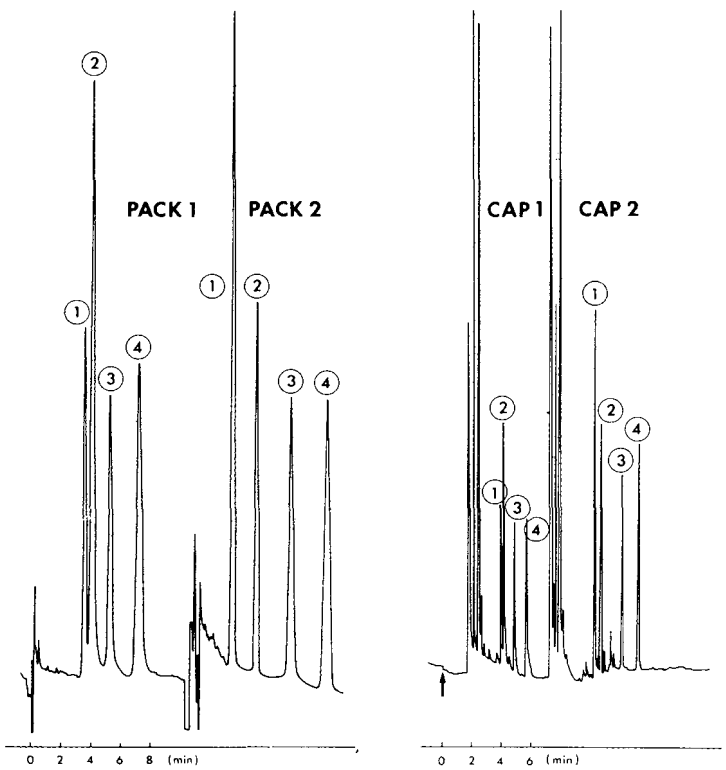

Fig. 5. Gas chromatograms of 30 ng of: nortriptyline (1), desipramine (2), maprotiline (3) and desmethylclomipramine (4) before (PACK 1, CAP 1) and after (PACK 2, CAP 2) derivatization with HFBA either on packed (PACK) or capillary (CAP) columns.

DISCUSSION

The simplicity of the procedure, which consists of a single *n*-hexane—isoamyl alcohol extraction, makes it particularly useful for routine analysis; the most common tricyclic antidepressants and their desmethyl congeners can be measured under the same GC conditions, using the respective internal standards, as indicated in the methodology.

All of the secondary amines are relatively polar molecules and when analysed by GC with semi-polar stationary phases such as SP-2250 (OV-17), tend to give tailing peaks. Derivatization with HFBA reduced the polarity of the compound and improved peak symmetry, hence, the sensitivity of the analysis (Fig. 5). As shown in Table I the chromatographic parameters such as the number of theoretical plates and peak symmetry factors improved considerably after derivatization of the secondary amines. Furthermore, formation of the derivatives minimized the possibility of column adsorption, especially at low concentrations. The reaction with HFBA was fast and complete within 5—10 min. The GC—MS analysis confirmed the structures of the compounds analysed and the formation of the heptafluorobutyric amide derivatives of the secondary amines. A prolonged reaction time should be avoided because it was found that IMI, DMI, CLOMI and DECLOMI can decompose and give rise to the formation of other peaks if the reaction time is more than 50—60 min. This was probably due to a reaction of HFBA on the side-chain of the drugs, as described [15] for DMI.

The results obtained with the packed and capillary columns show that both are reliable systems for the measurement of plasma concentrations of tricyclic antidepressants and their desmethyl metabolites at low therapeutic concentrations.

The drugs which are currently given in associated therapy with tricyclic antidepressants, such as benzodiazepines (diazepam, desmethyldiazepam, oxazepam, chlordiazepoxide) and phenothiazines (chlorpromazine, levomepromazine and thioridazine), were analysed using both systems (packed and capillary columns) under the analytical conditions used for tricyclic antidepressants. We found that with the SP-2250 packed column, chlorpromazine and levomepromazine could interfere with the analysis of DECLOMI-HFBA, and that with the SE-30 capillary column levomepromazine interfered with MAPRO-HFBA. These interferences, however, could be minimized by introducing a temperature program in the GC analysis.

The higher resolution of the GC peaks obtained with the capillary column makes this a superior technique for the determination of tricyclic antidepressants at very low plasma concentrations, or where there are interferences from concomitantly prescribed drugs or endogenous compounds. The capillary column appears to be particularly suitable for pharmacokinetic studies.

Analysis with the conventional packed column, however, is more simple and less expensive; also if one considers that a dual gas chromatograph enables one to work with two columns (which is not possible with a capillary column using a solid injector system), one can reduce the time of analysis by half. Thus, the packed column is more suitable for routine purposes such as monitoring plasma levels during chronic administration of tricyclic antidepressants.

In conclusion, we believe that the analytical methods described in the present report are suitable for the measurement of tricyclic antidepressants in plasma, both in pharmacokinetic studies and in routine therapeutic monitoring.

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REFERENCES

1 M. Åsberg, B. Crönholm, F. Sjöqvist and D. Tuck, Brit. Med. J., 3 (1971) 331.
2 R.A. Braithwaite, R. Goulding, G. Theano, J. Bailey and A. Coppen, Lancet, i (1972) 1297.
3 A.H. Glassman, M. Shostak, S.J. Kantor and J.M. Perel, Psychopharmacologia, 11 (1975) 27.
4 L.F. Gram, N. Reisby, I. Ibsen, A. Nagy, S.J. Dencker, P. Bech, G.O. Petersen and J. Christiansen, Clin. Pharmacol. Ther., 19 (1976) 318.
5 G. Burrows, B. Davis and B. Scoggins, Lancet, ii (1972) 619.
6 A. Coppen, S. Montgomery, K. Ghose, V.A. Rama Rao, J. Bailey, J. Christiansen, P.L. Mikkleson, H.M. van Praag, F. van de Poel, E.J. Minsker, V.G. Kozulia, N. Matussek, G. Kungkunz and A. Jorgenson, Lancet, i (1978) 63.
7 A. Jorgensen, Acta Pharmacol. Toxicol., 36 (1975) 79.
8 D.N. Bailey and P.I. Jatlow, Clin. Chem., 22 (1976) 777.
9 S. Dawling and R.A. Braithwaite, J. Chromatogr., 146 (1978) 449.
10 W.D. Mitchell, S.F. Webb and R.A. Padmore, Ann. Clin. Biochem., 16 (1979) 47.
11 R.A. Braithwaite and B. Widdop, Clin. Chim. Acta, 35 (1971) 461.
12 W.A. Garland, J. Pharm. Sci., 66 (1977) 77.
13 R.N. Gupta, G. Molnar and M.L. Gupta, Clin. Chem., 23 (1977) 1849.
14 A. Ros, J. Gas Chromatogr., 3 (1965) 252.
15 T. Walle, H. Ehrsson and C. Bogentoft, J. Chromatogr., Sci., 9 (1972) 509.

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CHROMBIO. 528

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROGUANIL, CYCLOGUANIL AND 4-CHLOROPHENYLBIGUANIDE USING HYDROPHOBIC PAIRING ION AND ITS APPLICATION TO SERUM ASSAY

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SUMMARY

A high-performance liquid chromatographic separation of proguanil, cycloguanil and 4-chlorophenylbiguanide is reported using a hydrophobic stationary phase and lauryl sulphate as pairing ion. It is suggested, on the basis of the behaviour of phenylbenzoate as an undissociated solute and the variation of retention with lauryl sulphate and sodium ion concentrations that the mechanism of separation is one of ion exchange. The biguanides can be detected in serum at concentrations in the region of 60 ng ml^{-1} and preliminary results are presented to show the variation of proguanil in serum over a 24-h period following ingestion of 200 mg orally.

INTRODUCTION

Proguanil hydrochloride (Paludrine, ICI) is widely used as a prophylactic agent against malaria. It is usually administered once daily as a 100-mg tablet although a 200-mg daily dose is recommended for parts of West Africa.

Proguanil is reported to be partially excreted unchanged from the body (40—60%) and partially metabolised to give cycloguanil as the main metabolite [1]. This metabolite has apparent antimalarial activity and it, rather than the parent drug, is thought to possess the prophylactic activity [2]. Subsequent metabolism results in the formation of another metabolite — 4-chlorophenylbiguanide [1].

Proguanil occasionally fails to provide a prophylactic effect despite strict adherence to the recommended dosage regime. This lack of prophylaxis has been attributed to acquired resistance by the plasmodia responsible [3]. In recent years apparent lack of activity or apparent toxicity of several drugs in humans has been well correlated with their pharmacokinetic parameters. The long-term

use of several drugs has been shown to result in the alteration of these parameters especially those relating to metabolism [4]. Since proguanil is administered on a daily basis over long periods of time and since metabolism apparently plays an important part in its activity there is a need for a detailed study of the pharmacokinetics of this drug and its metabolites.

The only pharmacokinetic data available [5] depend on relatively non-specific chemical assay. The method of high-performance liquid chromatography (HPLC) with its relative ease of sample preparation appears to offer the most suitable assay method for such a study. It is proposed to develop a chromatographic separation of these biguanides and to assess its suitability for serum assay of these compounds.

Since the advent of reversed-phase ion-pair chromatography [6—9] separations using this technique have been widely applied. Despite this, the mechanism of retention on hydrophobic phases using hydrophobic pairing ions is still under discussion [10—12] and relatively few data have been reported distinguishing between the ion-pairing concept and the alternative ion-exchange interpretation of retention [13—15]. It is proposed to examine the effect of lauryl sulphate and sodium ion concentrations on the separation of these compounds to obtain additional information on the mechanism of retention.

EXPERIMENTAL

Apparatus and materials

The liquid chromatograph used consisted of an Applied Chromatography System syringe pump linked to a Pye Unicam LC-3 ultraviolet detector. The chromatographic column (100 mm × 4.6 mm I.D.) was slurry packed at 200 bar with 5-μm Hypersil ODS (Shandon Southern, London, Great Britain). Syringe injection was used for the measurement of column capacity factors and for quantitative measurements a Rheodyne Model 7120 injection valve fitted with a 100-μl loop was used. The wavelength of measurement was optimised for proguanil at 247 nm and all measurements were made at ambient temperature. Acetonitrile was HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). All other chemicals used were obtained from BDH Chemicals (Poole, Great Britain). Proguanil, cycloguanil and 4-chlorophenylbiguanide were donated by ICI (Macclesfield, Great Britain).

Chromatography

A solution (20 μg ml^{-1}) in acetonitrile of the three biguanides was prepared containing phenylbenzoate (40 μg ml^{-1}) as a non-ionisable reference compound. The retention times of the four compounds were measured as a function of anion concentration over the range 0—0.909% w/v by adding volumes of 10% w/v sodium lauryl sulphate (SLS) solution in buffer together with an equal volume of acetonitrile to an eluent of acetonitrile—water (50:50) containing 0.175 *M* phosphoric acid and 0.0125 *M* sodium dihydrogen phosphate as a buffer. The pH of the eluent was 1.5. The retention times were also measured as a function of sodium counter ion concentration using a fixed SLS concentration of 0.7% w/v adjusted to pH 1.5 with sulphuric acid to which sodium sulphate solution

(1.33 *M*) was added together with an equal volume of acetonitrile to provide a range of sodium ion concentration from 0.07 to 0.313 *M*.

Calibration procedures for biguanides

Calibration curves for the three biguanides were run both in water and in bovine serum by spiking to provide concentrations in the range 80—450 ng ml^{-1} for each compound. The solutions in water were injected directly. The spiked serum was deproteinated by adding 0.25 ml of perchloric acid (60% w/v) to 1 ml of serum, shaking and centrifuging for 15 min. The clear supernatant was then injected. For quantitative measurements the eluent strength was modified to acetonitrile—water (75:25) and the SLS concentration to 2.5% w/v.

Determination of partition coefficients and dissociation constants

The partition coefficients for the three biguanides and phenylbenzoate were determined using an octanol—water system by measuring the absorbance of each compound at an appropriate wavelength in acid aqueous (adjusted to pH 1.5 with sulphuric acid) solution before and after equilibrating with a suitable volume of 1-octanol. The dissociation constants of the biguanides were determined by a spectrophotometric method [16]. A plot of pH against change in absorbance over that in the fully protonated form enabled the pK_a to be determined from the point of inflexion.

RESULTS AND DISCUSSION

Fig. 1 shows the separations obtained of the three biguanides and phenylbenzoate in the presence and absence of surfactant. It was noted that the order of elution of cycloguanil and 4-chlorophenylbiguanide was reversed on addition of surfactant.

Surfactant concentration

The results of increasing SLS concentration are shown in Fig. 2. The capacity ratios for the three biguanides are seen to increase linearly with increase in SLS concentration (R^2>0.956). The slopes of the lines are markedly different and are in the same order as the octanol/water partition coefficients, measured in acid solutions, for cycloguanil, 4-chlorophenylbiguanide and proguanil as 0.05, 0.11 and 0.33 respectively. The primary pK_a values of the bases at 20°C were determined as 4.2, 1.9 and 3.0 respectively and it is assumed that in the eluent used these compounds are appreciably protonated. The behaviour of the uncharged phenylbenzoate, showing decreased capacity ratio with increasing SLS concentration indicates that the surfactant is interacting directly with the stationary phase rather than in solution with the mobile phase.

Fig. 3 shows the effect of surfactant concentration on the reduced plate height (h). Data were obtained from chromatograms which allowed reliable measurement of column efficiency, i.e. where no overlap of peaks occurred. For proguanil and 4-chlorophenylbiguanide h is initially very large and tends, as the surfactant concentration increases, to the low constant value shown for phenylbenzoate which is independent of surfactant concentration. It would

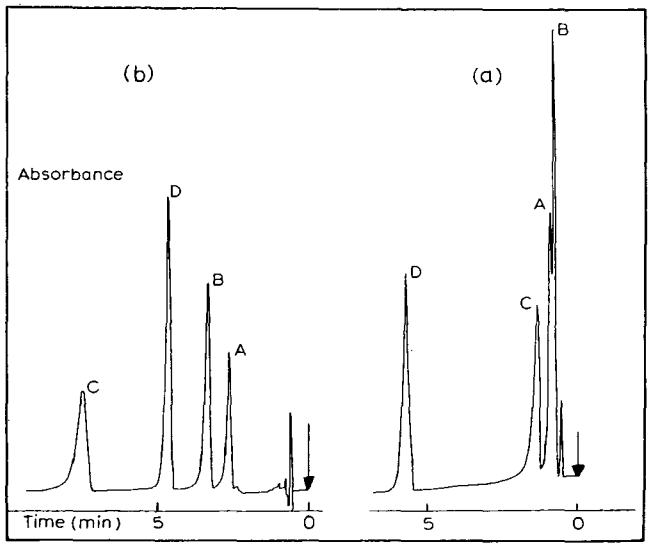

Fig. 1. Representative chromatograms to show the effect of sodium lauryl sulphate on retention. (a) No SLS; (b) 0.654% w/v SLS. Conditions: eluent acetonitrile—water (50:50) + 0.17 *M* H_3PO_4 + 0.0125 *M* NaH_2PO_4. Flow-rate, 1.5 ml min^{-1}; 10-μl syringe injection. Solute concentrations approximately 20 μg ml^{-1}. Full scale absorbance 0.08. Peaks: A, cycloguanil; B, 4-chlorophenylbiguanide; C, proguanil; D, phenylbenzoate.

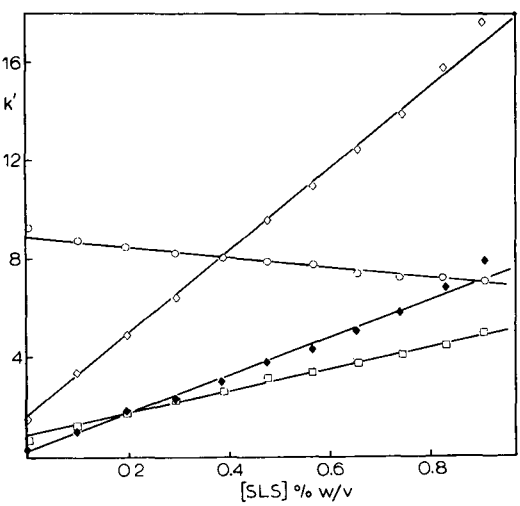

Fig. 2. Effect of surfactant concentration on capacity ratio (k') for cycloguanil (□), 4-chlorophenylbiguanide (♦), proguanil (◇) and phenylbenzoate (○). Chromatographic conditions as in Fig. 1.

appear from these results that high concentrations of surfactant are advantageous in that they render the putative ion-exchange mechanism of comparable efficiency to that obtained for uncharged solute species on reversed-phase systems. This is interpreted as evidence of an ion-exchange mechanism where

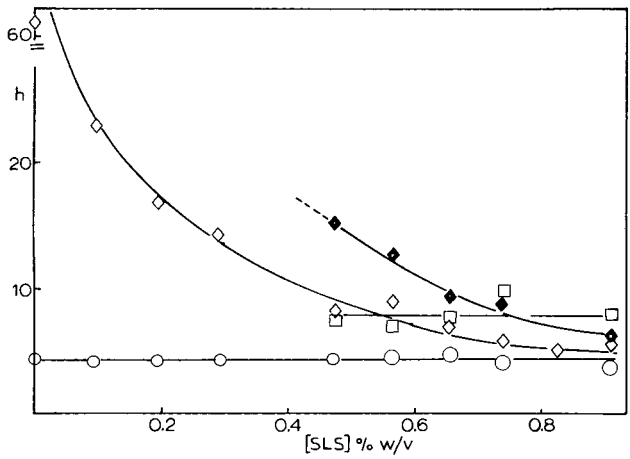

Fig. 3. Variation of reduced plate height (h) with surfactant concentration for cycloguanil (□), 4-chlorophenylbiguanide (♦), proguanil (◇) and phenylbenzoate (○). Chromatographic conditions as in Fig. 1.

an effectively low capacity resin is being formed at low surfactant concentration producing minimal retention with consequent peak broadening due to overloading of the ionic sites available. In the present results there appears to be no evidence of the complex dependence of h on surfactant concentration demonstrated for tryptophan in a similar chromatographic system [17]. The present results are similar to those obtained for sodium cromoglycate with alkylbenzyldimethylammonium chlorides [12]. These results favour an equilibrium of the ion-exchange type represented by

$$(P^-Na^+)_{org} + BH^+_{aq} \rightleftharpoons (P^-BH^+)_{org} + Na^+_{aq}$$

where P^- refers to the surfactant anion and BH^+ to the protonated base. It has been shown [18] that in such a system the distribution ratio of the drug between the organic and aqueous phases and thus the chromatographic capacity factor k' is given by an expression of the type

$$k' = K_{IE} \frac{[P^-Na^+]_{org}}{[Na^+]_{aq}} \quad (1)$$

K_{IE} being equilibrium constant for the ion-exchange reaction shown.

If the surfactant partitioned into the stationary phase is in equilibrium with that in the aqueous phase as is likely in the case of a surfactant where micellisation forms an alternative to the removal of surfactant from the aqueous phase then

$$k = \frac{[P^-Na^+]_{org}}{[P^-Na^+]_{aq}}$$

where k is an overall distribution coefficient for SLS between the aqueous and the organic phases and

$$k' = K_{IE}k \frac{[P^-Na^+]_{aq}}{[Na^+]_{aq}} \quad (2)$$

which is the situation observed in the present work. This may not be the case where a pairing ion is unable to form micelles in which case continuous partitioning of the surfactant into the stationary phase will occur and $[P^-Na^+]_{org}$ will be independent of $[P^-Na^+]_{aq}$.

Counter ion concentration

Eqn. 2 shows that at fixed $(P^-Na^+)_{aq}$ concentration, k' should vary as the reciprocal of $[Na^+]$. The results of varying the sodium ion concentration are shown in Fig. 4. The phenylbenzoate is seen to be unaffected by sodium ion while the column capacity factors for the protonated species are linearly related to $1/[Na^+]$. This result further supports the ion-exchange interpretation of retention and indicates competition for anionic sites between sodium and protonated base. It can also be observed in Fig. 4 that in the presence of a large concentration of added sodium ion the retention of all bases is greatly reduced and the elution order of cycloguanil and 4-chlorophenylbiguanide reversed to that found in low surfactant concentrations. In these experiments no significant change in h as a function of sodium ion concentration was observed.

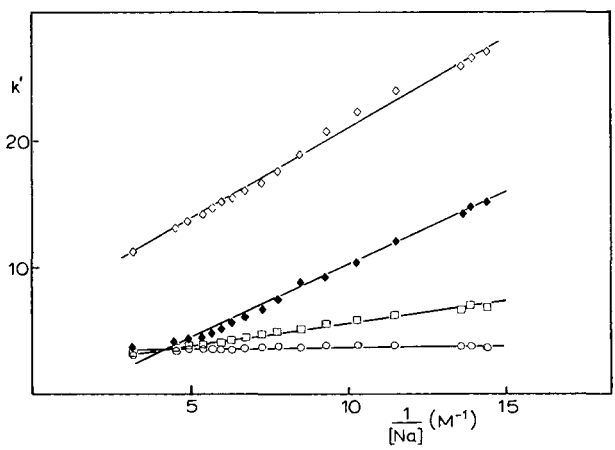

Fig. 4. Variation of the capacity ratio (k') with the reciprocal of sodium ion concentration for cycloguanil (□), 4-chlorophenylbiguanide (♦), proguanil (◇) and phenylbenzoate (○). Chromatographic conditions as in Fig. 1.

Serum determinations

The blank and spiked bovine serum after protein precipitation and injection produced chromatograms as shown in Fig. 5. The three bases are well resolved and, with the exception of cycloguanil, well removed from endogenous plasma constituents. The calibration curves of peak height against concentration (C) were linear and the regression lines represented as

Cycloguanil peak height (mm) = $0.268C$ (ng ml^{-1}) + 1.115 (R^2 = 0.990, S.D. = $5 \cdot 10^{-3}$)

4-Chlorophenylbiguanide peak height (mm) = $0.185C$ (ng ml^{-1}) + 0.53 (R^2 = 0.990, S.D. = $6 \cdot 10^{-3}$)

Proguanil peak height (mm) = $0.129C$ (ng ml^{-1}) + 0.294 (R^2 = 0.996, S.D. = $5 \cdot 10^{-3}$)

at a full scale absorbance of 0.01.

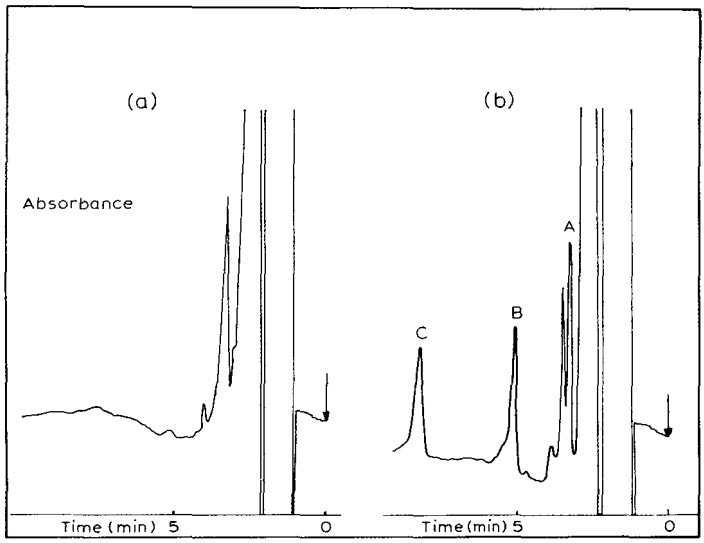

Fig. 5. Representative chromatograms to show the separation of biguanides in serum. (a) Blank bovine serum sample; (b) bovine serum sample containing 150 ng ml^{-1} of each biguanide. Peaks: A, cycloguanil; B, 4-chlorophenylbiguanide; C, proguanil. Conditions: eluent, acetonitrile—water (75:25) + 2.5% w/v SLS + 0.17 *M* H_3PO_4 + 0.0125 *M* NaH_2PO_4. Full scale absorbance 0.005, 100-μl valve and loop injection.

To estimate losses during the precipitation of protein comparable concentrations in water were injected directly. From the relative slopes of the lines obtained in water and in serum it was estimated that losses in the region of 10% were incurred during the deproteinating step.

The limit of detection for the three compounds was estimated to be in the region of 60 ng ml^{-1}. Attempts to improve this detection limit by selective extraction and concentration before injection were unsuccessful.

In vivo measurements

Since no information was available in the literature concerning human serum levels of proguanil following the ingestion of a prophylactic dose a trial run was made on a human volunteer. At varying times after ingestion of 200 mg proguanil blood samples were taken by venipuncture. Five ml of each sample were centrifuged after clotting and serum obtained. This was assayed by the above method. Specimen chromatograms are shown in Fig. 6. In these samples only proguanil could be detected and no clear evidence of either of the two accepted metabolites was found. The variation of serum proguanil with time is shown in Table I. This assay method was also applied to whole blood after lysing by repeated freezing and an increased proguanil concentration of 340 ng ml^{-1} was determined for the 1.5-h sample compared with 220 ng ml^{-1} for the serum alone.

In view of the paucacity of information on the pharmacokinetics of proguanil it is intended to apply the method described in a complete study. It appears that refinement of the method will be required if the presumably lower levels of the accepted metabolites are to be monitored.

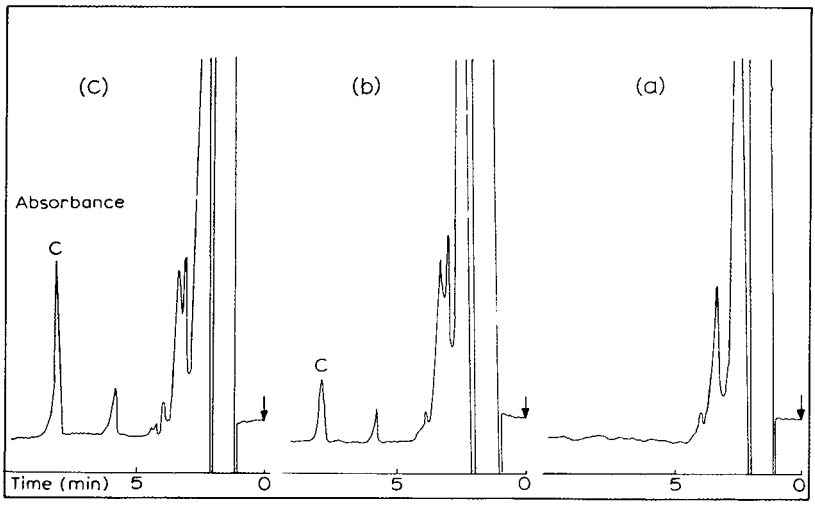

Fig. 6. Representative chromatograms of volunteer serum samples assayed after ingestion of 200 mg proguanil. (a) Blank serum sample; (b) serum after 0.5 h; (c) serum after 2.5 h. Chromatographic conditions as in Fig. 5. Peak C, proguanil.

TABLE I

RESULTS OF HUMAN SERUM DETERMINATION OF PROGUANIL AT VARIOUS TIMES AFTER INGESTION OF 200 mg PROGUANIL

Time (h)	0	0.5	1.5	2.5	3.5	24
Proguanil serum concentration (ng ml^{-1})	0	95	220	280	275	70

ACKNOWLEDGEMENTS

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REFERENCES

1 C.C. Smith, J. Ihrig and R. Menne, Amer. J. Trop. Med., 10 (1961) 694.
2 A.F. Crowther and A.A. Levi, Brit. J. Pharmacol. Chemother., 8 (1953) 93.
3 World Health Organisation, Technical Report Serial No. 296, 1965.
4 W.F. Bousquet, in J. Swarbrick (Editor), Current Concepts in Pharmaceutical Sciences, Lea Febiger, Philadelphia, PA, 1970, pp. 143—201.
5 B.G. Maigraith, M.M. Jaffey, A.R.D. Adams, W.H. Horner Adams and J.D. King, Ann. Trop. Med. Parasitol., 40 (1946) 493.
6 J.H. Knox and G.R. Laird, J. Chromatogr., 122 (1976) 17.
7 J.H. Knox and J. Jurand, J. Chromatogr., 125 (1976) 89.
8 D.P. Wittmer, N.O. Nuessle and W.G. Haney, Anal. Chem., 47 (1975) 1422.
9 S.P. Sood, L.E. Sartori, D.P. Wittmer and W.G. Haney, Anal. Chem., 48 (1976) 796.
10 P.T. Kissinger, Anal. Chem., 49 (1977) 883.
11 R.P.W. Scott and P. Kucera, J. Chromatogr., 175 (1979) 51.

12 E. Tomlinson, T.M. Jeffries and C.M. Riley, J. Chromatogr., 159 (1978) 315.
13 C. Horvath, W. Melander, I. Molnar and P. Molnar, Anal. Chem., 49 (1977) 2295.
14 E. Tomlinson, C.M. Riley and T.M. Jeffries, in E. Reid (Editor), Methodological Surveys in Biochemistry, Vol. 7, Blood Drugs and other Analytical Challenges, Ellis-Horwood, Chichester, 1978, p. 333.
15 J.C. Kraak, K.M. Jonker and J.F.K. Huber, J. Chromatogr., 142 (1977) 671.
16 H.H. Willard, L.L. Merritt and J.A. Dean, Instrumental Methods of Analysis, 4th ed., Van Nostrand, New York, 1966, p. 108.
17 C.M. Riley, E. Tomlinson, T.M. Jeffries and P.H. Redfern, J. Chromatogr., 162 (1979) 153.
18 J.H. Knox, High Performance Liquid Chromatography, Edinburgh University Press, Edinburgh, 1978, p. 60.

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DETERMINATION OF ISOFEZOLAC IN BIOLOGICAL FLUIDS BY REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive, and specific reversed-phase high-performance liquid chromatography assay was developed for the determination of 1,3,4-triphenylpyrazole-5-acetic acid (isofezolac) in plasma and urine. The assay involves extraction into diethyl ether from plasma buffered at pH 4.4. The organic phase is evaporated and the residue, dissolved in the mobile phase [acetonitrile—water—0.2 *M* phosphate buffer (pH 3) (65 : 15 : 20)] is chromatographed at a flow-rate of 1.5 ml/min. The drug is detected by its UV absorption (detection limit 100 ng/ml) or its very intense fluorescence (detection limit 10 ng/ml). Absolute analytical recoveries for isofezolac varied from 92.9 to 100.4%. The accuracy is ca. 1%. Each separation requires about 6 min. This method was applied successfully to the determination of isofezolac in humans for pharmacokinetic studies.

INTRODUCTION

Isofezolac, 1,3,4-triphenylpyrazole-5-acetic acid (Fig. 1), is a new drug selected on the basis of its anti-inflammatory, analgesic and antipyretic properties. This new non-steroid anti-inflammatory drug appeared to be as active as indomethacin in the various tests performed. Its activity is always greater than that of phenylbutazone and its ulcerogenic activity in the rat is 2.5 times weaker than that of indomethacin [1].

In order to perform pharmacokinetic studies, we have developed a sensitive technique for the determination of isofezolac in biological fluids. The method consists of a reversed-phase high-performance liquid chromatographic (HPLC) separation. The drug is detected by its UV absorption or its very intense fluorescence.

1-Phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid (Fig. 1) was used as internal standard.

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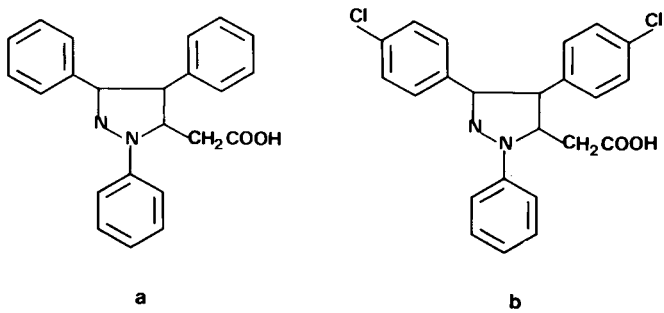

a b

Fig. 1. Chemical structures of isofezolac (a) and internal standard (b).

MATERIALS AND METHODS

Chemicals and reagents

Isofezolac and 1-phenyl-3,4-di-*p*-chlorophenylpyrazole-5-acetic acid (used as internal standard) were kindly supplied by Pharmuka (Gennevilliers, France).

All reagents were of analytical grade and used without further purification: diethyl ether (E. Merck, Darmstadt, G.F.R.), disodium citrate, 1 *N* sodium hydroxide, 0.1 *N* hydrochloric acid, orthophosphoric acid, potassium dihydrogen phosphate, acetonitrile and methanol (Prolabo, Paris, France), enzyme solution containing 100,000 units of β-glucuronidase per ml and 1,000,000 units of arylsulfatase per ml (I.B.F., Gennevilliers, France).

Buffer solutions

Citric acid—0.1 *M* disodium citrate buffer (pH 4.4) and 0.2 *M* phosphate buffer (pH 3) were stored at +4°C. For studies in which the pH of the eluent was varied, the pH was adjusted with a 10% solution of orthophosphoric acid or a 10% solution of sodium hydroxide in water. The phosphate concentration was maintained constant (0.02 moles/l) over the pH range investigated.

Stock solutions

Isofezolac stock solution (20 mg per 100 ml) and internal standard stock solution (20 mg per 100 ml) were prepared in methanol.

Apparatus

HPLC analyses were performed on a Chromatem 38 liquid chromatograph (Touzart et Matignon, Paris, France) operated at ambient temperature. A Jobin-Yvon Model J.Y. 3 D spectrofluorescence detector (Jobin-Yvon, Longjumeau, Paris, France) was operated at 273 nm for excitation and 335 nm for emission. The output of the detector was displayed on a recorder Model PE 1286 (Sefram, Paris, France) having a 1-V full-scale range. For UV detection, a Model 770 spectrophotometric detector (Spectra Physics, Santa Clara, CA, U.S.A.) was operated at 265 nm.

The column was 15 cm × 4.6 mm I.D. stainless steel, packed with LiChrosorb RP 8 (5 μm particle size; E. Merck) by a balanced density slurry technique [2]. A 20-μl loop injection valve Model 70-10 (Rheodyne, Berkeley, CA, U.S.A.) was used to introduce samples into the chromatographic system. The test of

the reversed-phase column was carried out with benzene, naphthalene and $NaNO_3$ as unretained compound. Under these conditions, the column efficiency was 3600 theoretical plates for benzene and 3150 for naphthalene.

Chromatographic conditions

The mobile phase, acetonitrile—water—0.2 *M* phosphate buffer (pH 3) (65:15:20), was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) under negative pressure, and degassed by ultrasonic vibration. The column was preconditioned with mobile phase until a stable recorder output was obtained. The flow-rate was then adjusted to 1.5 ml/min, generating a pressure of about 105 bar.

Extraction procedures

Plasma. To 1 ml of plasma transferred into a 45-ml screw-capped centrifuge tube, were added 1 ml of citrate buffer, 100 μl of internal standard solution (4 mg per 100 ml) and 15 ml of diethyl ether. After agitation and centrifugation (5 min at 3000 *g*), the organic phase was carefully pipetted out into a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C. The sample residue was dissolved in various volumes (0.5—3 ml) of mobile phase depending on the expected drug concentration; 20-μl aliquots were injected into the chromatograph.

Urine. In order to determine the total amount of isofezolac excreted in urine, the conjugated drug must be first hydrolysed. The best conditions for complete hydrolysis consisted of incubating 0.1 ml of urine, 1 ml of citrate buffer (pH 4.4), 100 μl of internal standard dilution (4 mg per 100 ml) and 0.1 ml of the enzyme solution at 37°C for 16 h. The sample was then treated as before. The residue was dissolved with 1—2 ml of mobile phase and 20-μl aliquots were injected into the chromatograph.

Calculations

The calculations of plasma and urine concentrations were always made by the internal standard method using peak area ratios.

RESULTS AND DISCUSSION

Chromatographic conditions

Reversed-phase liquid chromatography using bonded hydrocarbon stationary phases has proved to be an extremely versatile and easily used analytical technique for the analysis of ionogenic compounds [3—5]. Since isofezolac and the internal standard are compounds with a carboxylic function they can be determined by such a method. In order to achieve the best chromatographic conditions, the composition and pH of the mobile phase were varied.

Mobile phases studied included various ratios (v/v) of acetonitrile—water—phosphate buffer; namely 50:30:20, 55:25:20, 60:20:20, 65:15:20 and 70:10:20. The capacity factor (k') and resolution (Rs) of the two compounds were affected at higher acetonitrile concentrations (Fig. 2). When the acetonitrile concentration exceeded 65%, a loss of resolution between isofezolac

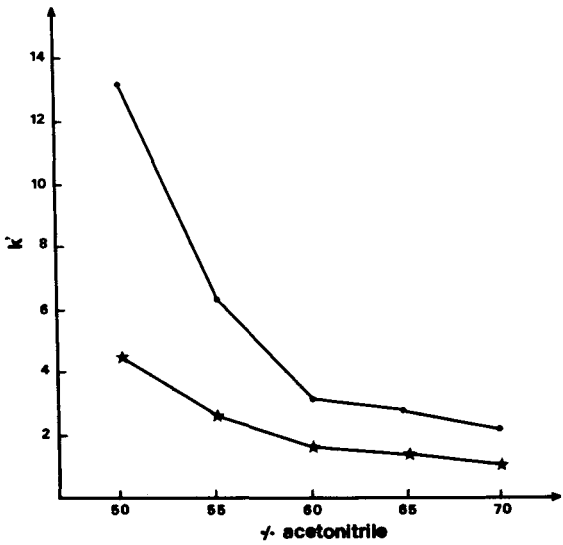

Fig. 2. Effect of the solvent acetonitrile content at pH 4 on capacity factors (k') of isofezolac (★) and internal standard (●).

and internal standard was observed. Moreover, the elution peak of isofezolac was near the peak of rapidly eluting plasma constituents.

At a lower acetonitrile concentration (50%) the resolution was very good (Rs = 11.2) but the elution time was more than 16 min. In order to optimize both resolution and elution time the amount of acetonitrile in the mobile phase was fixed at 65%.

The pH of the solvent system is the most critical factor influencing the reso-

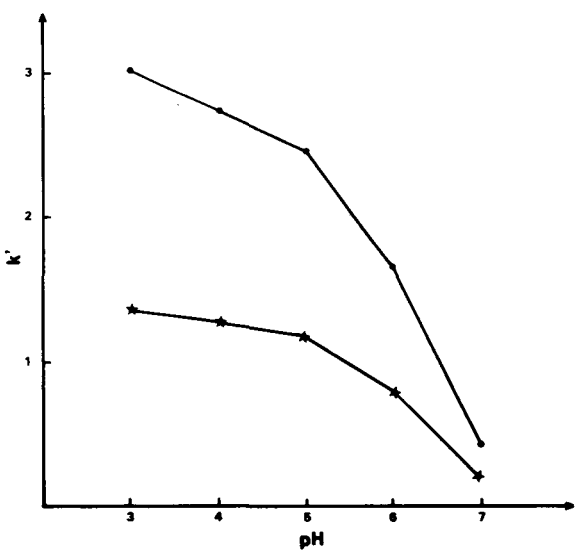

Fig. 3. Effect of variation of the solvent pH on capacity factors (k') of isofezolac (★) and internal standard (●). Mobile phase: acetonitrile—water—0.2 M phosphate buffer (65 : 15 : 20).

lution, as shown in Fig. 3. At pH > 5, the capacity factor decreased rapidly. Under these pH conditions, the two compounds are ionized and have a maximum affinity for the aqueous phase; thus they cannot be resolved. Excellent resolutions are achieved at pH < 5. The pH chosen for the separation of the compounds was pH 4. Resolution and column efficiency were very good (Rs = 7, N = 3090 theoretical plates for isofezolac). The total chromatographic time was less than 6 min. Moreover, at pH 4, the life of the column is very long.

Under the chromatographic conditions described, the retention times of isofezolac and internal standard were 2.7 and 4.5 min, respectively. Fig. 4B shows the chromatogram of an extract corresponding to 1 ml of human plasma containing 0.73 μg/ml isofezolac and 1 μg/ml internal standard. Fig. 4A shows the chromatogram of an extract of the same volume of a blank plasma from the same subject.

Fig. 5 shows the chromatogram of an extract corresponding to 0.1 ml of urine.

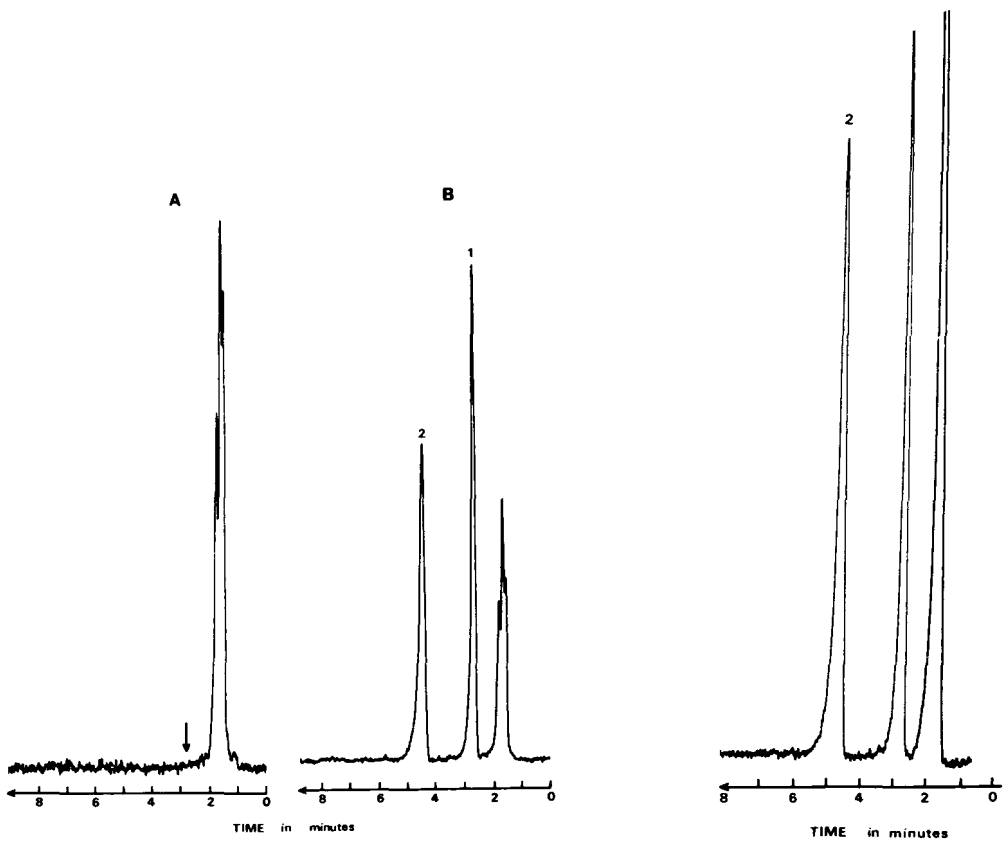

Fig. 4. Chromatograms of isofezolac in plasma. A, blank plasma sample (arrow indicates isofezolac elution position); B, plasma sample 2 h after a single oral dose of 50 mg. Isofezolac (peak 1) concentration = 0.73 μg/ml; internal standard (peak 2) concentration = 1 μg/ml.

Fig. 5. Chromatograms of isofezolac in urine (0—12 h collection). Isofezolac (peak 1) concentration = 16.53 μg/ml; internal standard (peak 2) concentration = 2 μg/ml.

Detection

Most of the pyrazole class of compounds exhibit very intense UV absorption and fluorescence. So monitoring of isofezolac plasma levels is possible with less than 1 ml of biological fluid, using either UV or fluorescence detectors.

Under the conditions of the assay (pH and solvents mixture) isofezolac and the internal standard give an absorption maximum at 265 nm (Fig. 6). Molar absorptivities are 18,600 for isofezolac and 21,800 for the internal standard. However, for pharmacokinetic studies, where a greater sensitivity is required, fluorescence detection must be preferred. Excitation and emission spectra are shown in Fig. 7. Isofezolac and the internal standard show the same excitation maximum at 273 nm. The emission maxima are 335 nm for isofezolac and 345 nm for the internal standard. In order to obtain greater sensitivity, the wavelengths chosen for the assay were 273 nm (excitation) and 335 nm (emission).

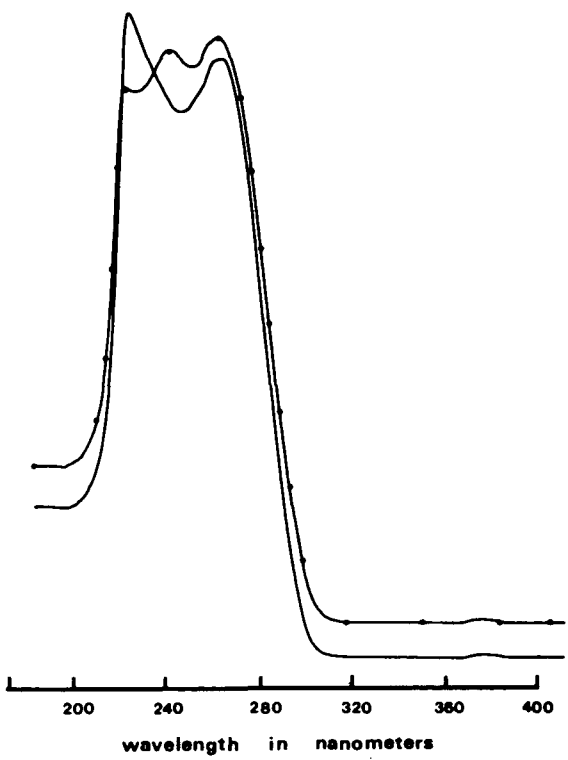

Fig. 6. UV absorption spectra of 2 μg/ml isofezolac (—) and internal standard (•—•—•) in acetonitrile—water—0.2 *M* phosphate buffer (pH 4) (65 : 15 : 20).

Repeatability and accuracy

Repeatability and accuracy of the assay were studied by measuring the concentrations of isofezolac in plasma samples spiked with isofezolac at concentrations of 0.2, 1 and 2 μg/ml. The results listed in Table I show that the repeatability and accuracy at lower concentrations are still very good and within the generally accepted limits for drug assays.

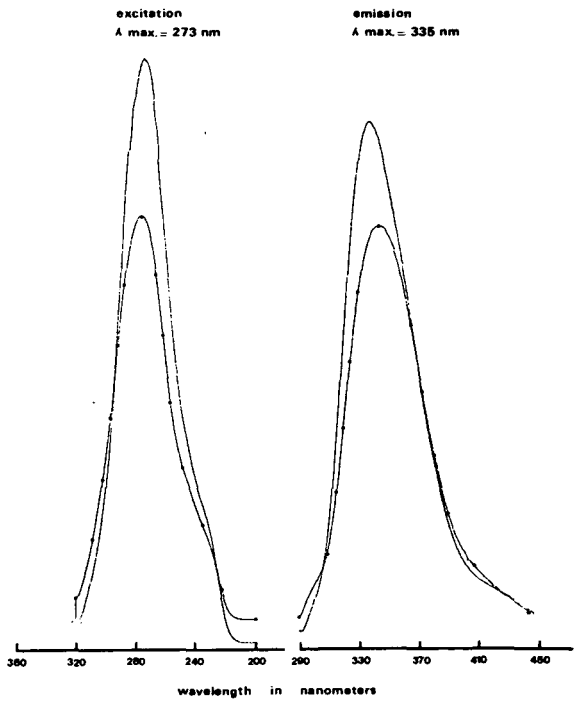

Fig. 7. Excitation and emission spectra of 200 μg/ml isofezolac (—) and internal standard (•—•—•) in acetonitrile—water—0.2 *M* phosphate buffer (pH 4) (65 : 15 : 20).

TABLE I

REPEATABILITY AND ACCURACY

Isofezolac added (μg/ml)	*n*	Isofezolac measured (μg/ml)*	Mean error	Relative error (%)	C.V.** (%)
0.5	5	0.203 (0.005)	0.002	1.5	2.34
1	5	0.994 (0.007)	0.003	0.6	0.66
2	5	1.986 (0.021)	0.009	0.7	1.04

*Standard deviation in parentheses.
**Coefficient of variation.

Recovery

The absolute analytical recovery from plasma of isofezolac and the internal standard was measured in the following way. The two compounds were added to plasma to achieve concentrations ranging between 0.2 and 2 μg/ml. The plasma samples were then assayed with the method described. Absolute analytical recovery was calculated by comparing the peak areas from plasma extracts

with the peak areas obtained by direct injection of the pure drug standards. As shown in Table II, the extraction efficiencies are quite comparable for the drug and internal standard.

TABLE II

ABSOLUTE ANALYTICAL RECOVERY OF ISOFEZOLAC AND INTERNAL STANDARD

Compound	Concentration (μg/ml)	Recovery (%)
Isofezolac	0.2	92.9
	1	92.9
	2	100.4
Internal standard	0.2	93.9
	1	94.1
	2	99.6

Linearity

The calibration curve was obtained by plotting the ratios of isofezolac peak area to that of internal standard, versus their respective concentrations in five different pools of plasma containing concentrations ranging from 0.2 to 2 μg/ml. The relation is linear over this range (r = 0.9999, slope = 0.7131, and intercept = 0.0063).

Detection limit

The described extraction procedure yields a relatively clean extract (Fig. 4A). Thus, isofezolac may be measured at the highest detector sensitivity. Under the conditions of this assay, the detection limit is about 10 ng/ml with fluorescence detector.

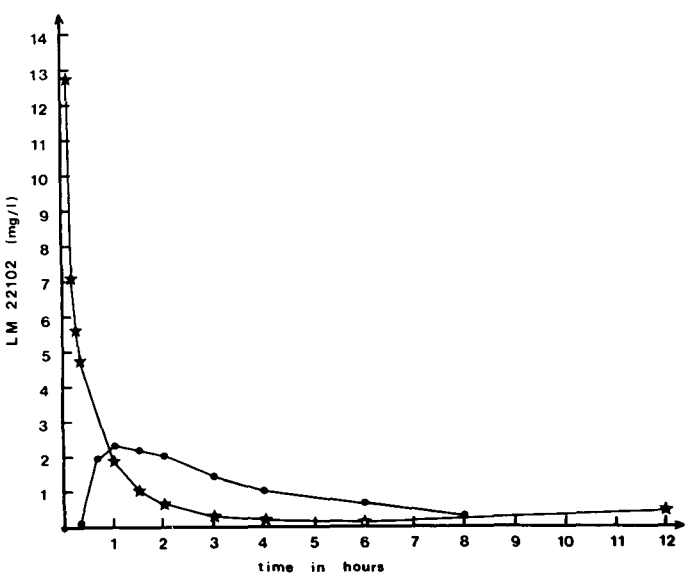

Fig. 8. Plasma decay of isofezolac after the administration of a single 50-mg dose of isofezolac. ★, Intravenous administration; ●, oral administration.

Clinical studies

A complete profile of the plasma isofezolac levels of one subject from a clinical study is shown in Fig. 8. This subject received one single intravenous dose of 50 mg of isofezolac and a week later a single oral dose of 50 mg. The half-life of isofezolac is about 1 h and the analytical method is able to measure plasma concentrations for more than 24 h, which is sufficient to achieve correct pharmacokinetic studies.

The proposed method is simple, sensitive, and rapid for the determination of isofezolac in biological fluids during pharmacokinetic studies.

ACKNOWLEDGEMENT

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REFERENCES

1 J. Mizoule, G. Lefur and A. Uzan, Arch. Int. Pharmacodyn., 238 (1979) 305.
2 B. Coq, C. Gonnet and J.-L. Rocca, J. Chromatogr., 106 (1975) 249.
3 P.J. Twitchet and A.C. Moffat, J. Chromatogr., 111 (1975) 149.
4 C. Horvath, W. Melander and I. Molnar, Anal. Chem., 49 (1977) 142.
5 J.L.M. van de Venne, J.L.H.M. Hendrikx and R.S. Deelder, J. Chromatogr., 167 (1978) 1.

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Biomedical Applications

CHROMBIO. 543

DETERMINATION OF THE ANTISPASMODIC AGENT ETHAVERINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Ethaverine can be measured in the plasma of human subjects by reversed-phase high-performance liquid chromatography employing UV detection. The limit of detection was 2 ng/ml, and the precision was ± 14, ± 6 and ± 2% at concentrations of 5, 25 and 50 ng/ml respectively. A peak mean plasma drug concentration of 20 ng/ml occurred at 1.5 h after single oral doses of a capsule formulation to human subjects, and declined with a half-life of 2.9 h.

INTRODUCTION

Ethaverine (Fig. 1) is an antispasmodic agent structurally and pharmacologically related to the benzylisoquinoline alkaloid papaverine; it is used as a smooth muscle relaxant and vasodilator and is claimed to be more potent and less toxic than papaverine [1]. Due to the rapid rate of metabolism of this type of compound, they are frequently administered as sustained-release formulations [2—5], which provide relatively lower blood concentrations of unchanged drug that are maintained, however, over longer periods of time [6]. In order to generate reliable pharmacokinetic data, accurate, precise, sensitive (<0.01 μg/ml) and specific methods of measurement of drug concentrations in

plasma are required. Very few methods are available for the measurement of this type of drug in biological fluids; early methods [7—10] were either non-specific or lacked sensitivity. Papaverine has been determined using ion-pair extraction followed by gas—liquid chromatography (GLC) with a sensitivity of 0.01 μg/ml when analysing a 5-ml plasma sample [11]. It has also been measured by gas chromatography utilising mass spectrometric detection (GC—MS), with a sensitivity of 0.005 μg/ml when analysing a 3-ml blood sample [12]. More recently, a column extraction procedure using Amberlite XAD-7 followed by GLC achieved this same level of sensitivity from 1 ml of whole blood [13]. Ethaverine has been measured by high-performance liquid chromatography (HPLC) in an adsorption mode with UV absorption detection at 254 nm [14]; a method sensitivity of 0.025 μg/ml could be achieved from 3-ml plasma samples.

This paper describes an HPLC method in the reversed-phase mode for the measurement of ethaverine from plasma. This mode of chromatography was preferred because of its high stability and reproducibility when analysing biological extracts. The method is simple, incorporates papaverine as internal standard and is more sensitive than any of the previously reported methods for this type of compound, achieving 0.002 μg/ml from a 2-ml plasma sample. The method can also be used for the analysis of papaverine from biological fluids in which case ethaverine could serve as an internal standard.

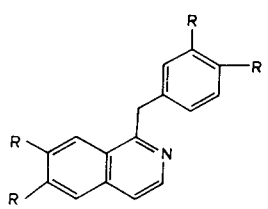

Fig. 1. Chemical structure of ethaverine (R = OC_2H_5) and papaverine (R = OCH_3).

EXPERIMENTAL

Materials

All reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Diethyl ether was freshly redistilled prior to use. Ethaverine hydrochloride [1-(3,4-diethoxybenzyl)-6,7-diethoxyisoquinoline hydrochloride] and papaverine hydrochloride [1-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline hydrochloride], used as internal standard (Fig. 1), were supplied by Dr. Kade Pharmazeutische Fabrik GmbH, Konstanz, G.F.R. Standard solutions of ethaverine HCl and internal standard were prepared in methanol at concentrations of 1 μg/ml and 2 μg/ml respectively and stored at 4°C.

Extraction procedure

Plasma samples (2 ml) were transferred into 10-ml pointed centrifuge tubes and spiked with internal standard (15 μl, containing 30 ng papaverine HCl). Sodium hydroxide solution (200 μl, 4 *M*) was added and the mixture was extracted by shaking it with diethyl ether (5 ml) using a vortex mixer. After

centrifugation of the extract at 2000 *g* for 10 min, the organic layer was transferred to another pointed centrifuge tube and the aqueous layer extracted with more ether (2 ml). After centrifugation, the organic layers were combined and extracted for 1 min by vortex mixing with hydrochloric acid (0.5 ml, 1 *M*). After centrigugation at 2000 *g* for 10 min, the ether layer was discarded and the aqueous acid layer made alkaline with sodium hydroxide solution (0.5 ml, 4 *M*). The alkaline solution was extracted with diethyl ether (5 ml) as previously described. The ether layer was transferred to a pointed centrifuge tube and evaporated to dryness under a stream of nitrogen at 37°C. The sides of the tube were rinsed with more ether to ensure that the residue was at the bottom of the tube, and the ether again evaporated. The residue was dissolved in methanol (25 μl) and aliquots injected into the chromatograph.

Apparatus

The liquid chromatograph consisted of a Waters M6000A pump (Waters Assoc., Northwich, Great Britain) coupled to a Cecil 212 variable-wavelength UV detector (Cecil Instruments, Cambridge, Great Britain) operated at 238 nm. Injection was by syringe (25 μl, Precision Sampling Corp., Baton Rouge, LA, U.S.A.) via a U6K universal injector (Waters Assoc.). The column was con-

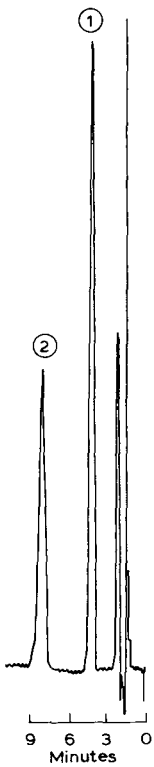

Fig. 2. Chromatogram of a standard mixture containing papaverine (1) and ethaverine (2). Column: 250 × 4.6 mm I.D., pre-packed with Partisil 10 ODS; flow-rate, 2 ml/min; solvent system: 65% (v/v) methanol—aqueous potassium dihydrogen orthophosphate (0.1% w/v), detector: UV at 238 nm, attenuation 0.01 a.u.f.s.

structed of stainless steel (25 cm × 4.6 mm I.D.), prepacked with Partisil 10 ODS (mean particle size 10 μm) (Whatman, Maidstone, Great Britain).

Chromatography was performed in a reversed-phase mode using a mobile phase of 65% (v/v) methanol in aqueous potassium dihydrogen orthophosphate (0.1%) with a flow-rate of 2 ml/min. The retention times of ethaverine and papaverine (internal standard) under these conditions were 8.5 min and 3.5 min respectively (Fig. 2).

Calibration procedure

Calibration lines of peak height ratio measurements of ethaverine HCl to internal standard against concentration of ethaverine HCl were constructed over the concentration range up to 50 ng/ml. Samples of blank (drug-free) plasma (2 ml) were spiked with ethaverine HCl at concentrations of 5, 15, 25, 35 and 50 ng/ml and with internal standard at a fixed concentration of 15 ng/ml. The samples were taken through the extraction procedure described previously.

Studies in humans

Plasma samples were obtained from six human volunteer subjects dosed orally with a sustained-release formulation of 30 mg ethaverine HCl and analysed by the foregoing procedures. The studies in volunteers were carried out under conditions similar to those described by Brodie et al. [15].

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration was repeated on five occasions. The precision of the method for the measurement of ethaverine HCl in plasma as indicated by the coefficient of variation of peak height ratio measurements of drug to internal standard (Table I) were ± 14%, ± 6% and ± 2% at 5 ng/ml, 25 ng/ml and 50 ng/ml respectively.

The coefficient of variation of peak height ratio measurements of a non-extracted mixture of ethaverine and internal standard analysed routinely was

TABLE I

RECOVERY AND PRECISION OF MEASUREMENT OF ETHAVERINE FROM PLASMA

Concentration of ethaverine HCl added to plasma (ng/ml)	Recovery* (%)	Coefficient of variation (%)
5	80	14
15	86	2
25	81	6
35	82	3
50	79	2
Mean	82 ± 3 S.D.	

*Means of 5 determinations at each concentration.

± 2% throughout the analysis of all the plasma samples analysed during a six-week period.

Accuracy

The calibration line for the measurement of ethaverine HCl in plasma was constructed from five replicate measurements at five concentrations over the range, and plots of peak height ratio against concentration were linear (y = 0.0271 x + 0.0210, correlation coefficient r = 0.9970) and the value of the intercept was shown to be not significantly different from zero ($P > 0.05$). The equation for the line forced through the origin was y = 0.0277 (± 0.0002 S.D.) x, where y is the peak height ratio and x is the concentration of ethaverine HCl (ng/ml). The accuracy of the method as defined by the 95% confidence limits of the least-squares regression line forced through the origin, i.e., taking the calibration line as an estimate of the concentration of ethaverine HCl in plasma, was ± 48%, ± 9% and ± 5% at 5 ng/ml, 26 ng/ml and 50 ng/ml respectively.

Recovery

The recovery of internal standard from plasma (15 ng/ml) was 86% ± 4 S.D. (n = 5). The mean recovery of ethaverine HCl from plasma over the concentration range 5—50 ng/ml was determined by comparison of peak height ratio measurements of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was 82% ± 3 S.D. (Table I).

Stability of ethaverine in plasma

The stability of ethaverine HCl in plasma under the storage conditions used was tested by storing spiked plasma samples (35 ng/ml) at −20°C for one

TABLE II

CONCENTRATIONS OF ETHAVERINE HCl (ng/ml) IN THE PLASMA OF SIX HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 30 mg

Figures in parentheses are repeat analyses up to one month after the first analysis.

Time (h)	Subject No.						Mean ± S.D.
	1	2	3	4	5	6	
0.5	<2	<2	<2	<2	<2	3	<2
0.75	6	12 (13)	<2	2	2	10	5 ± 5
1	19	35	<2	9	3	17	14 ± 13
1.5	25	36 (33)	<2	24	8	24	20 ± 13
2	24 (22)	29 (27)	<2	27	11	19	18 ± 11
3	18 (16)	21	2 (<2)	16 (18)	8 (9)	19 (20)	14 ± 7
4	18	12 (11)	32 (29)	13 (13)	5 (6)	15 (14)	16 ± 9
6	10	6	15	5	4	10	8 ± 4
8	5	3	6	3	3	6	4 ± 2
12	3	<2 (<2)	3	<2	<2	4	<2
16	<2	<2	<2	<2	<2	3	<2
24	<2	<2	<2	<2	<2	<2	<2
32	<2	<2	<2	<2	<2	<2	<2

month prior to analysis. Concentrations found (32 ng/ml ± 0.6 S.D.) indicated that a slight decomposition had occurred during this time period. The reproducibility of the method was tested by the re-analysis of a minimum of 10% of the samples up to one month after the first analysis. Concentrations of ethaverine HCl found were in very good agreement with results previously obtained (Table II).

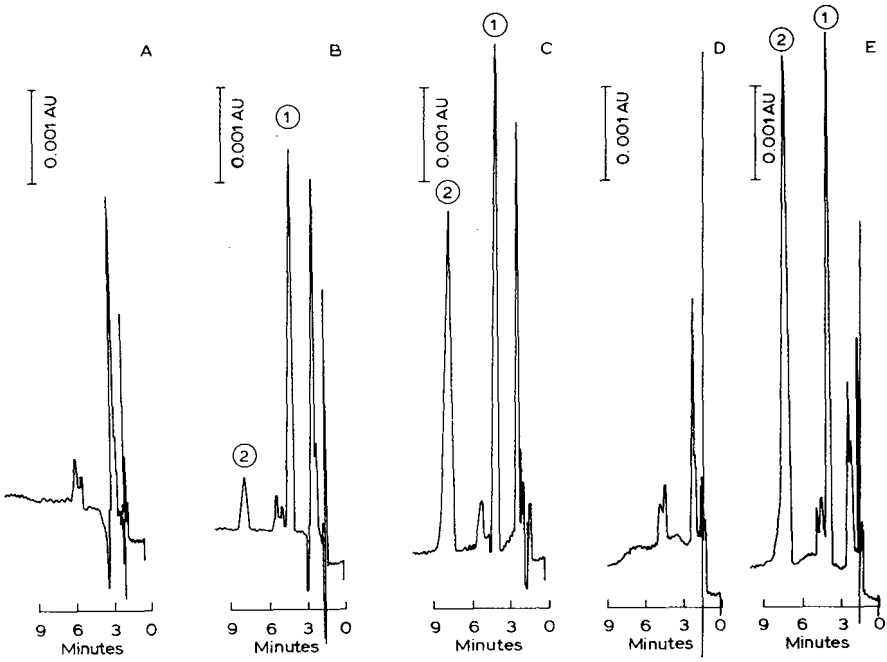

Fig. 3. Chromatograms of (A) control plasma extract used in calibration procedure; (B) control plasma spike containing internal standard (1) 15 ng/ml and ethaverine (2) 5 ng/ml; (C) control plasma spike containing internal standard (1) 15 ng/ml and ethaverine (2) 25 ng/ml; (D) predose plasma extract; (E) 1-h post-dose plasma sample containing ethaverine at a concentration of 35 ng/ml. Chromatographic conditions same as for Fig. 2.

TABLE III

HALF-LIVES OF THE TERMINAL LINEAR SECTIONS OF THE PLASMA ETHAVERINE CONCENTRATION—TIME RELATIONSHIPS

Subject	Half-life (h)	r^2
1	3.18	0.969
2	2.00	1.000
3	1.66	0.997
4	1.88	0.986
5	5.43	0.995
6	3.05	0.998
Mean ± S.D.	2.87 ± 1.41	

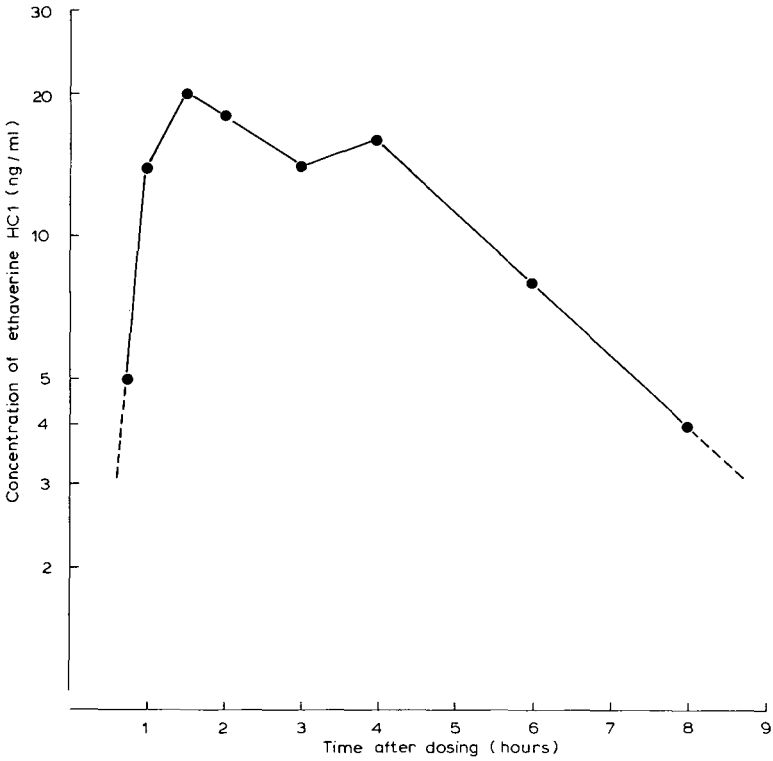

Fig. 4. Mean plasma concentrations of ethaverine HCl during 8 h after an oral dose of 30 mg of drug. Semi-logarithmic scale.

Limit of detection

Predose (blank) plasma samples taken from each subject showed no interfering peaks with similar retention times to either ethaverine or the internal standard (Fig. 3). The limit of detection of ethaverine under the experimental conditions used (signal-to-noise ratio = 5:1) with a 2-ml plasma sample was 2 ng/ml.

Concentrations of ethaverine (measured as the hydrochloride salt) in plasma

After single oral doses of capsules containing 30 mg of ethaverine (as the hydrochloride), a peak of mean concentrations of 20 ng/ml was reached at 1.5 h after dosing (Fig. 4, Table II). Mean concentrations remained between 14—20 ng/ml during 1—4 h after dosing, and thereafter declined to 4 ng/ml at 8 h. The mean half-life of ethaverine in plasma was 2.87 h ± 1.41 S.D. (n = 6) (Table III). After 8 h, mean concentrations of ethaverine were below the limit of detection.

REFERENCES

1 J.J. Lewis, An Introduction to Pharmacology, Livingstone, Edinburgh, 1965, p. 477.
2 E.R. Garrett, H. Roseboom, J.R. Green, Jr., and W. Schuerman, Int. J. Clin. Pharmacol., 16 (1978) 193.

3 G.C. Maggi, D. Cerchiari and G. Coppi, Arzneim.-Forsch., 27 (1977) 1214.
4 R.B. Miller, S.T. Leslie, F.M. Black and C. Boroda, Brit. J. Clin. Pharmacol., 5 (1978) 51.
5 B.Y. Lee, H. Sakamoto, F. Trainer, G. Brody and Y.W. Cho, Int. J. Clin. Pharmacol., 16 (1978) 32.
6 J.D. Arnold, J. Baldridge, B. Riley and G. Brody, Int. J. Clin. Pharmacol., 15 (1977) 230.
7 J. Axelrod, R. Shofer, J.K. Inscoe, W.M. King and A. Sjoerdsma, J. Pharmacol. Exp. Ther., 124 (1958) 9.
8 E. Mussini and A. Marzo, Biochem. Exp. Biol., 10 (1973) 331.
9 H.V. Street, J. Chromatogr., 29 (1967) 68.
10 N.D. Greenwood and I.W. Guppy, Analyst (London), 99 (1974) 1179.
11 D.E. Guttman, H.B. Kostenbauder, G.R. Wilkinson and P.H. Dubé, J. Pharm. Sci., 63 (1974) 1623.
12 J. De Graeve, J. Van Cantfort and J. Gielen, J. Chromatogr., 133 (1977) 153.
13 E. Zuccato, F. Marcucci and E. Mussini, J. Pharmacol. Meth., 1 (1978) 9.
14 E. Renier, K. Kjeldsen and M. Pays, Feuill. Biol., 20 (1979) 111.
15 R.R. Brodie, L.F. Chasseaud, T. Taylor, D.A. O'Kelly and A. Darragh, J. Chromatogr., 146 (1978) 152.

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DETERMINATION OF ESTRAMUSTINE AND ITS 17-KETO METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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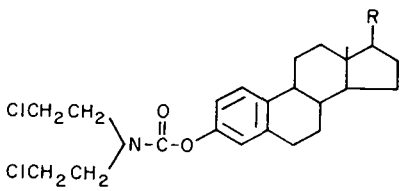
SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of estramustine and its 17-keto metabolite in plasma. The assay involves extraction of the compounds into hexane from plasma buffered to pH 9.0, the residue obtained by evaporation of the hexane extract is dissolved in the mobile phase hexane—ethanol (92.5:7.5) with HPLC analysis performed on a 5-μm silica gel column using a fluorescence detector with excitation at 195 nm and emission at wavelengths greater than 250 nm. The overall recoveries and limits of sensitivity for estramustine and the 17-keto metabolite are 74.7% and 40 ng/ml of plasma and 85.1% and 50 ng/ml of plasma, respectively. The method was used to obtain plasma concentration—time profiles in three subjects with prostatic cancer following oral administration of a single 7 mg/kg dose of estramustine phosphate.

INTRODUCTION

Estramustine phosphate (I), estradiol-3N-bis(2-chloroethyl) carbamate-17β-dihydrogen phosphate (Fig. 1), a nitrogen mustard derivative of estradiol-17β-phosphate has been shown to be effective in the treatment of prostatic carcinoma [1—5]. Studies on the distribution, absorption and metabolism of the drug in man, rat and dog have been reported [6—10]. In man, orally administered estramustine phosphate is rapidly dephosphorylated in the gastrointestinal tract to yield estramustine (II) [8]. Estramustine (II) and its 17-keto analogue (III), estrone-3N-bis(2-chloroethyl) carbamate, (Fig. 1) are the major metabolites found in plasma [9].

A radioimmunoassay was recently reported and used for the determination of intact estramustine phosphate following intravenous administration to dogs [10]. In addition, a gas—liquid chromatographic (GLC) method using flame ionization detection (FID) to quantitate II and III has been developed [9]. The



R	Compound
$-OP(OH)_2$ (O)	Estramustine Phosphate, I
$-OH$	Estramustine, II
$=O$	Estrone-3N-bis(2-Chloroethyl) carbamate, III

Fig. 1. Chemical structures of estramustine phosphate (I), estramustine (II) and estrone-3N-bis(2-chloroethyl) carbamate (III).

GLC assay utilizes selective extraction of the compounds into diethyl ether, purification of the extract by hexane washing, alumina chromatography, and GLC—FID of II and III as the silyl derivative and intact compound, respectively.

The present communication describes a high-performance liquid chromatographic (HPLC) assay with fluorescence detection capable of simultaneous measurement of the two unconjugated metabolites II and III in plasma. The assay is rapid and is performed directly on an extract of plasma and eliminates the time-consuming clean-up procedures and derivatization steps required for the GLC assay [9]. The HPLC assay has comparable sensitivity to the GLC assay [9] with the advantage that only 1 ml of plasma is required. The method was used to obtain the plasma concentration—time profiles of II and III in three subjects following single 7 mg/kg oral doses of estramustine phosphate (I).

EXPERIMENTAL

Column

The column used was a 25 cm × 4.6 mm I.D. stainless-steel prepacked column containing 5 μm Partisil PXS 5/25 silica gel (Whatman, Clifton, NJ, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector (Waters Assoc., Milford, MA, U.S.A.) and a Model FS-970 fluorescence detector (Schoeffel Instrument, Westwood, NJ, U.S.A.). A deuterium lamp was used to provide monochromatic excitation at 195 nm while fluorescence emission greater than 250 nm was measured through a UV 28 ultraviolet transmitting filter with a quartz window photo multiplier tube. The isocratic mobile phase was a mixture of hexane—ethanol (92.5:7.5) at a column head pressure of 1000 p.s.i. and a flow-rate of 1.5 ml/min. Under these conditions, the retention time of compound II was 7.2 (k' = 2.2) and that of com-

pound III was 5.4 min (k' = 1.4). The fluorescence detector attenuation was 0.1 μA full scale with a time constant of 3.0 sec. The chart speed was 1 cm/min on the 1.0-mV Omniscribe recorder (Houston Instrument, Austin, TX, U.S.A.). Under these conditions 140 ng of compound II and 200 ng of compound III per 20 μl injected give approximately full scale response. The minimum detectable amounts of both compounds is approximately 10 ng injected on column. A typical chromatogram for 90 ng of II and 80 ng of III per 20 μl injected is shown in Fig. 2.

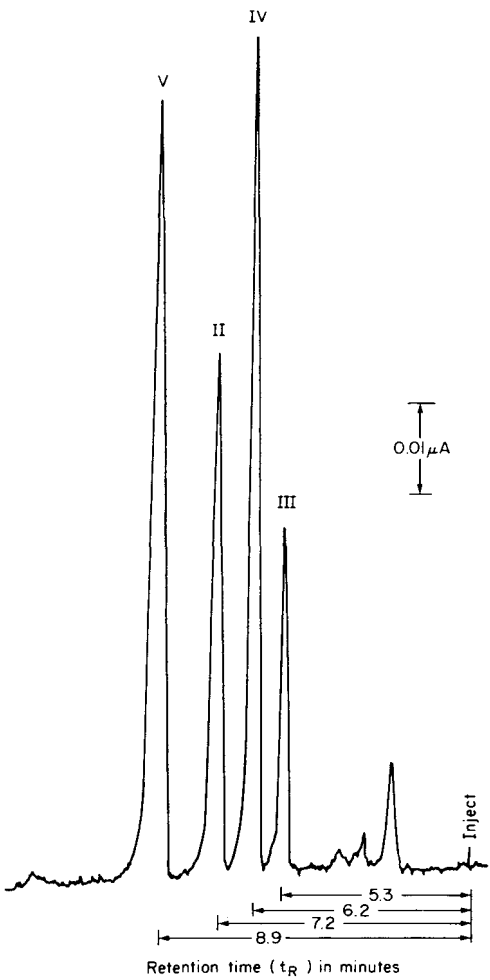


Fig. 2. Chromatographic separation of 90 ng estramustine (II), 80 ng estrone-3N-bis(2-chloroethyl) carbamate (III), 110 ng estrone (IV), and 20 ng estradiol (V).

Analytical standards

Compound II, [estramustine; estradiol-17β-3N-bis(2-chloroethyl) carbamate, $C_{23}H_{31}O_3NCl_2$, MW 440.41, m.p. 104–105°C] and compound III, [estrone-3N-bis(2-chloroethyl) carbamate, MW 439.40] of pharmaceutical grade purity (> 99%) are used as analytical standards.

Prepare stock solutions of compounds II and III in separate 50-ml volumetric flasks by dissolving 5.0 mg of each compound in 50 ml of ethanol. These stock solutions (containing 100 μg/ml) are used to prepare two sets of four mixed standard solutions (Nos. 1—4) containing 400, 600, 800 and 1000 ng of II and 500, 1000, 3000 and 5000 ng of III per ml of ethanol or hexane—ethanol (92.5:7.5). Aliquots (100 μl) of the ethanolic solutions are added to plasma to construct the calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery. Aliquots (20 μl) of the hexane—ethanol (92.5:7.5) solutions are injected as the external standards for establishing the HPLC parameters using fluorescence detection.

Reagents

All reagents are of analytical grade purity and are prepared in deionized, distilled water.

Borate—KCl—Na_2CO_3 buffer (1.0 M, pH 9.0). Dissolve 61.8 g of boric acid (H_3BO_3) and 74.6 g KCl per liter of distilled water. Dissolve 106 g of Na_2CO_3 per liter of distilled water. To 630 ml of the boric acid–KCl solution add 370 ml of the Na_2CO_3 solution to make 1 l of buffer solution. Shake well and adjust to pH 9.0 if necessary with the Na_2CO_3 solution. This solution is 1 *M* with respect to H_3BO_3—Na_2CO_3—KCl. The solution should be stored at 35–37°C to prevent crystallization of the salts from the solution. Other reagents included hexane (UV grade, Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and ethanol (200 Proof, Pharmaco, Publicker Industries, Philadelphia, PA, U.S.A.).

Assay

Into a 50-ml stoppered-centrifuge tube (PTFE No. 16, stoppered) add 100 μl of ethanol, 1.0 ml of unknown plasma, and 2.0 ml of 1 *M* borate—KCl—Na_2CO_3 buffer (pH 9.0) and mix well. Extract the samples with 12 ml of hexane by slowly shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.), centrifuge the samples in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon-IEC, Needham, MA, U.S.A.) at 5°C for 10 min at approximately 1200 *g*. Transfer a 10-ml aliquot of the hexane extract into a tapered 15-ml stoppered centrifuge tube (PTFE No. 13 stoppered), and evaporate to dryness at 50°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residue in 100 μl of hexane—ethanol (92.5:7.5) and inject a 20-μl aliquot for HPLC analysis. Along with the samples, a 1.0-ml specimen of control plasma and four 1.0-ml specimens of control plasma containing 100 μl of ethanolic solutions Nos. 1—4, equivalent to 40, 60, 80 and 100 ng of compound II and 50, 100, 300 and 500 ng of compound III per ml of plasma, are processed. These standards are used to establish the calibration curve for the direct quantitation of the unknowns using the peak height vs. concentration technique. Appropriate corrections for any changes in sample aliquots or serial dilutions used must be performed. Typical chromatograms of plasma extracts are shown in Fig. 3.

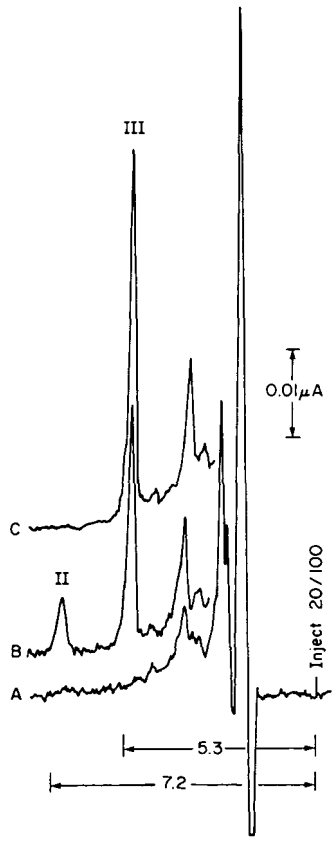


Fig. 3. Chromatograms for the HPLC analysis of hexane extracts of (A) control plasma, (B) control plasma containing 100 ng II per ml and 500 ng III per ml added authentic standard, and (C) human plasma 2 h post 7 mg/kg dose of estramustine phosphate.

Statistical evaluation of the method

The method was evaluated over a concentration range of 45.3–113.2 ng of II and 48.6—486 ng of III per ml of plasma. These concentration ranges were based upon the plasma levels of the two compounds measured in human subjects in a pilot study during the initial developmental stages of the assay. Four sets of four individual samples (total, 16 samples) of II and III in the above concentration ranges were added to 1 ml plasma. These spiked samples were then taken through the analytical procedure and the recovery data obtained are shown in Table I. The data for II and III were best described by linear least-square equations of the form $y = mx + b$ ($y = 0.0195\ x - 0.194$ and $y = 0.0150\ x - 0.0917$) with correlation coefficients r of 0.960 and 0.994, respectively indicating the high degree of linearity of the procedure for both compounds. The coefficients of variation for II and III over their respective concentration ranges were 5.7 and 5.6% respectively. The recovery of compounds II and III (after correction for 10/12 aliquot taken for analysis) calculated against pure standards of the compounds prepared in hexane—ethanol (92.5: 7.5) was 74.7 ± 6.0% and 84.9 ± 4.1%, respectively.

TABLE I

LINEARITY AND PRECISION OF THE HPLC ASSAY OF COMPOUNDS II AND III RECOVERED FROM PLASMA

Each determination was performed with four individual samples.

Concentration added (ng/ml)	Mean concentration found ± S.D.	Coefficient of variation (%)	Recovery (%)
*Compound II**			
45.3	46.3 ± 2.7	6.0	68.2
68.0	69.3 ± 1.1	1.6	77.3
90.6	84.7 ± 4.6	5.4	70.1
113.2	116.7 ± 11.7	10.1	83.2
Mean ± S.D.		5.7	74.7 ± 6.0
*Compound III***			
48.6	52.6 ± 2.4	4.5	80.0
97.2	101.6 ± 6.4	6.3	85.8
291.6	273.6 ± 21.6	7.9	82.7
486.0	495.5 ± 18.4	3.7	91.1
Mean ± S.D.		5.6	84.9 ± 4.1

*$y = 0.0195\,x - 0.194$; $r = 0.960$.
**$y = 0.0150\,x - 0.0917$; $r = 0.994$.

Specificity of the method

The specificity of the plasma assay for II and III was established by the absence of any interfering peaks occurring in the retention volume of these compounds following extraction of control (drug-free) human plasma from different sources and from treated subjects under the chromatographic separation conditions described above. Estramustine phosphate (I) is not extracted from plasma and does not give a chromatographic peak under the described conditions. Estrone (t_R = 6.2 min, k' = 1.8) and estradiol (t_R = 8.9 min, k' = 3.0), which are known metabolites in the dog [11], are chromatographically resolved from II and III (Fig. 1).

No suitable internal standard was found for this assay procedure. Of the many steroids evaluated as reference compounds, only equilenin, equilin and estradiol-3-methyl ether demonstrated similar fluorescence properties, however, these compounds were not chromatographically resolved from III under the chromatographic conditions described.

RESULTS AND DISCUSSION

Spectrofluorometric analysis of estrogens in urine typically measure the resultant fluorescence of these compounds generated in solutions containing a high percentage of concentrated phosphoric or sulfuric acid (excitation/emission, 410—450/500 nm) [12,13]. The native fluorescence of the estrogens (excitation/emission, 280/310 nm) has been used for the assay of estrogens in pregnancy urine [14]. HPLC assay of estrogens utilizing reversed-phase chromatography with UV detection for placental estriol in urine [15], normal-

phase chromatography with UV detection for estrogenic steroids in pregnancy urine [16], and ion-exchange chromatography with radiometric detection for estrogen conjugates in plasma [17] have all been recently reported.

The present study utilizes normal-phase HPLC and the measurement of the intrinsic fluorescence of the estrogen derivatives II and III for a highly sensitive and specific assay of the two components. A significant increase in fluorescence sensitivity in hexane—ethanol (92.5:7.5) was obtained by using a deuterium lamp at 195 nm as the excitation source compared to a xenon lamp. This wavelength (195 nm) coincides with the high-energy output range of the deuterium lamp and the UV absorbance maxima of the compounds, hence the fluorescence yield measured shows a dramatic increase in sensitivity. Reversed-phase chromatography of II (t_R = 7.4 min, k' = 3.8) and III (t_R = 8.4 min, k' = 4.4) is also possible in a solvent system of methanol–water (80:20) at this excitation wavelength. However, the overall sensitivity in this medium for estramustine (II) is only 25% of that measured in hexane—ethanol (92.5:7.5).

Application of the method to biological specimens

The HPLC assay described was applied for the analysis of plasma samples from three subjects with prostatic cancer following single oral administration of 7 mg/kg of estramustine phosphate (I). Peak levels of III ranging from 408 to 904 ng/ml were measured at 2-h post dosing and levels of III were measurable for 10—12 h (Table II). The levels of II were non-measurable ($\leqslant$ 40 ng/ml)

TABLE II

PLASMA LEVELS (ng/ml) OF III IN THREE HUMAN SUBJECTS FOLLOWING A SINGLE 7 mg/kg ORAL DOSE OF ESTRAMUSTINE PHOSPHATE (I)

Time (h)	Concentration of III (ng/ml)		
	Subject A	Subject B	Subject C
1	95	401	294
2	408	474	904
3	223	271	689
4	136	132	723
6	119	76	565
8	69	n.a.*	316
10	53	37**	232
12	n.a.*	29**	102

*n.a. = not analyzed.

**Analysis of 40/100 µl of reconstituted extract.

during the same experimental period which suggests that extensive oxidation of II at the 17-position occurs during its "first pass" through the liver and/or gastrointestinal tract. This procedure with minor modifications has recently been applied to the analysis of II, III, estrone, and estradiol in man following multiple dosing (8.6—15.4 mg/kg/day) of estramustine phosphate (I) [18].

REFERENCES

1 L. Andersson, F. Edsmyr, G. Jonsson and I. Konyves, Cancer Res., 60 (1977) 73.
2 G. Jonsson and B. Hogberg, Scand. J. Urol. Nephrol., 5 (1971) 103.
3 G. Jonsson, B. Hogberg and T. Nilsson, Scand. J. Urol. Nephrol., 11 (1977) 231.
4 A. Mittleman, S.H. Shukla and G.P. Murphy, J. Urol., 115 (1976) 409.
5 R. Nagel and C.P. Kolln, Brit. J. Urol., 49 (1977) 73.
6 R.Y. Kirdani, A. Mittleman, G.P. Murphy and A.A. Sandberg, J. Clin. Endocrinol. Metab., 41 (1975) 305.
7 R.Y. Kirdani, G.P. Murphy and A.A. Sandberg, Proc. Soc. Exp. Biol. Med., 155 (1977) 94.
8 G. Plym Forshell, J. Muntzing, A. Ek, E. Lindstedt and H. Dencker, Invest. Urol., 14 (1976) 128.
9 P.O. Gunnarsson, T. Nilsson and G. Plym Forshell, Urol. Res., (1979) in press.
10 B. Forsgren, P.O. Gunnarsson, S. Johansson and T. Kant, Acta Pharm. Suecica, 15 (1978) 200.
11 J. Carbone and R. Dixon, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 442.
12 S. Udenfriend, Fluorescence Assay in Biology and Medicine, Academic Press, New York, 1962, pp. 353—359.
13 C. Goutle-Coussieu, G. Habrioux, D. Eichenberger and M. Jayle, Clin. Chem., 25 (1979) 230.
14 J. Bramhall and A.Z. Britten, Clin. Chim. Acta, 68 (1976) 203.
15 G.R. Gottelli, J.H. Wall, P.M. Kabra and L.J. Marton, Clin. Chem., 24 (1978) 2132.
16 R.J. Dolphin and P.J. Pergande, J. Chromatogr., 143 (1977) 267.
17 P.I. Musey, D.C. Collins and J.R.K. Preedy, Steroids, 31 (1978) 583.
18 R. Dixon, M.A. Brooks and G. Gill, Res. Commun. Chem. Pathol. Pharmacol., 27 (1980) 17.

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Note

Approach to the quantitative analysis of nucleotides by gas chromatography—mass spectrometry

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Nucleotides are not easily amenable to gas chromatographic analysis [1] and for this reason the specificity and sensitivity of gas chromatography—mass spectrometry (GC—MS) with selected ion monitoring (SIM) [2] is rarely employable for nucleotide analysis. High-performance liquid chromatography (HPLC) has instead recently become a powerful and convenient method for the analysis of nucleotides [3]. The specificity and sensitivity of HPLC analysis of nucleotides may, however, be ultimately limited since UV detection is commonly employed [4]. In an effort to make nucleotides more amenable to GC—MS analysis with SIM, we have devised a general scheme for the analysis of nucleosides and nucleotides (so far, ribonucleoside monophosphates) from biological media.

The analytical scheme is evaluated with the analysis of nucleotide anabolites of the antineoplastic [5] and immunosuppressive [6] agent 6-mercaptopurine (6-MP). This drug must be taken up by cells and converted by hypoxanthine—guanine phosphoribosyl transferase (HGPRT) directly into 6-mercaptopurine riboside-5′-phosphate (MPRP), a potent inhibitor of nucleic acid synthesis. MPRP can then be methylated in vivo at the sulfur to give 6-methylmercaptopurine riboside-5′-phosphate (MMPRP), another active anabolite. These ribonucleoside monophosphates may be dephosphorylated in vivo to give the corresponding ribosides, 6-mercaptopurine riboside (MPR) and 6-methylmercaptopurine riboside (MMPR). This enzymatic dephosphorylation may be important in the development of clinical resistance to 6-MP [7]. It is important,

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therefore, that the analytical methodology devised should be capable of measuring both the nucleosides of 6-MP as well as the active nucleotides.

MATERIALS AND METHODS

Reagents

MPR, MMPR, other nucleosides, MPRP, acid phosphatase and 5′-nucleotidase were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium hydride, sodium methoxide, 2-mercaptoethanol, dimethylsulfoxide (DMSO) and methyl iodide were purchased from Aldrich (Milwaukee, WI, U.S.A.). Perchloric acid was obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.) and solvents were obtained from Baker (Phillipsburg, NJ, U.S.A.). Amberlite XAD-4 resin was purchased from Supelco (Bellefonte, PA, U.S.A.). Dideuterated nucleoside and nucleotide analogs were prepared as previously described [8].

Extraction from blood

Nucleosides and nucleotides were extracted from blood essentially by the method of Nelson et al. [9], which is itself a modification of Caldwell's procedure [10]. That is, one drop of 2-mercaptoethanol was added to 2.5 ml of blood to prevent oxidation of mercaptopurines. Five ml of 0.5 *M* perchloric acid solution were added to the 2.5 ml of whole blood and mixed thoroughly. The brown precipitate formed was removed by centrifugation and re-extracted with another 5 ml of 0.5 *M* perchloric acid. The perchloric acid extracts were combined and potassium hydroxide solution was added until the solution attained pH 6—6.5. The resulting precipitated potassium perchlorate was removed by centrifugation and washed with 5 ml of distilled water. The combined aqueous extracts were reduced in volume to ca. 1 ml.

Methanol-washed XAD-4 resin (40—80 mesh) was poured into a Pasteur pipette which contained a glass wool plug and had an attached reservoir. The column was then flushed with 20 ml of methanol followed by 40 ml of water. The 1-ml aqueous blood extract was added to the top of the XAD-4 column and the column was washed with 10 ml of distilled water to elute the nucleotide fraction. The column was then washed with 8 ml of a 20% aqueous ethanol solution. This eluted the nucleoside fraction which was then dried. To the eluted aqueous nucleotide fraction were added about 3 units of acid phosphatase and the solution was incubated at 37°C for 15 min. The solution was then poured onto another XAD-4 Pasteur pipette column. The column was washed with 10 ml of distilled water. The nucleosides which had been enzymatically produced from the nucleotides were then removed from the column with 8 ml of 20% aqueous ethanol and the eluate was dried.

Methylation of nucleosides

Nucleosides were either methylated by the methylsulfinyl carbanion/methyl iodide method [11] or by the use of sodium methoxide and methyl iodide. That is, for the latter method, the nucleosides were dissolved in 500 μl of DMSO in a 4-ml sample vial, and to this solution was added ca. 0.5 mg of sodium methoxide powder. After mixing well with shaking, 50 μl of methyl iodide were added. The vial was tightly closed with a PTFE-lined cap and

heated with shaking in a steam bath for 5 min. The reaction was then stopped by the careful addition of 500 μl of distilled water. The derivatized nucleosides were then extracted with 500 μl of chloroform which was subsequently washed with water to remove DMSO and dried with granular anhydrous sodium sulfate. If desired, the final volume of chloroform was reduced with a nitrogen stream before GC—MS analysis. This methylation procedure is essentially that which Bryant and Klein [12] used for the N-methylation of purines and pyrimidines.

GC—MS analysis

The permethylated nucleosides were analyzed on a Dupont 21-492 double-focusing mass spectrometer, equipped with a chemical ionization source and a four-channel selected ion monitoring system, and interfaced to a Varian 2740 gas chromatograph via an all-glass system with a jet separator. Methylated nucleosides were admitted to the mass spectrometer for selected ion monitoring via a 6-ft glass column packed with 3% OV-101 or 3% OV-17 on Supelcoport. Operating temperatures were: injection port, 280°C; column, 260°C; GC—MS interface and jet separator, 280°C; MS source, 220°C.

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed on Baker-flex silica gel IB2-F precoated sheets with 0.5 *N* ammonium sulfate–95% ethanol (1:1) as eluent. R_F values for MPRP and MPR were 0.68 and 0.75, respectively.

RESULTS AND DISCUSSION

The overall extraction and analysis scheme for nucleosides and nucleotides from tissue such as blood is outlined in Fig. 1. Nucleotides and nucleosides are rapidly separated from each other by XAD-4 resin [13] as illustrated in Fig. 2, which shows the elution of a spiked blood extract containing MPRP, MPR and MMPR. MPRP was eluted with ca. 10 ml of distilled water followed by elution of MPR with ca. 8 ml of 20% ethanol—water and finally elution of MMPR with 50% ethanol—water. The elution profiles were followed by passing the eluate through a quartz flow-cell placed in a Coleman 101 variable-wavelength UV spectrophotometer. MPRP and MPR were monitored at 321 nm while MMPR was monitored at 293 nm. By using 50% aqueous ethanol instead of 20%, the two ribonucleosides MPR and MMPR can be eluted together if desired.

The nucleoside monophosphates eluted from the XAD-4 column were cleaved to their ribonucleosides by incubation with acid phosphatase which was added directly to the eluate. Cleavage is complete after 15 min at 37°C, as determined by TLC. Although pH 4 is optimum for acid phosphatase activity and the eluate can be adjusted to this pH if desired, the enzyme is still active enough at more neutral pH to effectively cleave all the nucleoside monophosphate. Therefore, since elution of nucleotides was by distilled water, pH adjustment was not necessary. Note that buffers are also avoided. There is the distinct possibility that acid phosphatase may not be specific for monophosphates and can cleave higher phosphates to the riboside which would inflate the measurement of the amount of monophosphate present. Hence, instead of using acid phosphatase we have used 5′-nucleotidase, which is a much more

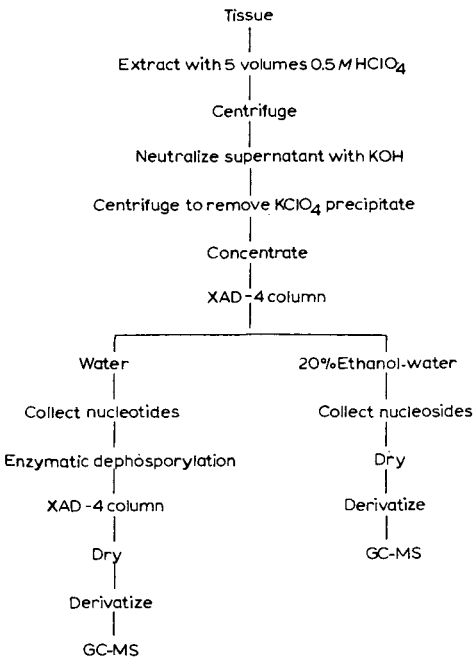

Fig. 1. Extraction, separation, derivatization and analysis scheme for tissue nucleosides and nucleotides.

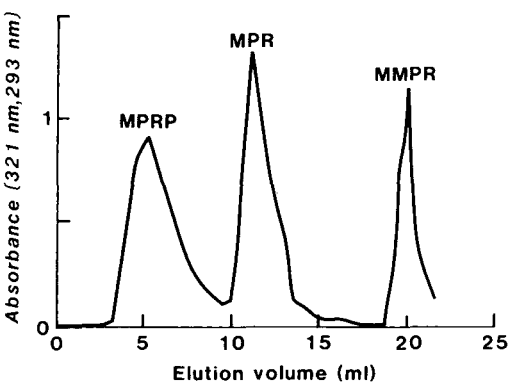

Fig. 2. Separation of mercaptopurine nucleosides and nucleotides on a 0.5 cm × 4.0 cm bed of Amberlite XAD-4 resin. Mercaptopurine riboside-5′-monophosphate (MPRP), mercaptopurine riboside (MPR), and methylmercaptopurine riboside (MMPR) were added to whole blood and extracted with perchloric acid. The neutralized, concentrated extract (1.5 ml) was applied to the column and eluted with 10 ml of distilled water, followed by 8 ml of 20% ethanol—water and finally with 50% ethanol—water. MPRP and MPR were monitored at 321 nm while MMPR was monitored at 293 nm.

specific enzyme. pH 9 is optimal for this enzyme but again it is active enough to easily cleave all the 5′-monophosphate to nucleoside at neutral pH and at 37°C for 15 min.

After enzymatic dephosphorylation, the resulting nucleoside was very effectively cleaned up for permethylation derivatization by passing the solution through another small XAD-4 column. After adding the solution to the column, it was first washed with distilled water which removed water-soluble material from the column before the nucleosides were removed with 20% aqueous ethanol.

Permethylation can be accomplished by the methylsulfinyl carbanion/methyl iodide procedure [11]. This is an efficient reaction for most nucleosides. However, the overall derivatization procedure is quite time-consuming since a total of 2 h is required for the overall reaction before extraction of the product. Further, fresh reagent solution must be prepared at intervals. We therefore investigated the possibility of using a simpler methylation procedure. Bryant and Klein [12] used sodium methoxide and methyl iodide in DMSO to N-methylate purine and pyrimidine bases in a convenient and rapid procedure. We have found that the same procedure is effective for the permethylation of nucleosides. The methylation yield was not quite as consistently good as with the methylsulfinyl carbanion method as illustrated in Fig. 3. The gas chromatograms are of an equimolar mixture (ca. 1 μg/μl) of uridine, deoxyadenosine, adenosine and MPR derivatized by the two permethylation procedures. The overall yield of these reactions, including extraction, for MPR relative to a standard solution of permethylated MPR was approximately 80% for the methylsulfinyl carbanion method and about 65% for the sodium methoxide method. However, the convenience and speed of the latter method may outweigh the slightly lower derivatization yield. The sodium methoxide method generally worked as well as the methylsulfinyl carbanion method with adenosine, deoxyadenosine, thymidine, uridine, cytidine and deoxycytidine, MPR, and 5-fluorodeoxyuridine (5-FU), but not with guanosine or deoxyguanosine.

Permethylated nucleosides were analyzed by GC—MS using chemical ioniza-

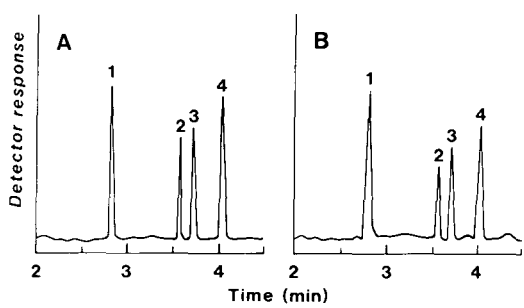

Fig. 3. Gas chromatograms of a mixture of uridine (1), deoxyadenosine (2), adenosine (3), and mercaptopurine riboside (4), derivatized by the methylsulfinyl carbanion/methyl iodide method (A) and the sodium methoxide—methyl iodide method (B), respectively. The traces are recordings of the total mass spectrometric ion current obtained when the derivatized mixtures were injected on a 6-ft. glass column packed with OV-101 and operated at 180—300°C at 20°/min.

tion and selected ion monitoring of the protonated molecular ions [14]. Although isobutane was routinely used for chemical ionization it is worth noting that the response of the protonated molecular ion was decreased to 63% when methane was used as the reagent gas. When ammonia was used the response increased to 143% relative to isobutane. Therefore, ammonia appears to be the most suitable reagent gas for this type of analysis.

Fig. 4 is an illustration of the effectiveness of the overall extraction, derivatization and analysis procedure. 12.5 nmoles (4.8 μg) of MPRP-5',5'-d_2 were added to 5 ml of whole blood along with 5 nmoles (1.8 μg) of MPRP. This represented a concentration of MPRP of 1 nmole/ml or 1 μM. After extraction and derivatization, which was carried out on two separate 2.5-ml blood samples, 2 μl were taken from a final combined volume of 20 μl of chloroform and injected on the GC—MS system to produce the SIM trace of Fig. 4C. Traces for standard permethylated MPR-5',5'-d_2 (Fig. 4B) and an extracted and derivatized blood blank (Fig. 4A) are also illustrated. It should be noted that the response for 1 nmole/ml from 5 ml of whole blood was obtained with only 10% of the final solution, without the instrument being set at its limit of sensitivity (noiseless baseline), and using the slightly less sensitive reagent gas isobutane. Although this was an extraction from a spiked blood sample and may not reflect the real situation, it is apparent that the nM range is within easy reach.

We are currently pursuing a number of modifications of the procedure discussed to increase the overall sensitivity and to minimize the time of analysis.

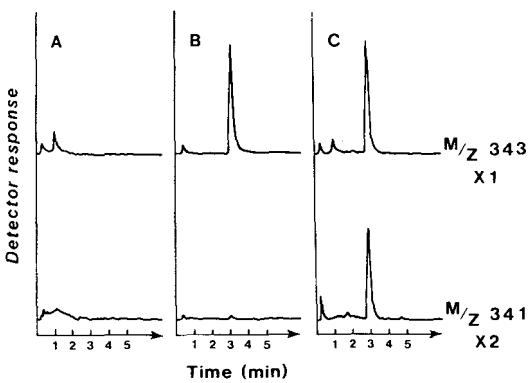

Fig. 4. GC—MS SIM traces of m/z 341 and 343. (A) Extracted blood blank; (B) dideuterated mercaptopurine riboside monophosphate (MPRP-5',5'-d_2) enzymatically dephosphorylated and permethylated by the sodium methoxide—methyl iodide procedure; (C) 1 nmole/ml of MPRP with 2.5 nmoles/ml of MPRP-5',5'-d_2 added to 5 ml of whole blood and extracted, chromatographed, derivatized (sodium methoxide—methyl iodide method), and analyzed as described in the text.

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REFERENCES

1 J.A. McCloskey, in P.O.P. Ts'o (Editor), Basic Principles in Nucleic Acid Chemistry, Vol. 1, Academic Press, New York, 1974, Ch. 3.
2 F.C. Falkner, B.J. Sweetman and J.T. Watson, Appl. Spectrosc. Rev., 10 (1975) 51.
3 P.R. Brown, High Pressure Liquid Chromatography — Biochemical and Biomedical Applications, Academic Press, New York, 1973.
4 R.A. Henry, J.A. Schmidt and R.C. Williams, J. Chromatogr. Sci., 11 (1973) 358.
5 A.R.P. Paterson and D.M. Tidd, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Part II, Springer-Verlag, New York, 1974, pp. 384—403.
6 G.B. Elion and G.H. Hitchings, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Part II, Springer-Verlag, New York, 1974, pp. 404—425.
7 A.C. Sartorelli, M.H. Lee, M. Rosman and K.C. Agrawal, Pharmacological Basis of Cancer Chemotherapy, Williams and Wilkins, Baltimore, MD, 1975, p. 643.
8 I. Jardine and M.M. Weidner, J. Labelled Compds. and Radiopharm., in press.
9 D.J. Nelson, C.J.L. Bugge, H.C. Krasny and T.P. Zimmerman, J. Chromatogr., 77 (1973) 181.
10 L.C. Caldwell, J. Chromatogr., 44 (1969) 331.
11 D.L. von Minden and J.A. McCloskey, J. Amer. Chem. Soc., 95 (1973) 7480.
12 W.F. Bryant and P.D. Klein, Anal. Biochem., 65 (1975) 73.
13 T. Uematsu and R.J. Suhadolnik, J. Chromatogr., 123 (1976) 347.
14 C. Pantarotto, A. Martini, G. Belvedere, A. Bossi, M.G. Donelli and A. Frigerio, J. Chromatogr., 99 (1974) 519.

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Biomedical Applications

CHROMBIO. 563

Note

Detection of tuberculostearic acid in biological specimens by means of glass capillary gas chromatography—electron and chemical ionization mass spectrometry, utilizing selected ion monitoring

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Tuberculostearic acid [(*R*)-10-methyloctadecanoic acid] is regarded a characteristic constituent of microorganisms of the order *Actinomycetales*. By using selected ion monitoring (SIM), the acid has been demonstrated to be present in several mycobacterial and nocardial species [1—3], and in sputum specimens from patients with pulmonary tuberculosis [2, 3]. The latter observation implies a possible way of using SIM for the diagnosis of such infections.

When using electron ionization (EI) mass spectrometry (MS), SIM allows the methyl ester of tuberculostearic acid to be determined quantitatively down to approximately 20 pg, monitoring at *m/z* 312 (= M) [1]. In earlier studies [3] it was sometimes found that sputum specimens from patients with pulmonary tuberculosis contained tuberculostearic acid in amounts close to the detection limit of the instrument. When such small amounts of the acid are present in clinical specimens, there is no proof of identity other than the chromatographic retention time. Therefore it is important that the gas chromatographic (GC) system used should possess optimal separation qualities.

In the present investigation we assessed the gain in sensitivity in the detection of tuberculostearic acid by using EI—SIM analysis of the *tert.*-butyldimethylsilyl (t-BDMS) ester [4], as compared with the corresponding methyl ester [1—3]. The former derivative yields spectra having ions of a very high intensity at *m/z* $(M - 57)^+$ upon electron impact [4]. Furthermore, we investigated the chemical ionization (CI) mass spectra of methyl tuberculostearate, using methane and isobutane as reactant gases, and compared the sensitivity

for its detection with that of EI. The GC system developed uses glass capillary columns to optimize the separation of various methyl-branched C_{18} positional isomers possibly present in clinical specimens.

EXPERIMENTAL

Organisms

One strain of *Mycobacterium tuberculosis*, H37Rv, was tested.

Sputum samples

Sputum specimens (2—4 ml) were collected from eight patients with pulmonary tuberculosis. All sputa contained acid-fast rods detectable by microscopic studies of Ziehl-Nelseen stained smears. For comparison, sputum samples from six patients with non-tuberculous pneumonia were also used.

Standards and reagents

The solvents and reagents used were analytical grade. Three per cent methanolic hydrochloric acid was prepared by adding 5 ml of acetyl chloride to 100 ml of dry methanol. 2- and 10-methyloctadecanoic acids were synthesized [3], while 16- and 17-methyloctadecanoic acids were purchased from Larodan Lipids (Malmö, Sweden). Imidazole, N,N-dimethylformamide and t-BDMS chloride were obtained from Merck (Darmstadt, G.F.R.), Mallinckrodt (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland), respectively.

Preparation of organisms and sputum specimens

The strain of *M. tuberculosis* studied was cultured on Proskauer-Beck medium, autoclaved, washed and lyophilized as previously described [5].

The sputum specimens were digested with 5 ml of aqueous sodium hydroxide (4% v/v) at 37°C for 15 min with occasional shaking. The pH was then adjusted to 7.0 with diluted hydrochloric acid and the preparations autoclaved and lyophilized.

Extraction and derivatization procedures

The reference acids were esterified using 3% methanolic hydrochloric acid at 80°C for 20 h. Also, 10-methyloctadecanoic acid (tuberculostearic acid) was derivatized to its t-BDMS ester as previously reported [4].

The lyophilized sputum specimens and mycobacterial cells were extracted using 2 ml of chloroform—methanol (2:1, v/v) at room temperature overnight. The organic solutions were then evaporated to dryness and the residues esterified using 2 ml of methanolic hydrochloric acid as described above. The methanolic phases were evaporated to dryness, 100 μl of *n*-hexane were added and the solution used for GC—mass spectrometric (MS) analysis.

In some studies, 4 μg of lyophilized cells of *M. tuberculosis* were added to each of two sputum specimens from patients with non-tuberculous pneumonia before being lyophilized and derivatized.

Gas chromatography—mass spectrometry

A Varian MAT Model 112 GC—MS combination, equipped with a glass capil-

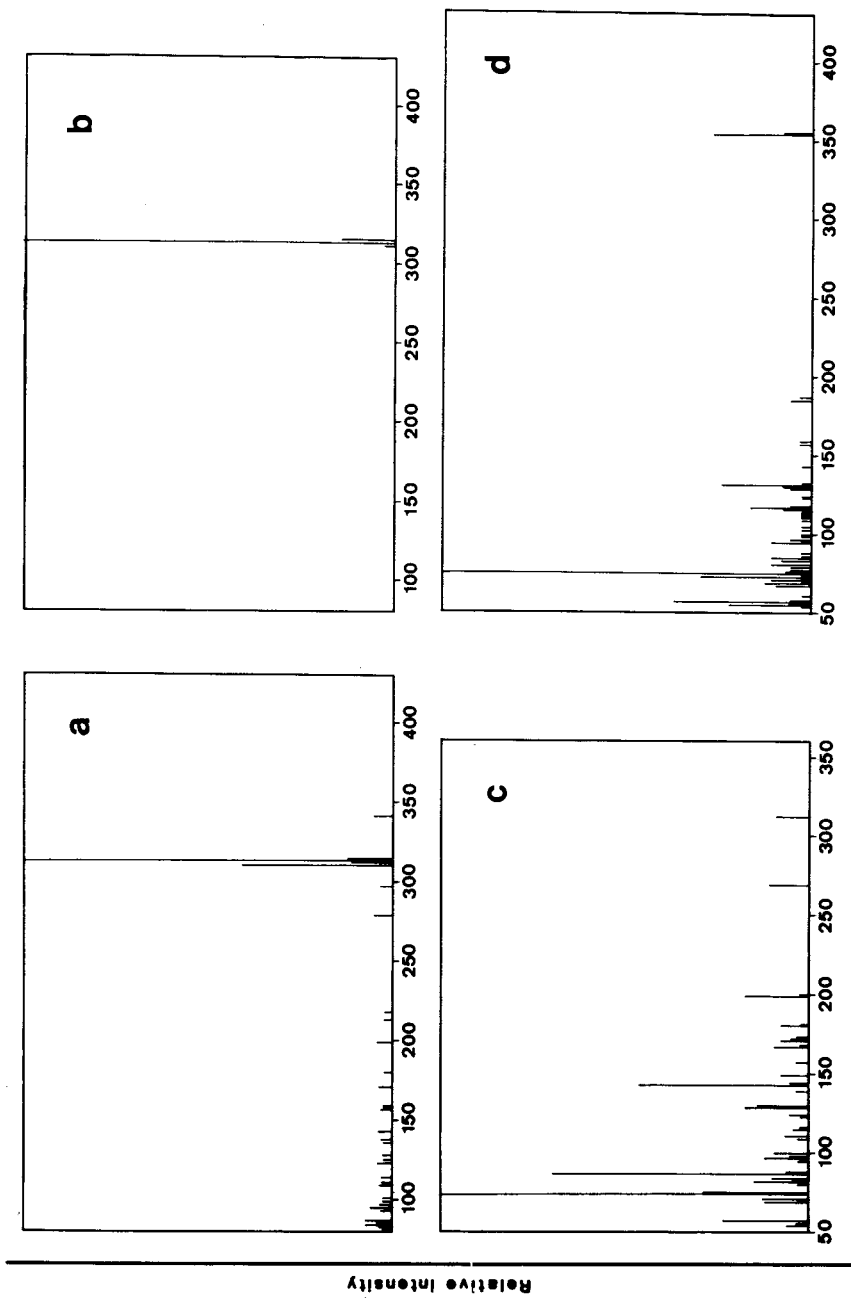

Fig. 1. Mass spectra of derivatized tuberculostearic acid. (a) CI methane spectrum of methyl ester; (b) CI isobutane spectrum of methyl ester; (c) EI spectrum of methyl ester; (d) EI spectrum of t-BDMS ester. The esters were prepared from the corresponding free acids as described in the text. See also text for specifications of GC—MS conditions.

lary column (25 m × 0.25 mm I.D.) coated with OV-17, was used in all EI studies. The analyses were performed using both split (split ratio 1:20) and splitless injections. The nitrogen carrier gas flow-rate was 2 ml/min through the column. The column temperatures were 210 and 240°C for the analysis of methyl and t-BDMS esters, respectively. The electron energy was 70 eV and the ion source temperature 250°C.

Studies using CI were carried out on a Finnigan Model 4021 GC—MS system, equipped with a 20-m glass capillary column coated with SE-30. Injections were performed at a split ratio of 1:25 and the temperature of the column was programmed from 80 to 230°C using a temperature increase of 20°/min. The carrier gas flow-rate (nitrogen) was 2 ml/min and the electron energy 150 eV at 0.3 torr of reactant gas. The ion source temperature was 250°C.

The SIM analyses were carried out using single ion detection monitoring at *m/z* 312 (EI) and *m/z* 313 (CI) when studying the methyl esters, and at *m/z* 355 (EI) for the t-BDMS ester.

RESULTS AND DISCUSSION

Mass spectra

Mass spectra of derivatized tuberculostearic acid, using CI and EI, are shown in Fig. 1. In the high mass range, the spectra of the methyl ester obtained by CI are dominated by the intense ion of *m/z* 313 $(M + 1)^+$ (Fig. 1a, b). Their relative abundance is considerably larger than at *m/z* 312 $(M)^+$, using EI (Fig. 1c). Variations of the reactant gas in the CI experiments caused only minor changes in the corresponding spectra (Fig. 1a, b). The spectrum representing the t-BDMS ester exhibited an intense ion at *m/z* 355 (Fig. 1d).

Estimation of detection limit

When the EI—SIM technique was employed, the detection limit of the methyl ester of tuberculostearic acid (monitored at *m/z* 312) was 5 to 10 times higher than that of the t-BDMS ester (monitored at *m/z* 355). Roughly the same increase in sensitivity was gained when focusing at *m/z* 313 $(M + 1)^+$, employing isobutane as reactant gas in the CI—SIM mode. The signal-to-noise ratio when using isobutane was approximately twice that found when employing methane. The detection limit was estimated to approximately 1 pg both for the methyl ester as detected by CI—SIM (isobutane) and for the t-BDMS ester, using EI.

Separation of C_{19} isomeric fatty acids

Of the isomeric C_{19} acids present in bacterial cells, the 2-, 16-, and 17-methyl-substituted C_{18} acids, and the straight-chain C_{19} acid, can be expected to be by far the most common. Fig. 2 shows a fragmentogram obtained by single ion detection, monitored at *m/z* 312 (EI) of the corresponding methyl esters, including methyl tuberculostearate. The analysis was carried out using a wall-coated glass capillary column with split injection, employing OV-17 as stationary phase. No difficulties were encountered in establishing the identity of methyl tuberculostearate by the GC retention time.

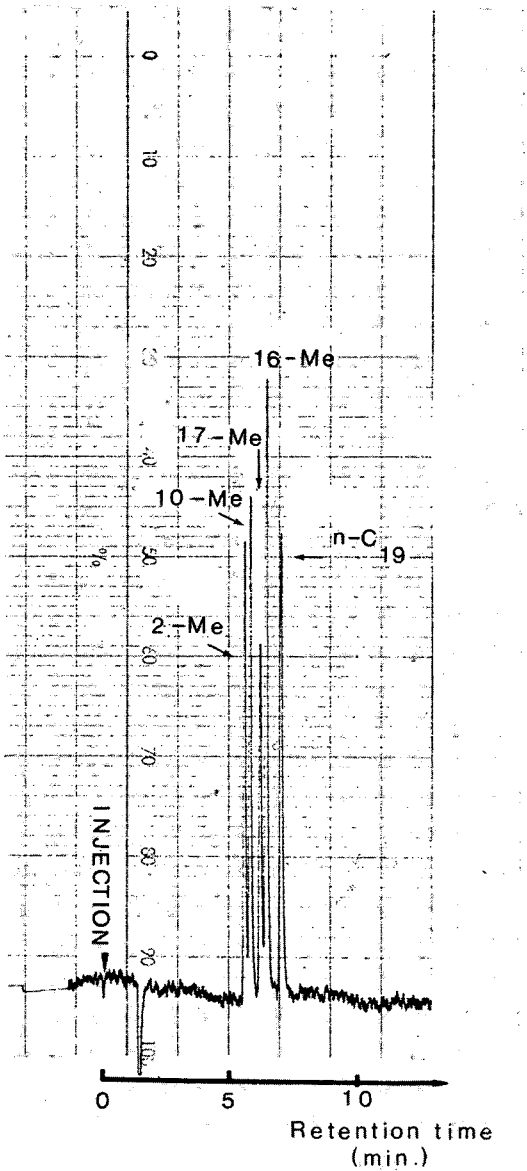

Fig. 2. EI mass fragmentogram of a mixture of equal amounts of the methyl esters of 2-, 10-, 16-, and 17-methyloctadecanoic acid and *n*-nonadecanoic acid monitored at *m/z* 312. A 25-m glass capillary column with OV-17 as stationary phase was used at an isothermal column temperature of 210°C. Each peak represents about 300 pg of methyl ester.

Analysis of sputum specimens

Mass fragmentograms representing sputa from patients with tuberculosis (Fig. 3a) and with pneumonia with no evidence of tuberculosis (Fig. 3c), employing EI—SIM of methylated samples, monitoring at *m/z* 312, are shown. For comparison, a mass fragmentogram of one of the latter specimens to which

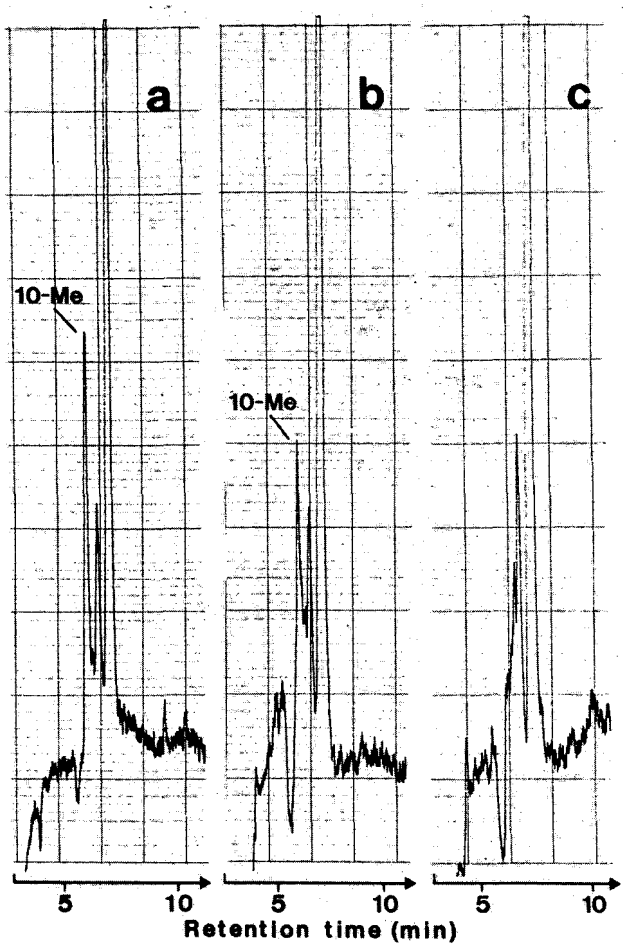

Fig. 3. EI mass fragmentograms of sputum specimens from a patient with tuberculous (a) and non-tuberculous pneumonia supplemented (b) and not supplemented (c) with 50 pg of authentic tuberculostearic acid, using splitless injections. The sputum specimens had been digested and derivatized as described in the text. The fragmentograms were recorded on a 25-m glass capillary column coated with OV-17 using single ion detection, monitoring at *m/z* 312.

50 pg of authentic tuberculostearic acid had been added is also shown (Fig. 3b). The fragmentograms obtained in CI—SIM analyses, focusing at *m/z* 313 $(M + 1)^+$, were similar, although the use of CI gave a far better sensitivity than EI. All analyses were carried out with splitless injection to avoid loss of material. Tuberculostearic acid was demonstrated in sputum samples from eight patients with pulmonary tuberculosis but not in sputa from six patients with non-tuberculous pneumonia.

The analytic procedure used seems well fitted for quantitative determination of tuberculostearic acid present in such a complex biological material as a sputum specimen. Thus it was found that the mass fragmentographic peak of methyl tuberculostearate in analyses of sputa from non-tuberculous patients

to which a given amount (4 μg) of mycobacteria (*M. tuberculosis*) had been added, was in size equal to that found when analysing the same amount of isolated bacteria of this species. The significance of the demonstration of tuberculostearic acid in clinical specimens for the rapid diagnosis of tuberculosis and other mycobacterial infections, is at present under evaluation.

The present investigation indicates that a glass capillary column system with OV-17 as stationary phase can be used to advantage for the determination of tuberculostearic acid in biological specimens. Furthermore, the detection sensitivity of the system can be enhanced by a factor of 5 to 10 by using EI of the t-BDMS ester or CI of the methyl ester, compared with analyses employing EI of the latter derivatives in SIM analyses. However, preparation of the t-BDMS ester is more laborious than methylation, and also seems to give lower yields. The almost exclusive formation of quasimolecular ions in the CI mode gives fragmentograms not complicated by additional fragments from cleavage of other ionized large molecules present in the sample.

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REFERENCES

1 L. Larsson, P.-A. Mårdh and G. Odham, J. Chromatogr., 163 (1979) 221.
2 G. Odham, in G.R. Waller (Editor), Biochemical Applications of Mass Spectrometry, First Suppl. Vol. Wiley-Interscience, New York, 1980, Ch. 8, in press.
3 G. Odham, L. Larsson and P.-A Mårdh, J. Clin. Invest., 63 (1979) 813.
4 G. Phillipou, D.A. Bigham and R.F. Seamark, Lipids, 10 (1975) 714.
5 L. Larsson and P.-A. Mårdh, J. Clin. Microbiol., 3 (1976) 81.

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Biomedical Applications

CHROMBIO. 529

Note

Automated ion-exchange chromatography in the detection of aspartylglucosaminuria

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Aspartylglucosaminuria (AGU) is an inherited neurovisceral storage disease associated with mental retardation and resulting from low activity of a lysosomal enzyme, 4-L-aspartylglycosylamine amidohydrolase (EC 3.5.1.26) [1—3]. Up to 14 different glycoasparagines have been found [4—7] in the urine of AGU patients. All glycoasparagines have a common structure of β-Gal-(1→4)-β-GlcNAc-Asn [8] and are homogeneous as judged by paper chromatography [9]. The main abnormal urinary metabolite in affected persons is aspartylglucosamine, i.e. 2-acetamido-1-N-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (GlcNAc-Asn) [9]. Screening for AGU in clinical laboratories takes place by demonstration of the presence of GlcNAc-Asn in urine either by gas chromatography—mass spectrometry [8, 10—13], gel chromatography [6, 9], paper chromatography and paper electrophoresis [4, 7, 9], thin-layer chromatography (TLC) [14, 15], or enzymatic determinations [16].

Since amino acid analysis is the most common method used for diagnosis of inborn errors of metabolism it is important that also AGU can be detected with an amino acid analyzer. It can not directly be used for the detection of AGU, though, because the glycoasparagines cannot be separated from other fast-eluting, highly acidic amino acids and peptides while using the lithium citrate buffer system for physiological fluids [17]. However, when the interfering compounds are removed by TLC before analysis, the suspected peaks become clearly detectable with slight modifications of the routine procedure [18]. The present paper describes a sensitive and relatively simple method for the determination of glycoasparagines in the urine of AGU patients.

EXPERIMENTAL

Samples

Urine samples from patients with AGU were obtained from Tampere Central

Hospital, Tampere, Finland (one male), and from Rinnekoti Institution for the Mentally Retarded, Majalampi, Finland (one female). The clinical findings included deepening mental retardation and structural abnormalities. The diagnosis was confirmed by the demonstration of aspartylglucosamine in the urine by various chromatographic and electrophoretic techniques.

Thin-layer chromatography

One hundred microlitres of urine were applied between the spots of a reference solution of 2-acetamido-1-N-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (Vega-Fox Biochemicals, Tucson, AZ, U.S.A.) on silica gel plates (Merck DC Alufolien Kieselgel 60; Merck, Darmstadt, G.F.R.). The plate was developed in *n*-butanol—acetic acid—water (50:25:25) for a distance of 10 cm. A glass plate was placed to cover the urine sample area and the TLC plate was sprayed with ninhydrin (Merck). The plate was heated as described by Palo and Savolainen [14, 15]. The area on the chromatogram of the urine sample corresponding to the reference substance was scraped off and collected into a conical centrifuge tube. The materials were extracted with 1 ml of distilled water. After centrifugation at 1000 *g* the supernatant was evaporated to dryness at 343°K with a rotary vacuum evaporator.

Amino acid analysis

The sample was dissolved in 100 μl of sample dilution buffer (lithium citrate, pH 2.2) [19]. Fifty microlitres of the sample were injected into a Beckman Multichrom M amino acid analyzer. Lithium citrate buffer (Durrum Pico-Buffer System IV, Pierce Eurochemie, Rotterdam, The Netherlands) was used according to the method of Perry et al. [17] with resin AA 20 from Beckman (Munich, G.F.R.) in a 0.4 cm × 24 cm water-jacketed glass column at a temperature of 305°K. The pH of the buffer A was lowered to 2.5 with concentrated HCl. The ninhydrin reagent [19] and buffer flow-rates were both 10 ml/h. One analysis lasted 90 min, including 25 min regeneration with lithium hydroxide and a 40-min equilibration period with the modified buffer A.

RESULTS AND DISCUSSION

In a laboratory concerned with the amino acid analysis of urine specimens it is customary to run either paper or thin-layer chromatograms of samples before the automated amino acid analysis. This reveals any qualitative or quantitative abnormalities in ninhydrin-staining properties of the spots. Aspartylglucosamines give a grayish-blue or grayish-green colour, and thus the absorbance at 440 nm is higher than at 570 nm. Most amino acids (with the exception of proline and hydroxyproline) have a higher absorbance at 570 than at 440 nm as do the other reference substances that elute in the region of very acidic amino acids (Fig. 1).

Fig. 2 illustrates an amino acid analysis with aspartylglucosamines from the urine of a patient with AGU. The glycoasparagines appear as two fairly broad peaks with retention times of 10.5 and 23.5 min. The latter peak includes 1-N-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine, as assessed by co-migration of the reference substance, but also other related glycoasparagines (Fig. 2C).

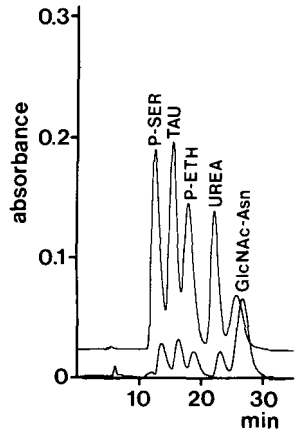

Fig. 1. Amino acid analysis of a reference mixture containing 100 μmol/l orthophosphoserine (P-Ser), taurine, ethanolamine phosphate (P-Eth) and 5 mmol/l urea, together with 0.1 g/l 2-acetamido-1-N-(4'-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (GlcNAc-Asn). The absorbances are measured at 570 nm (upper trace) and 440 nm (lower trace). The improved resolution is due to reduced buffer flow and pH.

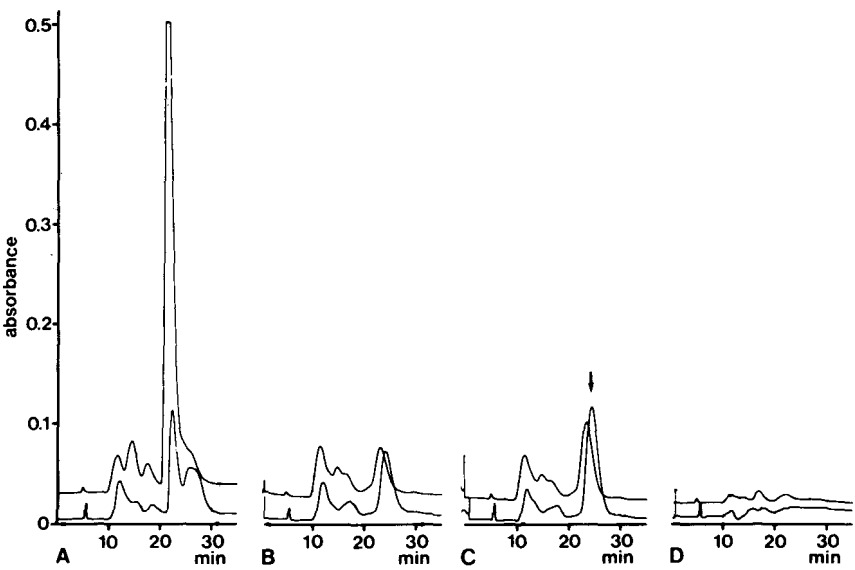

Fig. 2. Amino acid analysis of urine specimens. (A) Sample of a patient with aspartylglucosaminuria. (B) The same sample as in A after removal of interfering substances by TLC as described in the text. The aspartylglucosamines appear as two peaks with retention times of 10.5 and 23.5 min. (C) The same sample as in B with 5 μg of added GlcNAc-Asn (arrow). (D) A normal urine sample after the TLC procedure.

The structural identity of the first peak has not yet been established. Using Beckman Unichrom, Palo and Mattsson [20] detected only one peak in urine. It was close to the taurine peak, but it could not be detected in one brain biopsy specimen of a patient with AGU [21], although at the beginning of this amino acid chromatogram there are two unidentified peaks which could contain the first eluting glycoasparagine.

The procedure presented above is applicable to the analysis of glycoasparagines present in the urine of AGU patients. After co-migration with the reference compound in two different chromatographic systems the substances are most likely identical. The linear range of this procedure is from 0.01 to 1.6 g/l. If that amount is exceeded the sample must be further diluted with the sample dilution buffer (pH 2.2). The sensitivity of the amino acid analyzer or the smallest amount which can be visualized on TLC is 0.01 g/l, which is well exceeded by the GlcNAc-Asn content of urines determined by Maury [13] to range from 0.14 to 0.28 g/l. The recovery of the reference compound from thin-layer plates analysed by the described method was 73 ± 13% (S.D.; n = 10). Since many clinical laboratories do not have access to gas chromatograph—mass spectrometer systems for their definitive identification and quantitation, the method described above is easily applicable to determinations of glycoasparagines in biological specimens of patients with suspected aspartylglucosaminuria.

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REFERENCES

1 J. Palo, Thesis, University of Helsinki, 1966.
2 J. Palo, Acta Neurol. Scand., 43 (1967) 573.
3 F.A. Jenner and R.J. Pollitt, Biochem. J., 103 (1967) 48P.
4 J. Palo, R.J. Pollitt, K.M. Pretty and H. Savolainen, Clin. Chim. Acta, 47 (1973) 69.
5 K. Sugahara, S. Funakoshi, I. Funakoshi, P. Aula and I. Yamashina, J. Biochem. (Tokyo), 78 (1975) 673.
6 K. Sugahara, S. Funakoshi, I. Funakoshi, P. Aula and I. Yamashina, J. Biochem. (Tokyo), 80 (1976) 195.
7 R.J. Pollitt and K.M. Pretty, Biochem. J., 141 (1974) 141.
8 M. Akasaki, K. Sugahara, I. Funakoshi, P. Aula and I. Yamashina, FEBS Lett., 69 (1976) 191.
9 A. Lundblad, P.K. Masson, N.E. Nordén, S. Svensson, P.-A. Öckerman and J. Palo, Eur. J. Biochem., 67 (1976) 209.
10 K. Sugahara, M. Akasaki, I. Funakoshi, P. Aula and I. Yamashina, J. Biochem. (Tokyo), 82 (1977) 1499.
11 K. Sugahara, M. Akasaki, I. Funakoshi, P. Aula and I. Yamashina, FEBS Lett., 78 (1977) 284.
12 P. Maury and J. Kärkkäinen, Clin. Chim. Acta, 91 (1979) 75.

13 P. Maury, J. Biol. Chem., 254 (1979) 1513.
14 J. Palo and H. Savolainen, J. Chromatogr., 65 (1972) 447.
15 J. Palo and H. Savolainen, Ann. Clin. Res., 5 (1973) 156.
16 K. Sugahara, K. Nishimura, P. Aula and Y. Yamashina, Clin. Chim. Acta, 72 (1976) 265.
17 T.L. Perry, D. Stedman and S. Hansen, J. Chromatogr., 38 (1968) 460.
18 P. Kontro, K.-M. Marnela and S.S. Oja, Brain Res., 184 (1980) 129.
19 Beckman Multichrom-M Accelerated Micro Amino Acid Analyzer Instruction Manual, Beckman Instruments GmbH, Munich, G.F.R., 1975.
20 J. Palo and K. Mattsson, J. Chromatogr., 50 (1970) 534.
21 J. Palo, P. Riekkinen, A.U. Arstila and S. Autio, Neurology, 21 (1971) 1198.

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Biomedical Applications

CHROMBIO. 556

Note

Rapid high-performance liquid chromatographic method for quantitation of 3-methylhistidine

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The amino acid 3-methylhistidine is closely associated with skeletal muscle metabolism. The vast majority of the 3-methylhistidine formed in the body is present in skeletal muscle [1]. Methylation of the histidine occurs after its incorporation into the peptide chains of actin and myosin [2—4] and after catabolism of these proteins the liberated 3-methylhistidine is not recycled but quantitatively excreted in the urine of animals [5] and man [6]. The total amount of 3-methylhistidine excreted in the urine has therefore been proposed as an index of muscle protein catabolism [5].

Previous analyses for 3-methylhistidine in biological materials have predominately relied upon ion-exchange chromatography [7] and automatic amino acid analysis [8—10].

The importance of 3-methylhistidine as an index of muscle protein turnover shares considerable interest by many investigators, however, the time required for the analysis of 3-methylhistidine is relatively long and so it limits the application of this method for clinical studies. This report describes a simple, rapid high-performance liquid chromatographic (HPLC) technique which utilizes a reversed-phase separation with ion-pairing and post-column fluorescence derivatization for the analysis of 3-methylhistidine.

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EXPERIMENTAL

Materials

All reagents used were of highest purity available (A.C.S. certified grade). Boric acid, β-mercaptoethanol, potassium hydroxide (Fisher Scientific, Pittsburgh, PA, U.S.A.), *o*-phthalaldehyde (Eastman Kodak, Rochester, NY, U.S.A.), Brij 35, 30% solution (Pierce, Rockford, IL, U.S.A.), and ethanol (U.S. Industrial Chemicals, New York, NY, U.S.A.), were used in preparing the derivatization reagent. L-3-Methylhistidine, L-1-methylhistidine and L-histidine were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium hexanesulfonate was provided from the Peptide Synthesis Group (Massey University, Palmerston North, New Zealand).

Methods

The *o*-phthalaldehyde reagent was prepared by dissolving 30 g of boric acid in 1.0 l of deionized water, adjusting the pH to 10.4 with potassium hydroxide pellets, filtering through a 0.45-μm aqueous filter (Millipore Bedford, MA, U.S.A.) and adding 1.0 ml of 30% (w/v) aqueous solution of Brij 35, then transferring the solution into a dark glass bottle. Separately, a solution containing 600 mg of *o*-phthalaldehyde and 200 μl of β-mercaptoethanol in 10 ml of ethanol was added to the borate solution and stored under nitrogen. The reagent was prepared daily.

A stock solution of 5 m*M* sodium hexanesulfonate, pH 3.2 (pH adjusted with glacial acetic acid) in deionized water was degassed prior to use as the mobile phase in the chromatograph.

A standard solution of 3-methylhistidine was prepared in deionized water and analyzed by HPLC. When not in use, the solution was stored at 4°C.

Urine was collected from three children and one adult for 24 h in toluene and aliquots were stored at −15°C until analysis. Following the addition of 10% solution of trichloroacetic acid to the urine (1:9, v/v), the sample was centrifuged, the supernatant adjusted to pH 3.5 with 10% trichloroacetic acid and aliquots were injected into the chromatograph for the separation and quantitation of 3-methylhistidine.

Apparatus

A Model 6000A solvent delivery system and U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) were used with the HPLC system. The fluorescence detector was a Model 420 using an excitation filter of 340 nm and an emission filter of 440 nm (Waters Assoc.). The HPLC column used was stainless steel (300 × 4 mm I.D.) μBondapak C_{18}, particle size 10 μm, with a guard column, 2 in. long and 0.5 ml volume capacity, packed with Bondapak Phenyl/Corasil, particle size 37—50 μm and a post-column mixing chamber, all from Waters Assoc.

The emission of 440 nm was monitored at a chart speed of 5 mm/min on a 10-mV linear recorder B-5000 OmniScribe (Houston Instrument, Austin, TX, U.S.A.). The peak heights were measured.

A schematic diagram of post-column fluorescence derivatization is given in Fig. 1.

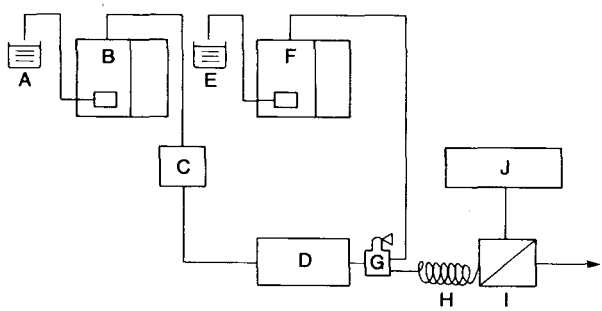

Fig. 1. Schematic diagram of post-column fluorescence derivatization system. A, reservoir (5 m*M* sodium hexanesulfonate); B, pump; C. injector; D, μBondapak C_{18} column; E, reservoir (*o*-phthalaldehyde); F, pump; G, mixing chamber; H, stainless-steel loop; I, fluorescence detector; J, recorder.

RESULTS AND DISCUSSION

The analysis of histidine and its metabolites in the presence of a large number of other materials present in urine, requires the addition of hydrophobic ion-pairing reagents to the mobile phase. At pH 3.2 hexanesulphonate significantly increases the retention of histidine, 1- and 3-methylhistidine. This increase is presumably due to ion-pairing of cationic groups formed at pH 3.2 from the α-amino- and imidazole N-groups in the sample molecules, with the hexanesulphonate in the mobile phase. Such an association has previously been shown to cause an increase in retention of amino acids on reversed-phase chromatography [11].

The use of sodium hexanesulphonate allows excellent resolution of histidine and the similar structural isomers 1- and 3-methylhistidine. This resolution is dependent, however, on the carbon-chain length of the sulphonate as the other members of the C_4—C_8 series did not give as good a separation, particularly in the urine samples.

3-Methylhistidine was identified on the basis of retention time by comparison with standards. As is shown in Fig. 2, good separation of 3-methylhistidine is obtained from other components present in human urine. For further confirmation of chromatographic peaks, work is in progress to utilize high-resolution mass spectrometry.

3-Methylhistidine added to urine was analyzed with good precision at concentrations comparable to those in samples of biological fluids. Repeated injections of 0.1—10.0 μg of 3-methylhistidine gave an overall coefficient of variation less than 5%. Recovery of 3-methylhistidine added to urine samples was at least 99%.

A linear response was observed for concentrations of 3-methylhistidine ranging from 100 ng to 10 μg injected into the column, thus, providing a more than adequate range for the analyses of 3-methylhistidine levels at concentrations found in biological materials (approximately 50—100 ng/μl urine).

The excellent sensitivity of this procedure is partly due to the fact that isocratic conditions can be used to separate histidine and its metabolites from the other urinary components. In addition, the analysis can be carried out

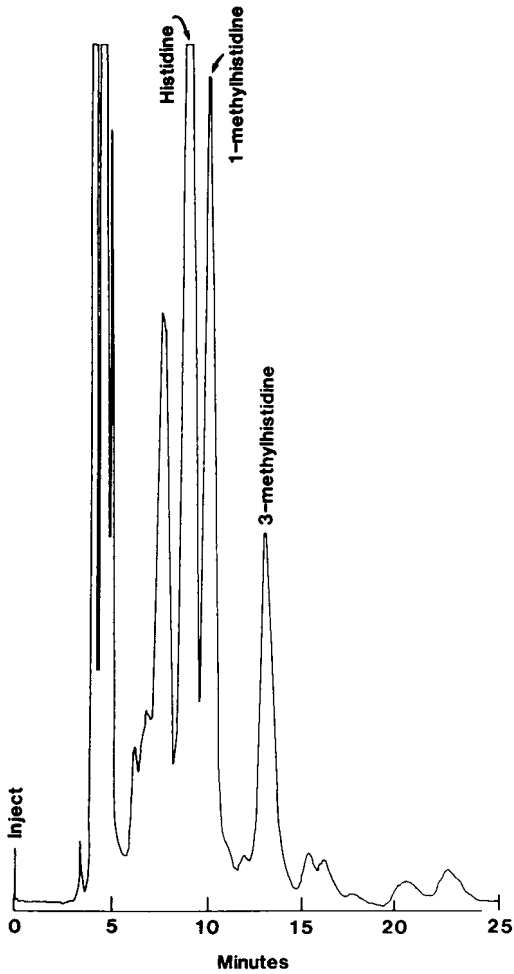

Fig. 2. Reversed-phase HPLC separation of 3-methylhistidine of urine. Sample injected, 5 μl; column, μBondapak C_{18} (300 × 4 mm I.D.) and mobile phase, 5 m*M* sodium hexanesulfonate at a flow-rate of 0.8 ml/min. Post-column derivatization occurs at the mixing chamber with *o*-phthalaldehyde (1 ml/min). Separation and quantification is via fluorescent detector Model 420 excitation 340 nm, emission 440 nm, and a linear chart recorder.

rapidly using the 30-min conditions shown in Fig. 2. Any non-polar contaminants which were retained on the column was washed with acetonitrile at the end of each day's analyses.

The reversed-phase partition mode of HPLC with post-column derivatization with *o*-phthalaldehyde and fluorescence detection for separation, provides a rapid (16-min baseline separation), highly sensitive, simple and quantitative method for the analysis of 3-methylhistidine at the nanogram level.

ACKNOWLEDGEMENTS

We are indebted to Dr. David Harding (Massey University) for synthesizing

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REFERENCES

1 L.N. Haverberg, P.T. Omstedt, H.W. Munto and V.R. Young, Biochim. Biophys. Acta, 405 (1975) 67.
2 M. Reporter, Biochemistry, 8 (1969) 3489.
3 M.F. Hardy and S.V. Perry, Nature (London), 223 (1969) 300.
4 V.R. Young, B.S. Baliga, S.D. Alexis and H.N. Munro, Biochim. Biophys. Acta, 199 (1970) 297.
5 V.R. Young, S.D. Alexis, B.S. Baliga and H.N. Munro, J. Biol. Chem., 247 (1972) 3592.
6 C.L. Long, V.R. Young, J.M. Kinney, H.N. Munro, L.N. Haverberg and J.W. Geiger, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 33 (1974) 691.
7 H.H. Tallan, W.H. Stein and S.J. Moore, J. Biol. Chem., 206 (1954) 825.
8 J.V. Benson, M.J. Gordon and J.A. Patterson, Anal. Biochem., 13 (1965) 265.
9 C.L. Long and J.W. Geiger, Biochem. Med., 12 (1975) 267.
10 I.B. Holbrook, E. Gross and M.H. Irving, Brit. J. Nutr., 41 (1979) 15.
11 C.M. Riley, E. Tomlinson, T.M. Jefferies and P.H. Redfern, J. Chromatogr., 162 (1979) 153.

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Biomedical Applications

CHROMBIO. 559

Note

Release of insulin analogues in man, stimulated with glucose

In vitro and in vivo studies

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The circulating insulin immunoreactive material was characterized by disc gel electrophoresis and it was found that the so-called proinsulin fraction was heterogenous [1—3]. Also, Haën et al. [4] reported that the heterogeneity of proinsulin-size immunoreactive insulin (IRI) in circulation was more marked than that of insulin-size material. Moreover, Kimmel and Pollock [5] and Elliott et al. [6] suggested the presence of an abnormal insulin in circulation.

The presence of two groups of insulin was reported in human serum after extraction and gel filtration in an earlier report [7]. The present studies were undertaken to elucidate the presence of the insulin analogues in an incubation medium and incubated human pancreas, and to clarify the levels of these insulin analogues in human sera, obtained from normal adults, well controlled diabetic and poorly controlled diabetic patients.

MATERIALS AND METHODS

Subjects

A piece of human pancreatic tissue was obtained from a patient with localized esophageal cancer (53 years old, male) and from one with gastric cancer (69 years old, male) as the surgical specimen.

Four healthy adults (age 34—70 years old, fasting blood sugar level: 89—96 mg/dl), four well controlled diabetics (age 47—71 years old, fasting blood sugar level: 118—149 mg/dl) and two poorly controlled diabetics (age 54 and 50 years old, fasting blood sugar levels: 203 and 228 mg/dl respectively) were studied. There were no significant differences in obesity among these three groups.

Incubation

About 50 mg (wet weight) of the pancreatic tissue was incubated for 10 min at 37°C (95% O_2–5% CO_2) in Krebs Henselite buffer solution containing 3.0 mg/ml of glucose. Afterwards, the pancreatic tissue was incubated in the medium containing 0.6 mg/ml of glucose for an additional 30 min. After the incubation, the pancreas was incubated for another 4 h at 37°C (95% O_2—5% CO_2) in the medium containing 3.0 mg/ml of glucose.

Tests

Oral glucose tolerance tests (O-GTT) (50 g) were given to all normals and diabetics. The serum samples taken at fasting and 60 min after O-GTT were frozen at —20°C until used.

Extraction and gel filtration

After the incubation, the medium was withdrawn and frozen until used, and the pancreatic tissue was homogenized by Polytron PT 10-35 Kinetmatica for 1 min at setting 7 in the siliconized glass tube with an acid—ethanol mixture (350 ml 99.5% ethanol + 7 ml concentrated hydrochloric acid + 153 ml distilled water). Afterwards, extraction and gel filtration of the samples were the same as in the previously reported method [7].

Assay for IRI and calculations

The assay procedure and the calculation were performed as in the earlier report [7]. The results were expressed as mean ± S.D.

RESULTS AND DISCUSSION

In the incubation study of human pancreas, two peaks of insulin were detected both in the medium and in the pancreatic tissue in the case of 4 h incubation as shown in Fig. 1, but in the case of the first 10-min incubation, the trace of Peak I of insulin was recognized, besides Peak II of insulin as shown in Fig. 2. When the pancreas was directly stimulated with 3.0 mg/ml glucose for 10 min, Peak II of insulin was markedly released into the medium, compared with Peak I of insulin. But in the 4-h incubation, the ratio of Peak I to Peak II of insulin in the medium was higher than that in the pancreatic tissue. From these data, Peak II of insulin is released from the human pancreas by the rapid change of glucose stimulation. On the contrary, maintained in the same condition for 4 h, the pancreatic tissue releases not only Peak II but Peak I of insulin. The proportion of Peak I of insulin in the medium, especially, is increased under the stable concentration of 3.0 mg/ml glucose for 4 h.

The serum samples of fasting and 60 min after O-GTT were gel filtrated after extraction, and the insulin contents of each fraction were assayed in normals, well controlled and poorly controlled diabetic patients. Two groups of insulin were apparently shown in all serum samples as reported elsewhere [7]. But the fraction of proinsulin-like component was not revealed.

Total insulin quantity of each group was calculated as shown in Table I. In normal adults, the calculated fasting values of Peak I of insulin and Peak II of insulin were 0.05 ± 0.01 and 0.01 ± 0.01 p*M*/ml, respectively. The level of Peak

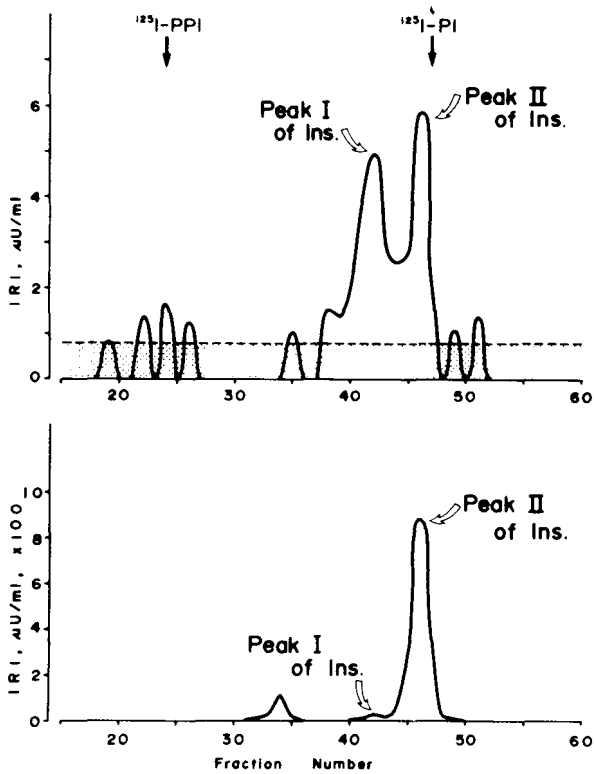

Fig. 1. Elution profiles of extracted human insulin on the Bio-Gel column with 4.05 ml fraction size obtained from incubation medium containing 3.0 mg/ml glucose for 4 h at 37°C (95% O_2—5% CO_2) (above) and from the pancreatic tissue incubated in the same medium under the same conditions (below). Pancreatic tissue was obtained from a patient with localized esophageal cancer (53 years old, male). PPI = porcine proinsulin; PI = porcine insulin. Dotted area shows detection level of IRI.

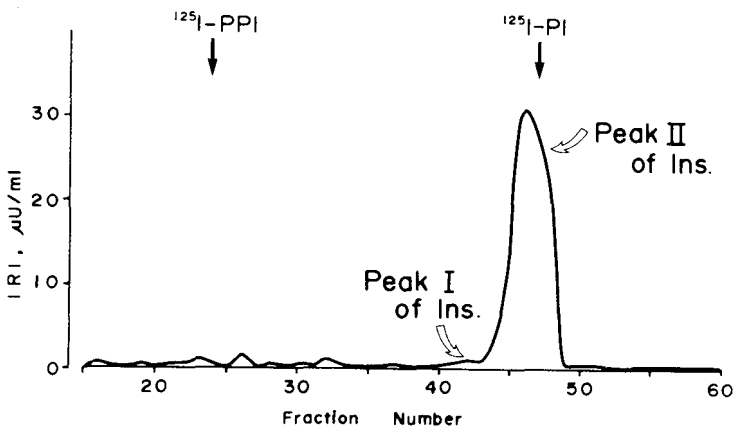

Fig. 2. Elution profile of extracted human insulin on the Bio-Gel column with 4.05 ml fraction size obtained from incubation medium containing 3.0 mg/ml glucose for 10 min at 37°C (95% O_2—5% CO_2) with pancreatic tissue obtained from a patient with localized esophageal cancer (53 years old, male). PPI = porcine proinsulin; PI = porcine insulin. Detection level is 0.1 μU/ml for IRI.

TABLE I

LEVELS OF INSULIN OBTAINED AT FASTING AND 60 MIN AFTER GLUCOSE LOADING

F, fasting; n.s., not significant. These values were corrected with the recovery rate of extraction (0.833) and gel filtration (0.647).

Case	*n*	Maximum BS level after 50-g O-GTT (mg/dl)	Time (min)	Peaks (p*M*/ml)	
				Peak I	Peak II
Normals	4	139.7 ± 25.9*	F	0.05 ± 0.01	0.01 ± 0.01
		$p < 0.02$		n.s.	$p < 0.05$
			60	0.07 ± 0.02	0.05 ± 0.03
Well controlled diabetics	4	279.0 ± 40.3	F	0.04 ± 0.01	0.01 ± 0.00
		$p < 0.02$		$p < 0.01$	$p < 0.01$
			60	0.10 ± 0.01**	0.08 ± 0.02**
Poorly controlled diabetics	2	453.0 ± 7.0*	F	0.04 ± 0.01	0.00 ± 0.00
				n.s.	n.s.
			60	0.05 ± 0.01**	0.02 ± 0.01**

*Significant difference ($p < 0.001$).
**Significant difference ($p < 0.05$).

I of insulin at the fasting state was observed to be higher than that of Peak II of insulin. At 60 min after 50 g O-GTT, the calculated values of Peak I of insulin and Peak II of insulin were 0.07 ± 0.02 and 0.05 ± 0.03 p*M*/ml, respectively. Peak II of insulin was elevated after glucose load ($p < 0.05$), but Peak I of insulin was not changed.

In well controlled diabetics, the levels of Peak I and Peak II of insulin were 0.04 ± 0.01 and 0.01 ± 0.00 p*M*/ml, respectively, at the fasting state. The level of Peak I of insulin obtained was also higher than that of Peak II of insulin at the fasting state. At 60 min after O-GTT, the calculated values of Peak I and Peak II of insulin were 0.10 ± 0.01 and 0.08 ± 0.02 p*M*/ml, respectively. In this group, both Peak I and Peak II of insulin were elevated in the serum after the stimulation of glucose ($p < 0.01$). Well controlled diabetics showed better response to glucose loading than normals in both insulin peaks, especially in Peak I of insulin. It was first noted by Yalow and Berson [8] that the total insulin secretory response in diabetics with glucose loading was significantly greater than normals and this has been subsequently confirmed by many laboratories [9—13]. The increase of total insulin in diabetics should be responsible in part for increments of Peak I of insulin, though Peak I of insulin was not increased by the stimulation of glucose in normals.

In poorly controlled diabetics, the values of Peak I and Peak II of insulin were 0.04 ± 0.01 and 0.00 ± 0.00 p*M*/ml, respectively at the fasting state. The level of Peak I of insulin was observed to be higher at the fasting state than that of Peak II of insulin. The fasting serum level of Peak I of insulin was 0.04—0.05 p*M*/ml amongst the three groups. At 60 min after glucose loading, the

total amounts of Peak I and Peak II of insulin were 0.05 ± 0.01 and 0.02 ± 0.01 pM/ml, respectively. In poorly controlled diabetics, increments in Peak I and Peak II of insulin were not observed. There were significant differences in the calculated values of Peak I and Peak II of insulin at 60 min after glucose loading between well controlled and poorly controlled diabetics ($p < 0.05$). It seems that the Peak II reservoir to glucose stimulation is restricted in poorly controlled diabetic patients.

The fraction of proinsulin-like material was not detected in the experiment of incubation and the study of human serum. These facts are quite compatible with the level of proinsulin in human plasma, reported by Turner and Heding [14].

From the above mentioned results, the following conclusions are suggested. (1) There are two groups of insulin not only in circulating human sera but in incubation medium and incubated human pancreas. (2) In normals, Peak II of insulin is released by glucose loading, but Peak I of insulin is not. (3) In a well controlled diabetic patient, both Peak I and Peak II of insulin are secreted by the same stimulation. Therefore, the secretion mechanism of Peak I of insulin could be different from that of a normal. (4) In poorly controlled diabetics, neither Peak I nor Peak II of insulin are released by the same loading. In this case, the impairment could be mainly in the secretion mechanism of Peak II of insulin. (5) The insulin secretion from human B-cell could firstly be hyperresponsive to the loading of glucose in the early stage of diabetes mellitus; according to the progress of the disease, the function of B-cell might be impaired gradually.

Further studies of Peak I and Peak II of insulin could lead to a better understanding of the mechanisms regulating the synthesis and secretion of insulins and its patho-physiological roles in man.

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REFERENCES

1 N.R. Lazarus, R.A. Gutman and L. Recant, Anal. Biochem., 40 (1971) 241.
2 N.R. Lazarus, R.A. Gutman, J.C. Penhos and L. Recant, Diabetologia, 8 (1972) 131.
3 R.A. Gutman, N.R. Lazarus and L. Recant, Diabetologia, 8 (1972) 136.
4 C.D. Haën, J.M. Little, J.M. May and R.H. Williams, J. Clin. Invest., 62 (1978) 727.
5 J.R. Kimmel and H.G. Pollock, Diabetes, 16 (1967) 687.
6 R.B. Elliott, D. O'Brien and C.C. Roy, Diabetes, 14 (1965) 780.
7 K. Kakita, M. Horino, H. Oyama, A. Tenku, S. Matsumura and S. Nishida, J. Chromatogr., 181 (1980) 248.

8 R.S. Yalow and S.A. Berson, Diabetes, 9 (1965) 254.
9 M. Perley and D.M. Kipnis, Diabetes, 15 (1966) 867.
10 J.D. Bagdade, E.L. Bierman and D. Porte, J. Clin. Invest., 46 (1967) 1549.
11 H. Seltzer, E.W. Allen, A. Herron, Jr., and M. Brennan, J. Clin. Invest., 46 (1967) 323.
12 G. Raeven and J. Dray, Diabetes, 16 (1967) 487.
13 E. Cerasi and R. Luft, Acta Endocrinol., 55 (1967) 305.
14 R.C. Turner and L.G. Heding, Diabetologia, 13 (1977) 571.

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Biomedical Applications

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Note

Determination of urinary quinolinic acid by high-performance liquid chromatography

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Quinolinic acid is a compound of interest as a tryptophan metabolite and precursor of the nicotinamide moiety of NAD(H). Quinolinic acid has been quantitated in the urine of vitamin B_6-deficient humans [1], guinea pigs, hamsters and rats [2]. Elevated urinary excretion of quinolinic acid by humans and rats fed high levels of leucine in the diet has been reported [3]. Consequently, some workers have implicated excess dietary leucine as a causative factor in the pathogenesis of pellagra. Our laboratory is currently studying this problem and it was for this purpose that a simple and accurate method for the quantitation of urinary quinolinic acid using high-performance liquid chromatography (HPLC) was developed.

Methods for quantitating urinary quinolinic acid are numerous. Henderson's microbiological method [4] measured niacin-active compounds before and after autoclaving urine in glacial acetic acid. Autoclaving in acid decarboxylates quinolinic acid in the 2-carbon position yielding nicotinic acid. Therefore, quinolinic acid was estimated by difference. Quinolinic acid has also been directly determined microbiologically after separation by paper chromatography [5].

Heeley et al. [6] described a method which involves a partial separation of quinolinic acid from other urinary compounds by Dowex anion- and cation-exchange chromatography, decarboxylation by heating in acid, and quantitation spectrophotometrically by reacting the nicotinic acid with cyanogen bromide and *o*-toluidine. McDaniel et al. [7] described a similar method with a modification in the pre-cleanup of the urine which involved absorbing quinolinic acid onto Norit A followed by elution with ammonium hydroxide. Quino-

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linic acid has been derivatized to the diester with ethereal diazomethane and analyzed by gas—liquid chromatography [8]. Lastly, Crawford et al. [9] measured urinary quinolinic acid using thick-layer chromatography.

MATERIALS AND METHODS

Quinolinic acid standard was purchased from Sigma (St. Louis, MO, U.S.A.). Other standards that were used were picolinic acid hydrochloride (E.H. Sargent & Co., Chicago, IL, U.S.A.) and nicotinic acid (Nutritional Biochemicals, Cleveland, OH, U.S.A.). All standards were dissolved in distilled water. All reagents used in the HPLC system were reagent grade. Methanol was glass distilled (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

The formate form of Dowex 1-X8, 200—400 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.) was prepared by sequentially washing 1-lb. (450-g) batches of resin in a column of 10 cm diameter with 4.0 l of 6 *N* hydrochloric acid, 4.0 l of distilled water, 16 l of 3 *N* sodium formate, 4.0 l of distilled water, 8.0 l of 3 *N* formic acid and finally with 12 l of distilled water.

Sample preparation

Twenty-four hour urine collections were made by normal human volunteers and the urine was preserved with a few milliliters of toluene. Aliquots of the urine were frozen at −4°C until analysis.

Two percent of the 24-h urine collection was pipetted in duplicate into 50-ml graduated tubes. To one duplicate was added 1 ml of standard quinolinic acid solution containing 0.5 μmoles/ml. The volumes of the samples were then brought up to 50 ml with distilled water.

Glass columns with reservoirs [10] were prepared by pipetting 15 ml of a 1:1 slurry (v/v) of Dowex 1-X8, 200—400 mesh (formate) resin into glass columns, 1.3 cm I.D. The resin bed heights were approximately 5.5 cm. The columns were washed with 30 ml of 3 *N* sodium formate in 3 *N* formic acid and then with 100 ml distilled water prior to loading samples.

Urine samples were poured onto the columns and the eluate discarded. The sample tubes were rinsed with 10 ml distilled water, the rinse poured onto the columns and the eluate discarded. The columns were then eluted with 50 ml of 0.08 *N* hydrochloric acid and the eluate discarded. About 97 ml of 0.15 *N* hydrochloric acid were passed through the columns and the eluate was collected in 100-ml graduated cylinders. The volumes of this eluate were brought up to 100 ml with distilled water. The urine samples and the 10 ml of water washings were eluted under 6.9×10^3 N m^{-2} pressure, while the rest of the chromatography was done with gravity flow.

A 20-ml aliquot of the final 0.15 *N* hydrochloric acid eluate was evaporated under vacuum on a flash evaporator in a water bath at 50°C. The residue was dissolved in 12 ml of distilled water and again flash evaporated in order to rid the sample of hydrochloric acid. The final residue was redissolved in 2 ml of the buffer used in the HPLC system and filtered with a 0.22-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

Apparatus and chromatographic conditions

A Perkin-Elmer 601 liquid chromatograph (Norwalk, CT, U.S.A.) was used for chromatographic analysis. A strong anion-exchange column (25 cm × 4.6 mm I.D.) prepacked with Partisil-10 SAX (10-μm diameter particle) (Whatman, Clifton, NJ, U.S.A.) was used. The column was wrapped with a heating coil and insulation so that the column temperature could be varied by a variable transformer and monitored by a thermo-couple. The detectors used were a Model 250 fixed-wavelength detector (254 nm) and a LC-55 UV/VIS variable-wavelength spectrophotometer, both from Perkin-Elmer. The flow cells of the spectrophotometers were connected in series. The dual pen recorder was set at 0.015 a.u.f.s. for most samples. A Rheodyne syringe loading sample injector was used. Samples were injected onto the column by 22-gauge (0.071 cm) Hamilton syringes and sample volumes ranged from 10 to 100 μl.

The column was operated isocratically with 0.06 *M* potassium phosphate buffer (pH 2.2)—methanol (9:1) as solvent. The flow-rate was 2.0 ml/min and the column temperature was constant but was varied from 30 to 50°C depending on the run. Therefore, the column pressure ranged from 41 to 76 bar. All buffers were filtered on 0.22-μm Millipore filters.

Absorbance was recorded at 254 nm and 272 nm. Quantitation of compounds was determined by peak height measurements.

RESULTS AND DISCUSSION

A representative chromatogram of a sample prepared from normal human urine is illustrated in Fig. 1A. Quinolinic acid added to the urine sample co-chromatographed with this peak (Fig. 1B) and had the same retention time as standard quinolinic acid which was directly injected (Fig. 1C and D). Also, standard quinolinic acid was directly added to a prepared urine sample and one peak resolved with a retention time equal to standard quinolinic acid. Quinolinic acid in distilled water was pretreated identically to urine samples. Only one peak resolved, thereby indicating that quinolinic acid was not decarboxylated by the sample preparation procedure.

Quinolinic acid peaks from a number of urine samples were collected and re-chromatographed. The major peak co-chromatographed with standard quinolinic acid. A peak also appeared at the solvent front due to tailing off of the initial unidentified compounds as seen in Fig. 1A. Because of the tailing off of the large initial peaks, baselines from which peak height measurements were made were drawn as shown in Fig. 1A.

The identity of the quinolinic acid peak was further established by comparing chromatographic and spectrophotometric properties of the peak of interest with standard quinolinic acid. The peak height ratios of urine samples and standards were determined by comparing absorbance at 272 nm (absorbance maximum of quinolinic acid) and 254 nm. Peak height ratios of absorbance at 272:254 nm for 13 analyses of samples (1.66 ± 0.036), recovery samples (1.64 ± 0.048) and standard quinolinic acid (1.64 ± 0.052) were identical. Urine samples and standard quinolinic acid behaved identically when column temperature and buffer strength were varied. Fig. 2A and B represent only a few of the many trials in which various solvents and temperatures were tested. Picolinic acid and nicotinic acid, two anionic pyridine compounds that

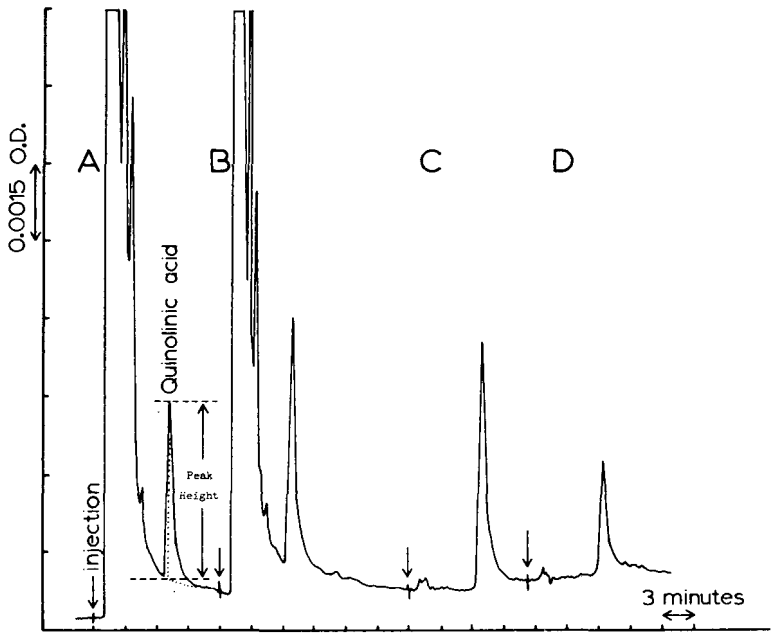

Fig. 1. Chromatograms of (A) normal urine sample and (B) urine sample with added quinolinic acid, both prepared as described in Sample preparation section; (C) 40 μl of quinolinic acid solution and (D) 20 μl of quinolinic acid solution (concentration = 0.1 μmole/ml). Conditions: column, Partisil-10 SAX, 25 cm × 4.6 mm I.D.; eluent, 0.06 *M* potassium phosphate buffer (pH 2.2)—methanol (9:1, v/v); flow-rate, 2 ml/min; column temperature, 38°C and pressure, 76 bar; detection, 272 nm.

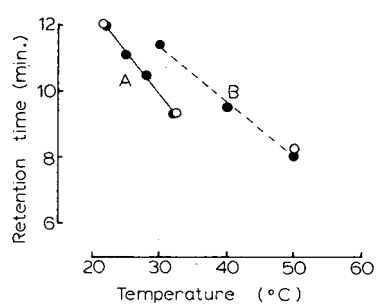

Fig. 2. Effect of temperature and buffer strength on retention time of quinolinic acid. (A) Conditions: column, Partisil-10 SAX; eluent, 0.08 *M* potassium phosphate buffer (pH 2.3)—methanol (9:1, v/v); flow-rate, 2 ml/min; detection, 272 nm. (B) Conditions, column, Partisil-10 SAX; eluent, 0.06 *M* potassium phosphate buffer (pH 2.35)—methanol (9:1, v/v); flow-rate, 2 ml/min; detection, 272 nm. Quinolinic acid peak from urine sample, •; peak from standard quinolinic acid, ○.

are excreted by humans, were tested. Both eluted at the solvent front.

Standard curves plotting peak height versus picolinate injected demonstrated excellent linearity (correlation coefficient = 0.999) in the range 0—10 nmoles. Urinary quinolinic acid was quantitated within the 2—8-nmole range by varying the volume of injected sample. Peak resolution, standard curves and the

quantitation or urinary quinolinic acid did not vary due to sample injection volumes. One ml of standard quinolinic acid solution (concentration = 0.5 μmoles/ml in distilled water) was added to the urine sample before treatment on Dowex 1 (formate) columns. More than 100 urine samples were analyzed and recoveries ranged between 95% and 105%. The percent of quinolinic acid recovered from urine was independent of the amount of quinolinic acid added to the urine sample as shown in Table I. Coefficient of variation (standard deviation × 100/mean) of replicate analyses was 7.2%. Freezing urine samples at −4°C and thawing them did not affect the quantitation of quinolinic acid. The same samples were analyzed four months apart with repeatable results.

This method was adapted for the analysis of rat urine. Rats fed a 10% casein diet and having a body weight of about 190 g excreted approximately 0.4 μmoles of quinolinic acid per 24 h.

TABLE I

RECOVERY OF QUINOLINIC ACID ADDED TO URINE SAMPLE

Conditions: column, Partisil-10 SAX; eluent, 0.06 *M* potassium phosphate buffer (pH 2.2)—methanol (9:1, v/v); flow-rate, 2 ml/min; column temperature, 37°C and pressure, 69 bar; detection, 254 nm; retention time, 10.2 min. Standard quinolinic acid was added to 2% of a 24-h urine collection from a normal male subject.

Amount of quinolinic acid added (μmoles)	Amount of quinolinic acid recovered (μmoles)	Recovery (%)
0	0.480	
0	0.506	
0.25	0.758	105
0.50	1.00	101
1.0	1.50	101
1.5	2.01	101

ACKNOWLEDGEMENTS

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REFERENCES

1 R.R. Brown, N. Yess, J.M. Price, H. Linkswiler, P. Swan and L.V. Hankes, J. Nutr., 87 (1965) 419.
2 J.K. Yeh and R.R. Brown, J. Nutr., 107 (1977) 261.
3 C. Gopalan and K.S. Jaya Rao, Vitam. Horm. New York, 33 (1975) 505.
4 L.M. Henderson, J. Biol. Chem., 181 (1949) 685.
5 W.B. Jakoby and D.M. Bonner, J. Biol. Chem., 191 (1951) 543.
6 A.F. Heeley, D.G. McCubbing and J. Shepherd, Arch. Dis. Childhood, 41 (1966) 562.
7 H.G. McDaniel, W.J. Reddy and R.R. Boshell, Anal. Biochem., 49 (1972) 373.
8 P. Toseland, Clin. Chim. Acta, 25 (1969) 185.
9 M.A. Crawford, I.L. Hansen and A. Lopez, Brit. J. Cancer, 23 (1969) 644.
10 R. Levine and R. Luft, Advan. Met. Disord., 2 (1965) 159.

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Note

Enzymatic color development of 3α-hydroxysteroids on thin-layer chromatograms for determination of excretion pattern of 3α-hydroxysteroids in patients with some adrenogenital syndrome

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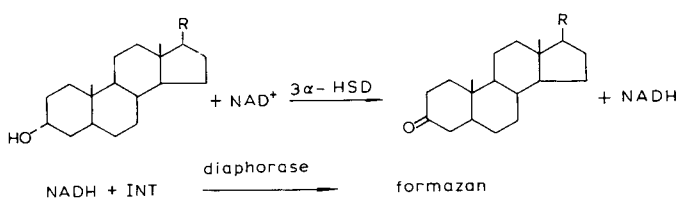
The differential diagnosis of adrenogenital syndrome is diffcult without the use of chromatographic techniques, but gas—liquid or column liquid chromatography are time-consuming and need high techniques of analysis.

The simpler methods, however, often do not provide adequate separation and identification of the individual steroid compounds and lack the accuracy of the original procedures, but many simple methods have been reported for the detection of steroids after chromatographic separation such as the Zimmermann reaction for 17-ketosteroids [1], some other chemical reactions and enzymatic color development of 3β-hydroxysteroids [2].

Although the paper chromatographic—enzyme spray technique for the detection of sugars is well known [3], in this paper a thin-layer chromatographic (TLC)—enzyme solution spray technique is described for the determination of the excretion pattern of 3α-hydroxysteroids in patients with some adrenogenital syndrome. The principle of the reaction is as follows:



The dye formed has a maximum absorption at 500 nm.

MATERIALS AND METHODS

3α-Hydroxysteroid dehydrogenase (3α-HSD) (from *Pseudomonas testosteroni*, EC 1.1.1.51) and β-NAD^+ were purchased from Nyegaard & Co. (Oslo, Norway). All steroids, β-glucuronidase (bacterial powder from *Escherichia coli*, EC 3.2.1.31), diaphorase (from *Clostridium kluyveri*, EC 1.6.99.2) and other reagents for color development were purchased from Sigma (St. Louis, MO, U.S.A.). To prepare the enzyme reagent for color development of 3α-hydroxysteroids on thin-layer plates: dissolve 6 mg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in 10 ml of 0.2 *M* K_2HPO_4 (pH 8.5) containing, per 10 ml, 1 U of 3α-HSD, 50 U of diaphorase and 5 μmol of β-NAD.

Preparation of sample

Pipette 5 ml of urine (in case of high excretion of urinary steroids, use 2 ml of sample urine) into a 40-ml tube and adjust to pH 6.5 with bromthymol blue paper as a indicator. Add 1 ml of β-glucuronidase (1,000,000 Fishman units/l), 1 ml of 0.5 *M* phosphate (pH 6.5) and a few drops of chloroform to the tube and mix well. Incubate the mixture for 24 h at 37° C, then adjust to pH 1 with 6 *M* sulphuric acid and saturate with 5 g of sodium chloride. Shake the solution with 20 ml of ethyl acetate for 5 min. After centrifuging, discard the urine layer and keep the ethyl acetate layer for another 24 h at 37° C to achieve complete solvolysis of the sample. Wash the ethyl acetate layer successively with 2 ml of sodium hydroxide (80 g/l), with concentrated sodium carbonate, and water. After centrifugal separation, transfer 15 ml of the ethyl acetate extract to a tube. Evaporate the ethyl acetate aliquots.

Thin-layer chromatography

To the dry residue, a few drops of chloroform are added, and the sample is applied to an activated thin-layer plate with marker dye (sudan III and isatin) and standards. The development of the thin-layer plate was performed with a solution of chloroform–methanol (9:1, v/v) for 60 min at 25° C. The distance of the front from the starting point was about 18 cm.

Color development of 3α-hydroxysteroids on thin-layer plates

Place the thin-layer plate on a heater at 37° C (or above a water bath at 40°C), and spray the enzyme reagent. Incubate for 30 min so that the zone of pink color can be seen. Then quantitative denistometric scanning at 500 nm can also be performed for the determination of 3α-hydroxysteroids in the sample. The instrument used for the assay is a dual-wavelength TLC scanner CS-910 (Shimadzu, Tokyo, Japan).

RESULTS

The absorption curve of the dye formed has a maximum at 500 nm.

Selectivity of 3α-HSD with some steroids

Selectivity of 3α-HSD was tested with some steroids (Table I). Phenolic hydroxy, 3β-hydroxy and 3-keto groups did not react with this enzyme.

Excretion patterns of 3α-hydroxysteroids

The excretion pattern of 3α-hydroxysteroids in patients with some adrenogenital syndrome are shown in Fig. 1 and each R_F value of the standards is listed in Table II.

Precision

The percentages of each fraction in five repeated assays on thin-layer plates were calculated and the average of each C.V. was 7.8% using 10 μg of each steroids, and a convenient range for quantitative analysis was 5—50 μg of each steroid in the residue of the extracts.

TABLE I

SELECTIVITY OF 3α-HYDROXYSTEROID DEHYDROGENASE

Each steroid of 25 μg per tube was determined with 2 ml of enzyme solution at 37° C for 20 min.

Steroid	Intensity of reaction (O.D. at 500 nm; 25 μg)
Etiocholanolone	0.560
Androsterone	0.525
Cortol	0.504
Tetrahydrocortisol, tetrahydrocortisone	0.490
Tetrahydro-11-deoxycortisol	0.470
Pregnanediol	0.190
Pregnanetriol	0.090
Cortisol	0.000
Dehydroepiandrosterone	0.000
Testosterone	0.000
Estriol	0.000

TABLE II

R_F VALUES OF SOME 3α-HYDROXYSTEROIDS

The development was performed by chloroform—methanol (9:1, v/v).

Compounds	R_F value
Marker dye	
Sudan III	0.82
Isatin	0.58
Steroids	
Androsterone	0.68
Etiocholanolone	0.67
11-Ketoandrosterone	0.63
Pregnanediol	0.56
11β-Hydroxyandrosterone	0.56
Tetrahydro-11-deoxycortisol	0.41
Pregnanetriol	0.37
Tetrahydrocortisone	0.33
Tetrahydrocortisol	0.22
Cortol	0.10

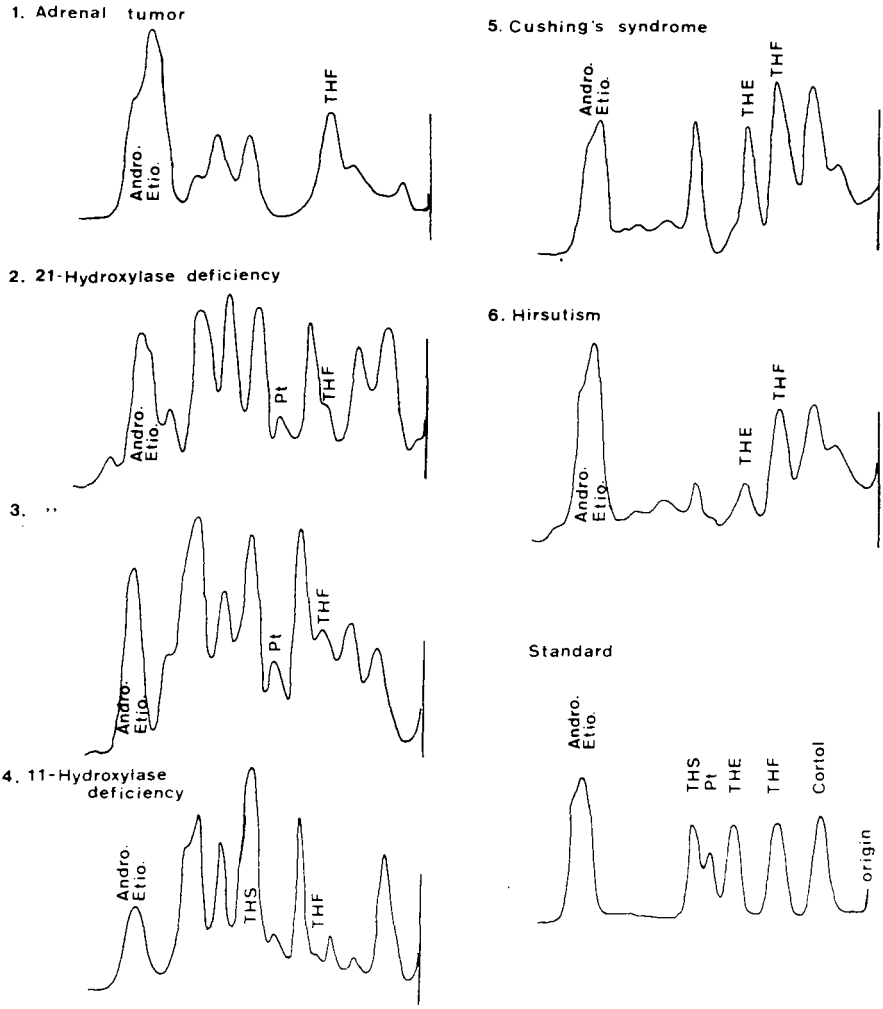


Fig. 1. Excretion pattern of 3α-hydroxysteroids by densitometric scanning. Samples are from 1, adrenal tumor; 2, 21-hydroxylase deficiency; 3, 21-hydroxylase deficiency; 4, 11-hydroxylase deficiency; 5, Cushing's syndrome; 6, hirsutism and some standard compounds. THF, tetrahydrocortisol; THE, tetrahydrocortisone; Andro., androsterone; Etio., etiocholanolone; Pt, 5β-pregnane-3α,17α,20α-triol.

DISCUSSION

The available techniques for quantitation of individual steroids are complicated and time consuming, but this method provides an estimate of groups of 3α-hydroxysteroids and is adequate for certain purposes being a simple and rapid procedure.

It is easily recognized that peaks of tetrahydro-11-deoxycortisol and some C_{21}-steroids can be seen between peaks of 17-ketosteroids and 17-hydroxycorticosteroids in patients with adrenogenital syndrome caused by enzyme

deficiency, so that this method can be used for the diagnosis of adrenogenital syndrome.

Color development with diaphorase, NAD and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride can also be used for the determination of certain steroids using certain hydroxysteroid dehydrogenases, e.g., NADH formed by the reaction of certain hydroxysteroid dehydrogenases such as 17β-hydroxysteroid dehydrogenase can be used for specific determination of the corresponding steroids.

The new detection method, TLC—enzyme spray technique, for 3α-hydroxysteroids is of value for the diagnosis of some adrenogenital syndrome.

REFERENCES

1 B. Hamman and M. Martin, J. Clin. Endocrinol. Metab., 24 (1964) 1195.
2 Y. Yamaguchi, J. Chromatogr., 163 (1979) 253.
3 J.H. Pazur and B.M. Romanic, J. Chromatogr., 169 (1979) 495.

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Biomedical Applications

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Note

Determination of nitromethaqualone in blood by electron-capture—gas chromatography

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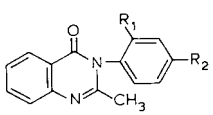
(Received December 6th, 1979)

Nitromethaqualone (I), the active compound from Parnox^{R}, or 2-methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3H)-quinazolinone is a non-barbituric hypnotic that has been used in Europe since 1967. Methaqualone (III), the active compound from Cateudyl^{R}, Mequalone^{R}, Mollinox^{R}, Noxybel^{R}, Revonal^{R}, Toquilone^{R}, and mecloqualone (IV), the active compound from Nubarène^{R}, are two other products of the quinazolinone series. Numerous papers describe the gas chromatographic analysis of methaqualone and mecloqualone in biological samples [1–7]. No procedure for the analysis of nitromethaqualone in biological samples has been described. The gas chromatographic method described by Daenens and Van Boven [8] for the identification of quinazolinones in illicit mixtures is not sensitive enough when applied to biological samples. The present paper describes the identification of nitromethaqualone in blood by gas—liquid chromatography with electron-capture detection after solvent extraction of the drug along with the previously added internal standard.



R_1	R_2		
OCH_3	NO_2	I	nitromethaqualone
H	NO_2	II	internal standard
CH_3	H	III	methaqualone
Cl	H	IV	mecloqualone

MATERIALS AND METHODS

Materials

The extraction solvents were of analytical grade. Nitromethaqualone for establishing the calibration graph was prepared by extraction of Parnox^{R} tablets [8] followed by careful recrystalisation (m.p. 193°C). The internal standard [2-methyl-3-(4′-nitrophenyl)-4(3H)-quinazolinone] was synthesized according to Grimmel et al. [9]. White crystals were obtained, m.p. 197°C.

Apparatus

A Becker 420 gas chromatograph, equipped with a $3.7 \cdot 10^8$ Bq ^{63}Ni pulse-frequency-modulated electron-capture detector was used; pulse period was 200 μsec, pulse width 0.5 μsec. The chromatograph was operated with a glass column (1.5 m × 2 mm I.D.), inactivated with dimethylchlorosilane in toluene and packed with 2% OV-17 on Chromosorb AW (100—120 mesh). The carrier gas was dry nitrogen at a flow-rate of 50 ml/min. The instrument settings were as follows: temperature of the oven 255°C, temperature of both injector and detector 280°C.

Assay of plasma samples

To 1.0 ml of human blood in a centrifuge tube were added 2.0 ml ammonia buffer (pH 9.2) followed by 0.5 ml of internal standard solution (0.01 mg% in water) and 1 ml of the extracting solvent consisting of a mixture of toluene—hexane—isoamyl alcohol (78:20:1). The blood was extracted for 20 min by means of a mechanical rotator. The layers were separated by centrifuging for 3 min at 1100 *g* and 1-μl aliquots of the organic phase used for injection into the gas chromatograph.

Quantitation

The concentrations of nitromethaqualone in blood were calculated with the aid of calibration graphs, which were prepared as follows. Methanolic solutions containing 10, 20, 40, 60, 80, 110, 140 and 200 ng of nitromethaqualone were evaporated and the residue dissolved in 1.0 ml of blood. The samples prepared were analysed by the procedure described above. The ratios of the peak heights of nitromethaqualone to that of the internal standard were plotted against the known concentrations of nitromethaqualone.

Recovery studies

We measured the absolute recovery of nitromethaqualone from blood and the internal standard as follows. Solutions of 25 and 50 ng of nitromethaqualone and internal standard, respectively in 1.0 ml of the extracting solvent were prepared. Carefully measured 2-μl aliquots of these solutions were chromatographed and the peak heights determined. To the prepared solutions were added 1 ml of blood, 2 ml of buffer and 0.5 ml of water. After mixing with a mechanical rotator for 20 min, the layers were separated by centrifuging. Again exactly 2-μl aliquots were injected in the gas chromatograph and the peak heights determined. Percentage recovery was calculated by comparing these peak heights with the peak height obtained by the injection of solution of the pure compound.

RESULTS AND DISCUSSION

The greater sensitivity of the electron-capture detector for nitromethaqualone along with the use of an internal standard makes the quantitative analysis of nitromethaqualone in blood relatively simple. Concentrations down to 1 ng/ml of blood can still be measured. The procedure was shown to be specific for nitromethaqualone as no interfering peaks from constitutents of normal blood in the same region as either nitromethaqualone or internal standard have been found. Fig. 1 shows typical gas chromatograms of an extract of a drug-free blood, an extract from a 1.0-ml blood sample to which 80 ng nitromethaqualone were previously added and an extract from a 1.0-ml blood sample collected from a volunteer after oral intake of 25 mg nitromethaqualone. According to the calibration graph there is a linear relation between the ratio of the peak height of nitromethaqualone to that of the internal standard and the concentration of nitromethaqualone up to 100 ng/ml of blood when 1-μl aliquots were injected. The concentrations of nitromethaqualone in blood samples can be read directly from the previously constructed calibration graph (Fig. 2).

Extraction yields for one single extraction are 99.1 ± 2% for both nitromethaqualone and the internal standard.

The good resolution from both the solvent peak and nitromethaqualone makes 2-methyl-3-(4′-nitrophenyl)-4(3H)-quinazolinone (II) a good choice as internal standard for nitromethaqualone assays.

The procedure has been applied to measure plasma concentrations of two volunteers after oral intake of 25 mg nitromethaqualone hydrochloride. Peak plasma levels of 65 and 135 ng/ml were observed after 120 and 90 min respectively. Fig. 3 shows blood concentration graphs after oral intake of 25 mg nitromethaqualone hydrochloride by those two volunteers.

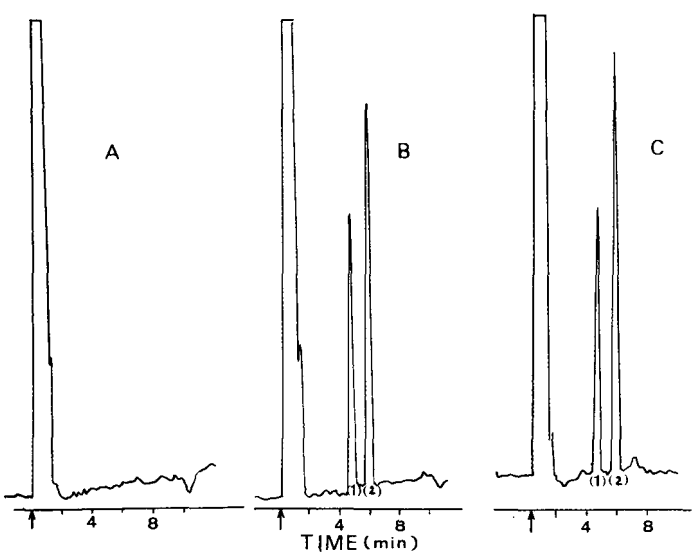


Fig. 1. Typical gas chromatograms of blood extracts from (A) drug-free blood without addition of internal standard; (B) blood to which 80 ng nitromethaqualone and 50 ng internal standard were added and (C) blood sample from a volunteer after intake of 25 mg nitromethaqualone. Peaks: 1 = internal standard, 2 = nitromethaqualone.

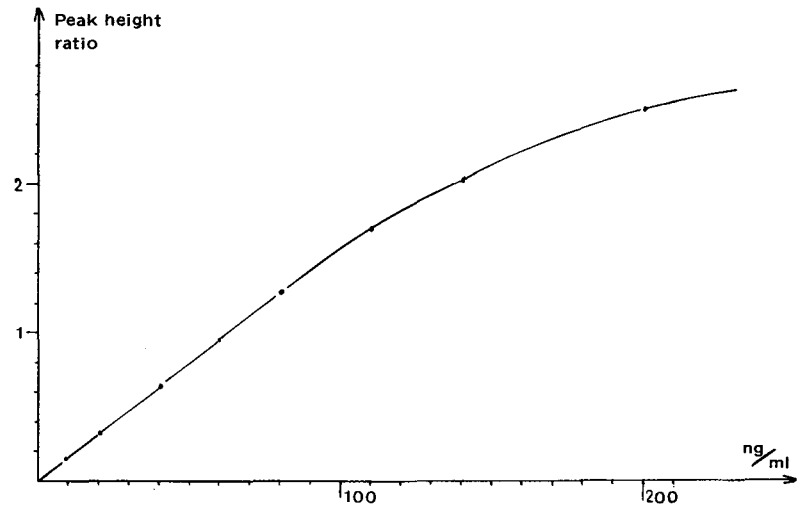


Fig. 2. Calibration graph for the determination of nitromethaqualone in blood.

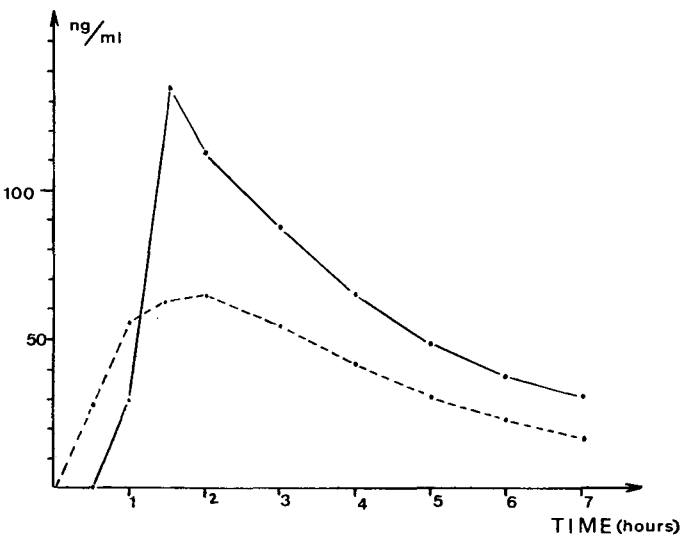


Fig. 3. Blood concentration graphs from two volunteers after oral intake of 25 mg nitromethaqualone hydrochloride.

REFERENCES

1 M.A. Evenson and G.L. Lensmeyer, Clin. Chem., 20 (1974) 249.
2 D.J. Berry, J. Chromatogr., 42 (1969) 39.
3 R.O. Bost, C.A. Sutheimer and I. Sunshine, Clin. Chem., 22 (1976) 689.
4 R. Bonnichsen, G.G. Fri, C. Negoita and R. Ryhage, Clin. Chim. Acta, 40 (1972) 309.
5 M. Mitchard and M.E. Williams, J. Chromatogr., 72 (1972) 29.
6 A. Proslie and B. Schubert, J. Legal Med., 67 (1970) 342.
7 M. Van Boven, G. Janssen and P. Daenens, Microchim. Acta, (1974) 603.
8 P. Daenens and M. Van Boven, J. Forensic Sci., 21 (1976) 552.
9 H.W. Grimmel, A. Guenther and J.F. Morgan, J. Amer. Chem. Soc., 68 (1946) 542.

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Biomedical Applications

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Note

Determination of midazolam in serum by gas chromatography with a nitrogen-selective detector

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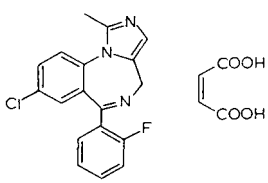
Midazolam maleate (I) is an investigational benzodiazepine which is similar in its clinical effect to diazepam. Because of its shorter half-life and water solubility, midazolam is especially suited for induction of anesthesia [1—3].

Using gas chromatography (GC) with an electron-capture detector, a procedure has been reported for the direct determination of midazolam in blood [4]. We report here a sensitive gas chromatographic procedure using a nitrogen-selective detector for the determination of midazolam in serum. Gas chromatography—mass spectrometry (GC—MS) was used to confirm the presence of the drug and the specificity of the procedure. Interferences and the use of different internal standards was also investigated.



(I)
Midazolam maleate

MATERIALS AND METHODS

Apparatus

We used a Hewlett-Packard Model 5840A gas chromatograph with dual nitrogen—phosphorus detectors. Coiled glass columns (1.2 m × 2 mm I.D.)

packed with 2% SP-2250 and 2% OV-101 on Chromosorb W HP (100—120 mesh) (Hewlett-Packard, Avondale, PA, U.S.A.) were used.

GC—MS analyses were performed on a Model 5985A quadrupole system (Hewlett-Packard, Palo Alto, CA, U.S.A.) used in the electron impact mode. The system consisted of a Hewlett-Packard 5840A gas chromatograph interfaced to the mass spectrometer. The glass column was 1.2 m × 2 mm I.D. packed with 2% SP-2250 Chromosorb W HP (100—120 mesh). The ionization energy was 70 eV. For selected ion monitoring (SIM) we used the gas chromatograph—mass spectrometer in the electron impact mode, with the electron multiplier voltage set at 3000 V and the dwell time for each ion being monitored was 100 msec.

The column temperature was 260°C for GC and 250°C for GC—MS. Helium was used as carrier gas with a flow-rate of 40 ml/min.

Reagents

All reagents were analytical reagent (AR) or spectral grade: *n*-heptane, isobutanol. Absolute ethanol was used. Anesthetic-grade diethyl ether (J.T. Baker, Phillipsburg, NJ, U.S.A.) was used. Sodium hydroxide, 0.5 and 4.4 mol/l; sulfuric acid, 1 mol/l; *n*-heptane—isobutanol (96:4, v/v); sodium sulfate, anhydrous.

Standards

Midazolam maleate and flurazepam dihydrochloride were obtained from Hoffmann-LaRoche (Nutley, NJ, U.S.A.).

Flurazepam, 1 mg/ml (internal standard). Dissolve 29.6 mg of flurazepam dihydrochloride in 25 ml of ethanol. Prepare a 20 μg/ml solution from the above.

Midazolam, 1 mg/ml. Dissolve 33.9 mg of midazolam maleate in 25 ml of ethanol. Prepare an intermediate solution of 0.1 mg/ml and aqueous standards from 0.1 to 5 μg/ml. Reconstitute lyophilized normal human serum to volume with the aqueous 0.1 to 5 μg/ml standards.

Procedure

A modification of our previously reported drug isolation procedure was used [5]. To 2 ml of serum containing midazolam, 0.3 ml of flurazepam internal standard (20 μg/ml) was added, and extracted at basic pH (4 ml of 0.5 mol/l sodium hydroxide) into 25 ml of *n*-heptane—isobutanol (96:4, v/v). The drugs were back-extracted into 4 ml of 1 mol/l sulfuric acid. The solution was made basic with 2.5 ml of 4.4 mol/l sodium hydroxide and back-extracted into 10 ml of diethyl ether. To the diethyl ether layer 1 g of anhydrous sodium sulfate was added. The mixture was shaken, filtered and then evaporated to dryness. The residue was dissolved in 25 μl of absolute ethanol and 1 μl was injected for analysis. The peak height ratio of midazolam to flurazepam (internal standard) was calculated for each sample, and then the concentration of the unknown was calculated using the closest standard. For GC—MS the ratios of the area counts at *m/e* 310/183, or 310/58 were used to calculate the midazolam concentration.

RESULTS

Chromatograms for a serum blank, standards, and patients' samples are given in Fig. 1. GC—MS of serum extracts confirmed the presence of midazolam *m/e* 310, 325, 312, 311 and the internal standard, flurazepam, *m/e* 86, 87, 58, 183. At 260°C the retention times for midazolam and flurazepam were 1.60 and 2.10 min respectively.

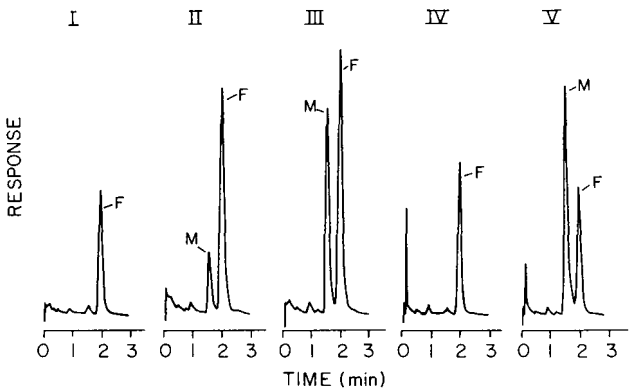


Fig. 1. Chromatograms of a serum blank, I; serum standards, II, III, and patient samples, IV, V, taken through the extraction procedure. Midazolam: II, 1 μg/ml and III, 3 μg/ml; patient serum: IV, prior to induction and V, with midazolam after induction. F = Flurazepam (internal standard) 2.6 μg/ml, M = midazolam. Conditions as in Procedure.

When the peak height value of sera containing 0.5, 1.0 and 3.0 μg of midazolam per ml, determined in duplicate, were plotted against concentration the resulting line had a slope of 1.54, a *y*-intercept of —0.27, a standard error of estimate of *Syx* of 0.07 and a correlation coefficient of 0.99. Sera containing 1, 3 and 5 μg/ml of midazolam determined on separate days ($n = 4$) gave a slope of 0.28, *y*-intercept of —0.03, *Syx* = 0.09 and a correlation coefficient of 0.98. The absolute recovery of sera containing 0.5, 1, 3, and 5 μg of midazolam per ml was 82 ± 7 ($n = 2$), 63 ± 7 ($n = 5$), 68 ± 6 ($n = 6$), and 70 ± 10% ($n = 5$) respectively. The absolute recovery of sera extracts in the 0.1 to 0.8 μg/ml range averaged 55 ± 9%. The relative within-run percent recovery using serum standards of 0.5, 1, 3, and 5 μg of midazolam per ml was 96 ± 20 ($n = 4$), 94 ± 8 ($n = 7$), 104 ± 16 ($n = 9$), and 106 ± 6% ($n = 5$) respectively. The within-run precision of sera standards containing 3 and 5 μg of midazolam per ml was 6% ($n = 7$ in each). The between-run precision at the 3 and 5 μg/ml concentration was 6 and 7% ($n = 5$ in each).

Prazepam and *p*-chlorodisopyramide were not well enough separated from midazolam, using either an SP-2250 or OV-101 column, to be used as internal standards. Although nitrazepam is well separated from midazolam, it did not chromatograph well. Flurazepam was therefore chosen as the internal standard since it is well separated from midazolam and shows good detector sensitivity. Since flurazepam is a commonly used benzodiazepine, patient serum blanks were run to verify the absence of flurazepam (Fig. 2).

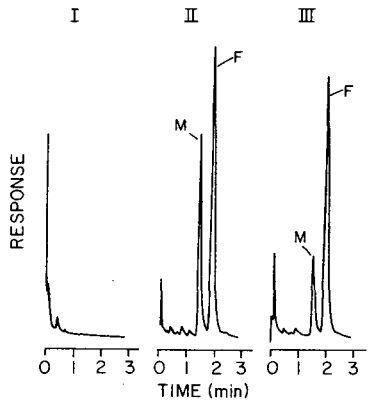


Fig. 2. Chromatograms of a patient's serum extracts before induction (I) and at 1—2 min (II) and 4—5 min (III) after induction. Midazolam concentration: (II) 1.8 and (III) 0.81 μg/ml. F = Flurazepam 2.6 μg/ml, M = midazolam.

Serum concentrations of six patients who were induced for anesthesia with midazolam prior to open heart surgery are given in Table I. Blood samples were collected at 0, 1—2, 4—5, and 13—14 min after induction and analyzed by this procedure.

TABLE I

PATIENT MIDAZOLAM CONCENTRATIONS

Total dose (0.2 mg/kg) infused within 15 sec and samples collected at specified times using opposite arm.

Patient	Dose (mg)	Midazolam concentration (μg/ml) Time (min)		
		1—2	4—5	13—14
1	16	4.6	0.81	0.56
2	15.3	1.2	0.52	0.28*
3	19.6	1.2	0.28	—
		3.0	3.74	3.20**
4	20.8	1.8	0.81	0.31
5	12.4	0.66	0.44	—
6	14	1.1	0.81	0.3

*GC—MS analysis gave 1.3, 0.66 and 0.30 μg/ml, respectively.
**Patient was also given diazepam. Serum diazepam concentrations are as indicated.

DISCUSSION

Prazepam interference can be eliminated by first extracting at pH 7.4 and then following this procedure. Prazepam as well as diazepam will remain in the organic phase while midazolam and flurazepam are extracted into the

sulfuric acid. Nordiazepam, which is the active metabolite of diazepam will interfere. The following most commonly prescribed benzodiazepines and metabolites will not interfere on a 2% SP-2250 column: oxazepam, chlordiazepoxide, norchlordiazepoxide, and diazepam. The tricyclic antidepressants, amitriptyline, doxepin, imipramine, their metabolites, as well as the commonly prescribed antiarrhythmics, quinidine, procainamide and disopyramide will not interfere with this procedure. In the presence of interferences, sera extracts can be analyzed by another technique such as GC—MS [6]. Using GC—MS in the SIM mode, good agreement was observed between GC and GC—MS (Table I). Masses monitored were *m/e* 310 and 325 for midazolam and *m/e* 58, 86, and 183 for flurazepam. Fig. 3 shows a SIM run of a urine standard which is 100 ng/ml in midazolam. Urine determinations were performed using the present extraction procedure followed by GC or GC—MS for analysis. Using GC—MS in the SIM mode, the detection sensitivity is 2—4 ng/ml.

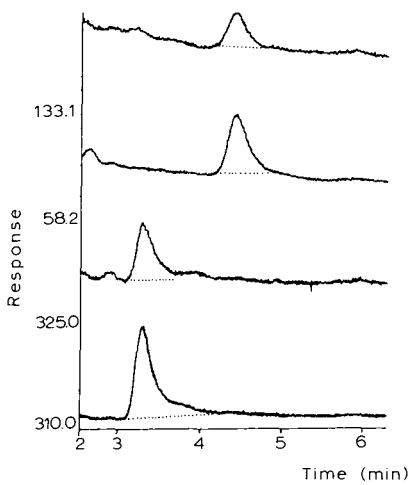


Fig. 3. GC—MS of a urine extract (SIM mode) containing 100 ng/ml of midazolam and 40 ng/ml of flurazepam as internal standard; *m/e* 310 and 325 for midazolam and 58 and 183 for flurazepam.

The results of six patients analyzed in this study (Table I) indicate that midazolam is rapidly distributed from the main compartment with the alpha phase half-life being less than 10 min. This results in low patient midazolam concentrations within a short period of time after induction. The sensitivity of the present GC procedure is less than 50 ng/ml with a relative recovery of 100% using serum standards.

ACKNOWLEDGEMENTS

The authors are indebted to J.G. Reves, M.D. and P.N. Samuelson, M.D., for providing us with the blood samples, and to Barbara Navare for running the GC—MS samples.

REFERENCES

1 C.R. Brown, G.H. Sarnquist, C.A. Canup and T.A. Pedley, Anesthesiology, 50 (1979) 467.
2 J.C. Reves, C. Guenter and C. Holcomb, Can. Anaesth. Soc. J., 25 (1978) 211.
3 R.J. Fragen, F. Gahl and N. Caldwell, Anesthesiology, 49 (1978) 41.
4 C.V. Puglisi, J.C. Meyer, L. D'Arconte, M.A. Brooks and J.A.F. de Silva, J. Chromatogr., 145 (1978) 81.
5 J. Vasiliades, C. Owens and D. Pirkle, Clin. Chem., 25 (1979) 311.
6 J. Vasiliades, C. Owens and F. Ragusa, Clin. Chem., 25 (1979) 1900.

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Biomedical Applications

CHROMBIO. 558

Note

Gas—liquid chromatographic determination of acephylline in urine

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Acephylline piperazine (Acepiphylline, Etaphylline^{R}) has been used for the treatment of asthma for over more than 30 years in many countries. Acephylline is N-7-theophylline acetic acid. Recently the pharmacokinetics, after oral and intravenous (i.v.) administration, have been described [1,2]. A rapid high-performance liquid chromatographic method for the determination of acephylline in human serum has been reported [3]. This method cannot be used for the determination of acephylline in urine since interfering peaks occur. A gas chromatographic method has been developed, which makes use of the OV-17 column. The internal standard used is N-7-theophylline propionic acid which is a structural analogue.

MATERIALS AND METHODS

Synthesis of N-7-theophylline acetic acid (acephylline)

Acephylline was synthesized by reacting sodium chloroacetate and sodium theophyllinate in water, as described by Ride et al. [4]. Identity and purity were confirmed by IR, NMR, HPLC thin-layer (TLC) and gas—liquid chromatography.

Synthesis of N-7-theophylline propionic acid

The internal standard (i.s.) was synthesized in a two-step synthesis. N-7-Theophylline-3-propionitrile was synthesized by reacting theophylline and acrylonitrile in the presence of Triton B. The nitrile is hydrolyzed in concentrated hydrochloric acid, resulting in N-7-theophylline propionic acid. The synthesis is described by Zelnik and Pesson [5]. Identity and purity were confirmed by IR, NMR, TLC, GLC and HPLC.

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Procedure

A 1.0-ml sample of urine was pipetted into a tube, containing 60 μl of 8 *M* hydrochloric acid (the pH has to be about 2) and 0.5 ml of the i.s. solution (150 μg/ml). The sample was extracted twice with 5 ml chloroform—isopropanol (20:1). The tubes were centrifuged for 10 min. The organic layers were separated using a glass syringe with a blunt needle, collected and mixed well.

The chloroform—isopropanol mixture was evaporated off at 70°C under nitrogen until a volume of 500 μl was reached. This was transported in a small glass ampoule. The chloroform—isopropanol mixture was evaporated to dryness. Then 100 μl of a 10% ethyl iodide solution in acetonitrile and 5—10 mg potassium carbonate were added. The ampoule was fused after replacement of air by nitrogen and heated for 1 h at 80°C [6]. From this mixture 1—2 μg was injected into the chromatograph (Perkin-Elmer 3920).

GLC conditions: injection temperature 300°C, column temperature 255°C, detection temperature 300°C; column (2 m × 3 mm I.D.), 3% OV-17 on Gas-Chrom Q (100—120 mesh) (Chrompack, Middelburg, The Netherlands); nitrogen flow-rate 40 ml/min; flame ionization detection.

The results were calculated with a Hewlett-Packard integrator, type 3380A.

RESULTS AND DISCUSSION

Chromatograms of urine samples demonstrate that no contamination peaks occur (Figs. 1 and 2). The retention time of acephylline is about 6 min. Dietary xanthines, caffeine, theophylline and theobromine do not interfere with the assay. The standard curve of acephylline added to urine was linear over the ranges 0—5, 0—10, 0—100 and 0—1000 μg/ml and passed through the origin. The correlation coefficients were $r = 0.968$ ($n = 5$, $P < 0.01$); $r = 0.975$ ($n = 12$, $P < 0.01$); $r = 0.991$ ($n = 15$, $P < 0.01$) and $r = 0.986$ ($n = 15$, $P < 0.01$) respectively.

Normal urine concentrations in patients and volunteers vary from 0 to 100 μg/ml after oral administration and to a maximum variation of 1000 μg/ml after i.v. administration. The within-run variation was determined by the repetitive injection of a sample derived from spiked urine with a concentration of 50 μg/ml. The recovery was 100.5% as could be calculated from the mean. The within-run variations were 5.0, 7.4 and 9.9%, expressed as the variation coefficient at 5, 10 and 100 μg/ml respectively, ($n = 10$, $n = 13$ and $n = 11$). The between-run variation was determined by a daily extraction of the 10 μg/ml urine samples and injection onto the gas chromatograph over two weeks. The between-run variation was 6.8%, expressed as the variation coefficient ($n = 10$). This shows that the between-run-variation is not different from the within-run-variation.

We compared a standard curve (0—100 μg/ml), using N-7-theophylline propionic acid as external standard, carrying out only the chromatographic procedure (correlation coefficient $r = 0.599$) with a standard curve, using this substance as internal standard, carrying out the extraction procedure as well (correlation coefficient $r = 0.999$). The results stipulate the important role of the internal standard and the large variation in the extraction efficiency of both acephylline and the internal standard.

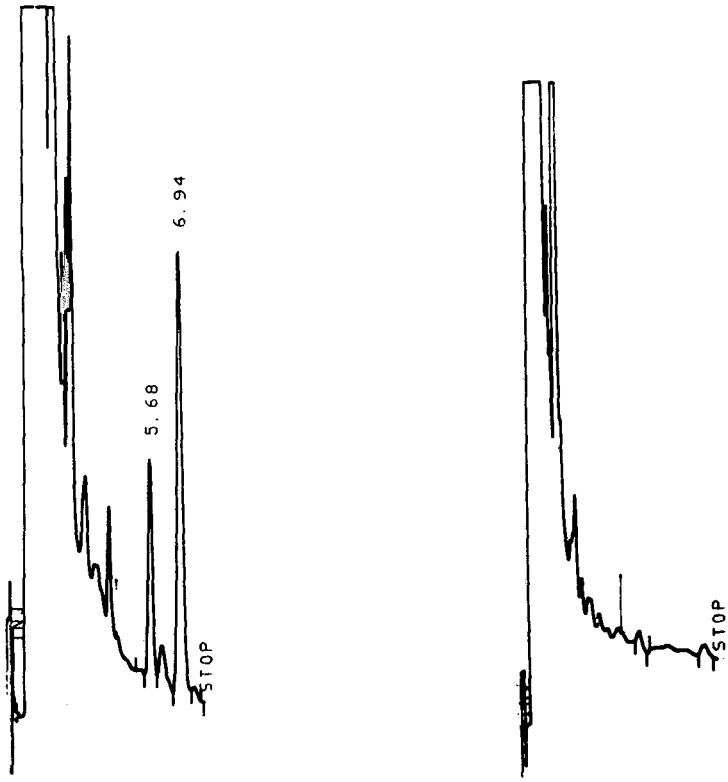

Fig. 1. Chromatogram of a urine sample containing acephylline, 25 μg/ml, retention time = 5.68 and internal standard, 50 μg/ml, retention time = 6.94.

Fig. 2. Chromatogram of a blank urine sample.

We determined urine samples obtained from volunteers and patients with this GLC method. We discovered the low absorption efficiency of acephylline and the rapid complete renal elimination [1,2]. Acephylline appears to be about 50% bound in the urine. These investigations will be continued, focussing on the absorption and elimination mechanisms.

REFERENCES

1 J. Zuidema and F.W.H.M. Merkus, Lancet, i (1978) 1318—1319.
2 J. Zuidema and F.W.H.M. Merkus, Pharm. Weekbl., 113 (1978) 605—610.
3 J. Zuidema and F.W.H.M. Merkus, J. Chromatogr., 145 (1978) 489—491.
4 S.M. Ride, A.M. Farghally and F.A. Ashour, Pharmazie, 32 (1977) 672—676.
5 R. Zelnik and M. Pesson, Bull. Soc. Chim. Fr., (1959) 1667—1669.
6 F.N. Ydenberg, Pharm. Weekbl., 110 (1975) 21—24.

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Biomedical Applications

CHROMBIO. 538

Note

High-performance liquid chromatographic determination of ceftizoxime, a new cephalosporin antibiotic, in rat serum, bile and urine

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Ceftizoxime [(6*R*,7*R*)-7-[(*Z*)-2-(2-imino-4-thiazolin-4-yl)-2-methoxyimino-acetamido]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid], a semi-synthetic cephalosporin derivative, is a new broad-spectrum antibiotic developed by Takaya et al. [1]. We required a simple and rapid method of determining ceftizoxime for pharmacokinetic studies in rats and dogs given [^{14}C]ceftizoxime.

Various methods of analysis have been used to determine cephalosporins in body fluids. Concentrations of antibiotic in the serum or other body fluids have traditionally been determined by microbiological assay rather than by chemical methods. However, high-performance liquid chromatography (HPLC) for the quantitative determination of cephalosporin antibiotics in biological fluids has recently been shown to have definite advantages over routine bioassay in rapidity, precision and specificity.

Cooper et al. [2] reported the use of high-speed ion-exchange liquid chromatography for the separation and analysis of cephalothin and deacetylcephalothin in human serum after ion-pair extraction. Rapid analysis of cefazolin in dog and human serum by HPLC has been reported by Wold [3]. In 1978, Nilsson-Ehle and Nilsson-Ehle [4] reported a liquid chromatographic assay of cefuroxime in serum. Direct HPLC determination of cephalexin in human urine has recently been reported by Nakagawa et al. [5].

In the present paper, we describe a method for determining ceftizoxime in rat serum, bile and urine by direct injection of deproteinized biological fluids into a reversed-phase liquid chromatographic column. This technique is more simple and rapid than microbiological assays and is reproducible and sensitive.

EXPERIMENTAL

Reagents and materials

Ceftizoxime (Fig. 1) was prepared by the Fujisawa Pharmaceutical Co. (Osaka, Japan). Acetonitrile of UV grade was used. All the other solvents and reagents were of analytical reagent grade and were used without further purification.

The following aqueous solutions were made in distilled water: 0.2 *M* KH_2PO_4, 0.02 *M* KH_2PO_4, 0.2 *M* H_3PO_4, 0.02 *M* H_3PO_4, 10% acetic acid and 1% acetic acid.

Blank dog serum was obtained from the fresh blood of male beagle dogs. Blank rat serum was obtained from male SD rats fasted overnight. The sera were pooled and stored at −20°C in glass containers until analyzed.

Apparatus

Analyses were made on Waters Assoc. liquid chromatograph equipped with a Model 440 absorbance detector (254 nm or 280 nm fixed wavelength), a Model 6000A pump, a U6K universal injector and a 10-mV recorder.

Chromatographic conditions

A 30 cm × 4 mm I.D. μBondapak Alkyl Phenyl analytical column (Waters Assoc., Milford, MA, U.S.A.; particle size 10 μm) and a 5 cm × 2 mm I.D. precolumn packed with Bondapak Phenyl Corasil (37—50 μm) were used for analysis.

Although a number of mobile phases were tested during the investigation, no single solvent system afforded simultaneous resolution of ceftizoxime and endogenous components of the serum, bile and urine. Therefore, mobile phases of 13% acetonitrile in 0.02 *M* KH_2PO_4—H_3PO_4 buffer (pH 2.6), 11% acetonitrile in 0.02 *M* KH_2PO_4—H_3PO_4 buffer (pH 2.6), and 13% acetonitrile in 1% acetic acid were used for serum, bile and urine samples, respectively. The mobile phase was de-aerated under vacuum for approximately 1 min before use.

Bile and urine samples were detected at 280 nm using sensitivity settings of 0.05—0.2 absorbance unit full scale (a.u.f.s.). Serum samples were detected at 254 nm using sensitivity settings of 0.01 and 0.05 a.u.f.s. The operating temperature was ambient, and the flow-rate was 2.0 ml/min. The chart speed was 0.5 or 1.0 cm/min.

Preparation of standard solutions

All stock solutions of ceftizoxime were prepared by diluting a 50 mg per 100 ml primary standard solution with distilled water to make 0.2, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 μg per 0.1 ml concentrations. The solutions were used

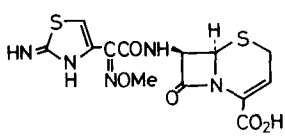

Fig. 1. Chemical structure of ceftizoxime.

for preparing standard curves and in reproducibility studies. The solutions were stored in glass containers at −20°C until analyzed.

Determination of standard curve

Serum. A 0.5-ml sample of serum was placed in a sample tube, and 0.1 ml of acetonitrile, 0.1 ml of 0.2 *M* KH_2PO_4—H_3PO_4 buffer (pH 2.6) and 0.1 ml of ceftizoxime standard solution (0.2—50 μg) were added. After about 10 min, the mixture was filtered through a 0.5-μm membrane filter. Then, 10 μl of the solution were injected into the liquid chromatograph equipped with a UV detector monitoring absorbance at 254 nm at sensitivity settings of 0.01—0.05 a.u.f.s. Standard curves were obtained by plotting peak height against concentration of ceftizoxime.

Bile. A 0.1-ml sample of rat bile was placed in a sample tube, and 0.4 ml of distilled water, 0.1 ml of acetonitrile, 0.1 ml of 0.2 *M* KH_2PO_4—H_3PO_4 buffer (pH 2.6) and ceftizoxime standard solution (1—50 μg) were added. After 10 min, the mixture was filtered through a 0.5-μm membrane filter. Then, 10 μl of the solution were injected into the liquid chromatograph equipped with a UV detector monitoring absorbance at 280 nm at a sensitivity setting of 0.05 a.u.f.s. The peak height was plotted against concentration of ceftizoxime.

Urine. Urine samples of 100 μl volume were diluted ten times with water; a 0.5-ml sample was then placed in a sample tube, and 0.1 ml of acetonitrile, 0.1 ml of 10% acetic acid and 0.1 ml of ceftizoxime standard solution (10—200 μg) were added. After about 10 min, the mixture was filtered through a 0.5-μm membrane filter, and 10 μl of the filtrate were injected into the liquid chromatograph equipped with a UV detector monitoring absorbance at 280 nm at sensitivity settings of 0.05—0.2 a.u.f.s. A standard curve was obtained by adding known amounts of ceftizoxime to the control urine. The peak areas or peak heights of ceftizoxime were plotted against the concentrations of ceftizoxime. Peak areas were obtained from the product of the maximum peak height and width at peak half-height.

RESULTS AND DISCUSSION

Separation

The liquid chromatograms of ceftizoxime from the spiked serum, bile and urine samples are shown in Figs. 2, 3 and 4, respectively. As shown in Fig. 2, the background peaks of the control serum have short retention times and are almost completely separated from those of ceftizoxime. Initially, the bile and urine samples were chromatographed under the same chromatographic conditions as the serum samples. Under those conditions, however, peaks interfering with certizoxime were present on the chromatograms from the control bile and urine. Therefore, a UV detector monitoring absorbance at 280 nm and different mobile phases were used to eliminate the interfering peaks from the chromatogram. The chromatograms of bile and urine samples obtained under these conditions are shown in Figs. 3 and 4. No interfering peaks appeared in any blank bile or urine samples at a retention time similar to that of ceftizoxime. Under these conditions, the retention times for ceftizoxime from serum, bile and urine were 5.0, 6.3 and 6.2 min, respectively.

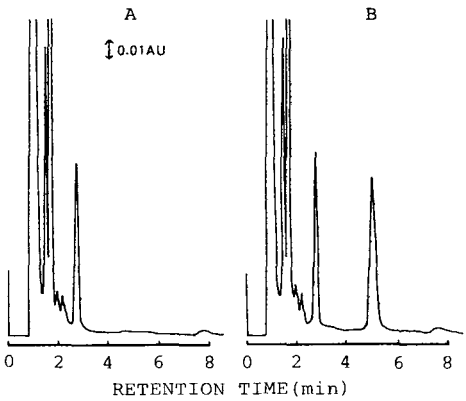

Fig. 2. Chromatograms of blank serum (A) and serum containing 20 μg of ceftizoxime per ml (B). Conditions: column, 30 cm × 4 mm μBondapak Alkyl Phenyl; mobile phase, acetonitrile—0.02 *M* KH_2PO_4—H_3PO_4 (pH 2.6) (13:87, v/v); flow-rate, 2.0 ml/min; detection, UV at 254 nm.

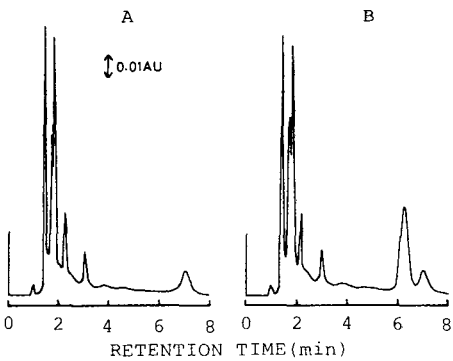

Fig. 3. Chromatograms of blank bile (A) and bile containing 100 μg of ceftizoxime per ml (B). Conditions: column, 30 cm × 4 mm μBondapak Alkyl Phenyl; mobile phase, acetonitrile—0.02 *M* KH_2PO_4—H_3PO_4 (pH 2.6) (11:89, v/v); flow-rate, 2.0 ml/min; detection, UV at 280 nm.

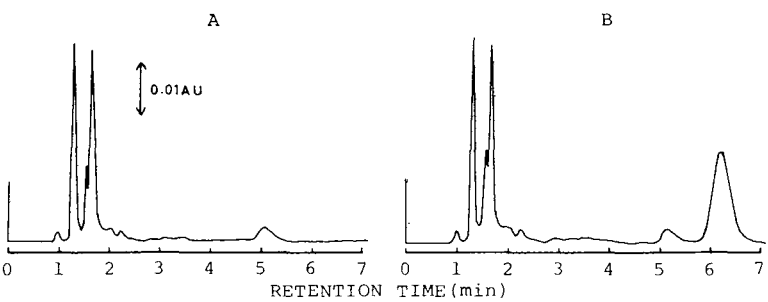

Fig. 4. Chromatograms of blank urine (A) and urine containing 1000 μg of ceftizoxime per ml (B). Conditions: column, 30 cm × 4 mm μBondapak Alkyl Phenyl; mobile phase, acetonitrile—1% acetic acid (13:87, v/v); flow-rate, 2.0 ml/min; detection, UV at 280 nm.

Stability of ceftizoxime

As the assay procedure is extremely simple and does not involve an evaporation step or alkalinity, the degradation of ceftizoxime, which is unstable in alkaline media, is avoided. Ceftizoxime in the serum, bile, urine and mobile phase solutions was stable for more than 6 h at room temperature and for 2 days at 4°C. Ceftizoxime standard solutions were stable for at least one month when the samples were stored at −20°C.

Recovery

Sample recovery of ceftizoxime from spiked solutions prepared with biological fluids was compared with that with water. The results are given in Table I. The peak of ceftizoxime in the spiked urine was low when determined by peak height measurement. It is presumed that this low peak was broadened by urinary components. These results suggest that the peak area measuring method is better than the peak height measuring method for the standard curve for urine.

TABLE I

RECOVERY OF CEFTIZOXIME FROM SPIKED BIOLOGICAL FLUIDS

Recovery = (biological fluid value/water control value) × 100 (n = 5 or 6). Chromatographic conditions are described under Experimental.

Biological fluid	Ceftizoxime range (μg/ml)	Peak measuring method	Recovery (mean ± S.D.)
Serum	0.2—5	Peak height	99.7±2.2
Serum	1—100	Peak height	101.8±5.0
Bile	10—500	Peak height	100.8±3.7
Urine	100—4000	Peak height	87.1±1.9
Urine	100—4000	Peak area	99.3±5.8

Standard curve

Standard curves were obtained by plotting peak height or peak area against concentration of ceftizoxime. A straight line was plotted by least-squares regression analysis, and its slope and intercept at the peak height or peak area was determined (Table II). The correlation coefficients (r) calculated from the regression lines were 0.9998 or better.

TABLE II

LINEAR REGRESSION DATA FOR CALIBRATION CURVES

Conditions for HPLC are given under Experimental.

Biological fluid	Concentration range (μg/ml)	Regression		Correlation coefficient (r)
		Intercept	Slope	
Serum	0.2—5	−0.015	1.004	0.9998
Serum	1—100	+0.047	0.371	0.9999
Bile	10—500	+0.016	0.222	0.9999
Urine	100—4000	+0.018	0.071	0.9998

The lower limit of sensitivity was 10 μg/ml for the bile sample and 100 μg/ml for the urine sample. The determination limit for serum samples can be decreased to 0.2 μg/ml by the use of sensitivity setting of 0.01 a.u.f.s.

Reproducibility

Reproducibility was obtained by adding known amounts of ceftizoxime to the serum and by comparing five samples with a single calibration curve. The results are given in Table III. The data in Table III demonstrate the usefulness of HPLC in the analysis of ceftizoxime in the serum.

The procedure described here was used to obtain an accurate and precise determination of ceftizoxime in dog serum and urine. In this case also was the good accuracy and precision obtained.

TABLE III

REPRODUCIBILITY OF CEFTIZOXIME DETERMINATION IN RAT SERUM

Actual ceftizoxime conc. (μg/ml)	0.60	3.00
Number	5	5
Mean analyzed conc. (μg/ml)	0.57	3.06
Percentage of actual conc.	96.7	102
S.D.	0.02	0.05
Range (μg/ml)	0.55—0.59	3.01—3.10
Coefficient of variation (%)	3.5	1.7

ACKNOWLEDGEMENT

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REFERENCES

1 T. Takaya, H. Takasugi, K. Tsuji and T. Chiba, Pat. Ger. Offen., 2,810,922 (1978); C.A., 90 (1979) 204116k.
2 M.J. Cooper, M.W. Anders and B.L. Mirkin, Drug Metab. Dispos., 1 (1973) 659.
3 J.S. Wold, Antimicrob. Ag. Chemother., 11 (1977) 105.
4 I. Nilsson-Ehle and P. Nilsson-Ehle, Clin. Chem., 24 (1978) 365.
5 T. Nakagawa, J. Haginaka, K. Yamaoka and T. Uno, J. Chromatogr., 147 (1978) 509.

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Biomedical Applications

CHROMBIO. 532

Note

High-performance liquid chromatographic determination of cyclosporin A in human plasma and urine

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Cyclosporin A (CyA; OL 27-400) is a new fungal cyclic polypeptide with great potential as an immunosuppressive agent in man [1, 2]. The compound has a molecular weight of 1202 and the structural formula is given in Fig. 1.

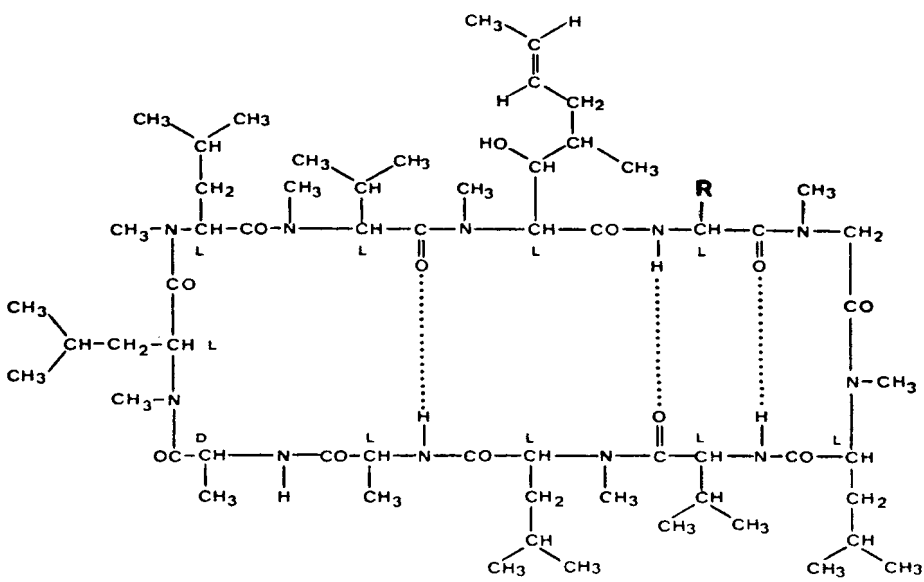

Fig. 1. Structural formula of cyclosporin A (R = CH_2—CH_3) and cyclosporin D [R = $CH(CH_3)_2$].

In this paper, a method for the determination of cyclosporin A in human plasma and urine is described, using a high-performance liquid chromatographic (HPLC) separation in a reversed-phase mode at 72°C and a UV absorption detector at 210 nm. At this wavelength, the compound has a molar extinction ϵ of 45,000 (ϵ_{max} = 66,000 at 195 nm). As an internal standard, cyclosporin D (CyD; 33-804) is used, because it differs only slightly from CyA in structure (Fig. 1) and chromatographic properties.

EXPERIMENTAL

Reagents

For the mobile phases in chromatography, methanol (Lichrosolv, Merck, Darmstadt, G.F.R.), acetonitrile (HPLC grade S, 50% minimal transmission at 205 nm, Rathburn Chemicals, Walkerburn, Great Britain), and water (demineralized and filtered on a Milli-Q-filter) were used. For the extraction, diethyl ether of analytical grade was used. For the development of the method, outdated heparinized blood bank plasma was used.

Standard solutions of cyclosporin A and cyclosporin D (internal standard) at a concentration of 2500 ng/ml each in methanol—water (1:49) were stored at 3°C throughout the study. Both compounds were supplied by Sandoz (Basle, Switzerland).

Extraction procedure

Conical glass centrifuge tubes (25 ml) were rinsed with diethyl ether and dried. One ml of human plasma (or urine) was added and spiked with a standard solution of CyA and of CyD (200 μl, containing 500 ng CyD). Water was added to a total volume of 2 ml and mixed with a vortex mixer during 5 sec. For the extraction, 10 ml of diethyl ether were added. The tubes were shaken mechanically for 20 min on a horizontal shaker (Model Sm 2, Bühler, Tübingen, G.F.R.) at 160 rpm and centrifuged for 40 min at 800 *g*. An 8-ml aliquot of the separated diethyl ether layer was then transferred to another conical centrifuge tube, which had been rinsed previously with diethyl ether. The diethyl ether extract was evaporated to dryness under vacuum for 45 min with a vortex evaporator (Buchler, Fort Lee, NJ, U.S.A.), starting at room temperature and ending at 50°C. The plasma residue, concentrated now at the bottom of the conical tube, was either stored at 3°C or dissolved in 120 μl of the mobile phase B (see under Chromatographic conditions) and mixed on a vortex mixer. An aliquot of 100 μl was injected into the chromatograph.

Chromatographic conditions

The liquid chromatograph consisted of two pumps (Altex Scientific, Berkeley, CA, U.S.A., Model 110A), which were operated with a gradient programmer (Altex, Model 420). A pressure filter (Altex, Model 110-40) reduced the pulsation and a dynamic mixer (Altex, Model 400) mixed the mobile phases of both pumps. Injection was made with a 100-μl syringe (Hamilton, Bonaduz, Switzerland) via a sample injector (Rheodyne, Berkeley, CA, U.S.A., Model 7120 with a 100-μl loop). The absorption at 210 nm was determined at a sensitivity of 0.02 a.u.f.s. with a UV detector (Uvicon LCD 725, Kontron,

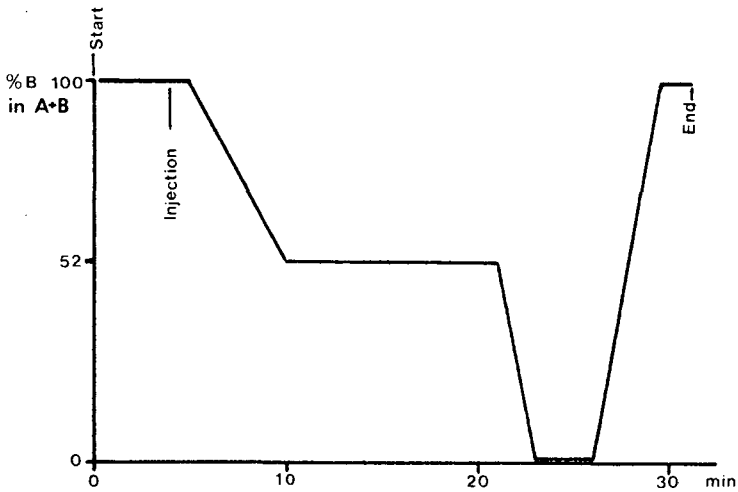

Fig. 2. Gradient profile of the mobile phases water—acetonitrile—methanol (50:750:200) (pump A) and (600:200:200) (pump B).

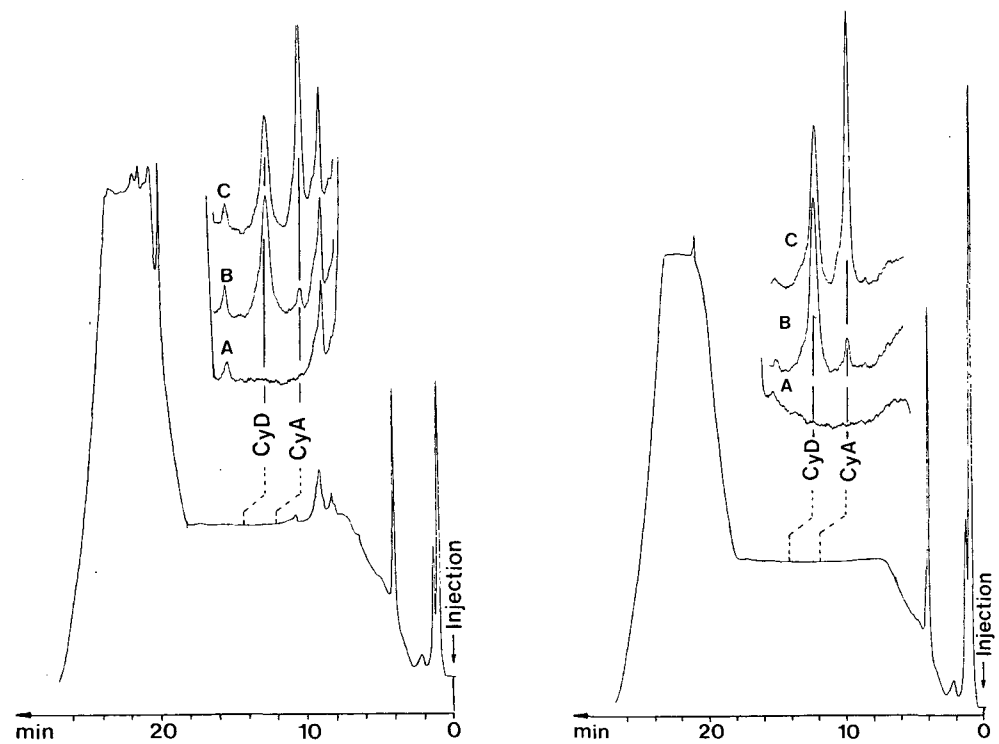

Fig. 3. Liquid chromatograms of (A) blank human plasma and (B) spiked with 50 ng/ml and (C) 500 ng/ml of cyclosporin A. The internal standard cyclosporin D was added at a concentration of 500 ng/ml. The lowest curve is trace A, 10 times attenuated.

Fig. 4. Liquid chromatograms of (A) blank human urine and (B) spiked with 50 ng/ml and (C) 500 ng/ml of cyclosporin A. The internal standard cyclosporin D was added at a concentration of 500 ng/ml. The lowest curve is trace A, 10 times attenuated.

Zürich, Switzerland), which was connected to a two-channel recorder (W + W, Tarkan 600, Kontron).

A stainless-steel column (12.5 cm× 0.3 cm I.D.) was slurry packed (Sandoz) with 5-μm particle size LiChrosorb RP-8 (Merck). It was operated at 72°C [3] inside a Plexiglas jacket (Knauer, Oberursel, G.F.R.), connected to a water circulating thermostat (Haake, Karlsruhe, G.F.R.). To ensure temperature equilibration of the mobile phase, a capillary of 1 m length was placed in front of the column (inside the jacket) [4, 5]. Chromatography was performed using a gradient of the profile given in Fig. 2. The mobile phases consisted of water—acetonitrile—methanol with a composition of 50:750:200 for pump A and of 600:200:200 for pump B. They were degassed with helium before use. With a flow-rate of 1.5 ml/min, the retention times are 12.2 min for cyclosporin A and 14.6 min for cyclosporin D (Figs. 3 and 4). The peak heights were determined graphically.

Human studies

Blood samples were withdrawn into heparinised tubes from two volunteer male human subjects who had received single oral doses of 600 mg cyclosporin A as an olive oil solution (24 ml). The samples were centrifuged, the plasma pipetted off and deep frozen until analysis.

RESULTS AND DISCUSSION

Concentrations of cyclosporin A in plasma and urine were determined from calibration curves for the peak height ratio of the drug CyA to the internal standard CyD (500 ng/ml) over the concentration range 0—2000 ng/ml. The plasma and urine samples, taken in triplicate throughout the extraction procedure described, gave the chromatograms shown in Figs. 3 and 4. For the ratio of the peak heights for CyA to CyD, the coefficients of variation (for 3 determinations) were ± 10, ± 6, ± 9, ± 7, ± 4, ± 1.5 and ± 2.5% for plasma and ± 16, ± 17, ± 7, ± 1, ± 5, ± 1 and ± 2% for urine at 25, 50, 100, 250, 500, 1000 and 2000 ng/ml, respectively. The correlation coefficients of 0.99993 for plasma and 0.99976 for urine indicate a good linearity of the calibration curve. The recovery of CyA was found to be 76 ± 5% in plasma and 104 ± 5% in urine at a concentration of 500 ng/ml.

As no interfering peaks were present in blank plasma and urine (Figs. 3 and 4), the detection limit is determined by the detector noise, which arises mainly from the pulsation of the pumps. Assuming double determinations for later applications, a concentration of 20 ng/ml in plasma or urine differed significantly from the blank according to the *t*-test.

The use of the internal standard CyD is essential for the good precision and linearity of the method. A considerably higher coefficient of variation was found in the upper concentration range taking the peak height of CyA alone compared to the ratio of CyA and CyD. In addition, the correlation coefficients were lower without the internal standard.

The analytical method was applied to plasma and urine samples from the two human subjects dosed with cyclosporin A. The results in Table I show that

TABLE I

PLASMA CONCENTRATIONS OF CYCLOSPORIN A AFTER A SINGLE ORAL DOSE OF 600 mg TO 2 HUMAN SUBJECTS

Time	Plasma concentration* (ng/ml)	
(h)	Subject A	Subject B
0	0	0
1	109 ± 6	0
2	554 ± 17	193 ± 6
4	335 ± 17	642 ± 29
6	176 ± 20	176 ± 8
8	119 ± 6	121 ± 5
24	<20	37 ± 7

*Mean ± standard deviation for $n = 2$.

plasma levels reach a maximum of about 600 ng/ml after 2 h and 4 h for subjects A and B, respectively. The plasma concentrations then decline with an apparent half-life of 5 h (subject A) and 8 h (subject B) for the terminal slope. From urine data, a cumulative excretion of 0.21 and 0.27% of the dose for unchanged drug was found between 0 and 24 h. Peaks from other compounds, known from a radiotracer study [6], were well separated from the peak of the parent compound, thus indicating a good specificity of the method.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. H. Keller, W. Pacha and G. Maurer for helpful discussions.

REFERENCES

1 A. Rüegger, M. Kuhn, H. Lichti, H.R. Loosli, R. Huguenin, C. Quiquerez and A. von Wartburg, Helv. Chim. Acta, 59 (1976) 1075.
2 R.Y. Calne, D.J.G. White, S. Thiru, D.B. Evans, P. McMaster, D.C. Dunn, G.N. Craddock, B.D. Pentlow and K. Rolles, Lancet, ii (1978) 1323.
3 F. Erni, personal communication.
4 R. Freiburghaus, personal communication.
5 R.J. Perchalski and B.J. Wilder, Anal. Chem., 51 (1979) 774.
6 G. Maurer, personal communication.

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Biomedical Applications

CHROMBIO. 561

Note

High-performance liquid chromatographic determination of chloramphenicol and its monosuccinate ester in plasma

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When used parenterally, chloramphenical is administered as a water-soluble salt of the monosuccinate ester of chloramphenicol. Because plasma samples obtained shortly after intravenous administration may contain both chloramphenicol and the monosuccinate pro-drug, and because urine has been reported to contain up to 30% of the chloramphenicol dose in the form of the unhydrolyzed monosuccinate ester [1], it is necessary that analytical methods be capable of distinguishing between these species. Furthermore, it is necessary that specimens be processed in a manner which avoids in vitro conversion of the monosuccinate pro-drug to chloramphenicol.

Several high-performance liquid chromatographic (HPLC) assays for chloramphenicol in biological fluids have been published [2—6]. One procedure [4], using drug extraction from biological fluids adjusted to pH 10.4, would be expected to effectively separate chloramphenicol and hemisuccinate ester in the extraction step, although there was no consideration of potential for error due to hydrolysis of the monoester pro-drug to chloramphenicol in pH 10.4 buffer. The present communication addresses the above question and also presents methodology for HPLC quantitation of both chloramphenicol and its monosuccinate ester pro-drug in plasma.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatographic system consisted of a Milton Roy Minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) equipped with a variable-wavelength spectrophotometric detector, Vari-chrom (Varian, Palo Alto, CA, U.S.A.) and a Houston Instrument OmniScribe recorder (Austin, TX, U.S.A.). A Rheodyne loop injector (Berkeley, CA, U.S.A.) with a 100-μl

sampling loop was used to introduce the samples onto a LiChrosorb C_8 reversed-phase column, 10 μm (Brownlee Labs., Santa Clara, CA, U.S.A.). During the development of the calibration curves, a Waters Assoc. WISP 710A automatic injector (Milford, MA, U.S.A.) was used.

Operation conditions

The variable wavelength UV-VIS detector was set at 280 nm. The sensitivity of the detector was 0.05 a.u./10 mV output. The flow-rate was held constant at 2.5 ml/min. The column pressure was between 2000 and 3000 p.s.i.

Mobile phase

The mobile phase, methanol—0.05% aqueous phosphoric acid (4:6), was prepared by direct admixture and degassed by water pump aspiration for 5 min.

Routine sample preparation

To 0.4 ml plasma in 16 × 125 mm culture tubes were added 50 μl ethylparaben (Aldrich, Milwaukee, WI, U.S.A.) (20 mg%) as internal standard, 1 ml of a 0.1 *M* citric acid—0.2 *M* Na_2HPO_4 (803:197) pH 3.0 aqueous buffer, and 5 ml anhydrous diethyl ether. The contents were mixed for 30 sec via gentle tube inversion to prevent gel formation and were then centrifuged for 3 min. The organic layer was transferred and evaporated to dryness via a gentle air stream at room temperature. The samples were then reconstituted with 100 μl of mobile phase, and 20-μl aliquots were chromatographed.

Pooled human plasma samples supplemented with chloramphenicol concentrations up to 40 μg/ml were also analyzed to establish standard calibration curves on a daily basis.

Standards for assay validation

Standard curves were developed for chloramphenicol (Aldrich) and the 3-monosuccinate ester of chloramphenicol. Chloramphenicol-3-monosuccinate was isolated by acidifying a solution of chloramphenicol sodium succinate for injection (Parke, Davis and Co., Detroit, MI, U.S.A.). The precipitate was collected and recrystallized from a water—methanol mixture.

Samples containing from 2.5 to 40.0 μg/ml chloramphenicol or chloramphenicol-3-monosuccinate and 20 μg/ml ethylparaben (internal standard) were prepared in pooled human plasma. Extractions of six samples of each of the five concentrations were done prior to chromatography for both the chloramphenicol and chloramphenicol-3-monosuccinate samples.

Direct HPLC injections of aqueous samples of the same concentrations of chloramphenicol and its 3-monosuccinate ester were also chromatographed to assess the plasma extraction recovery.

Stability of chloramphenicol-3-monosuccinate

Aliquots of 20 μl of a solution containing 1 ml chloramphenicol-3-monosuccinate aqueous (1 mg/ml), 1 ml ethylparaben aqueous (25 mg%), and 8 ml of citric acid—phosphate pH 3.0 buffer were chromatographed at 20-min intervals for seven injections.

Similarly, direct 20-μl injections of a solution buffered with 8 ml of 0.8 *M*

tris(hydroxymethyl) aminomethane (Tris) pH 10.4 buffer were also chromatographed.

RESULTS AND DISCUSSION

Typical chromatograms for chloramphenicol and chloramphenicol-3-monosuccinate aqueous standards are shown in Fig. 1. Under these chromatographic

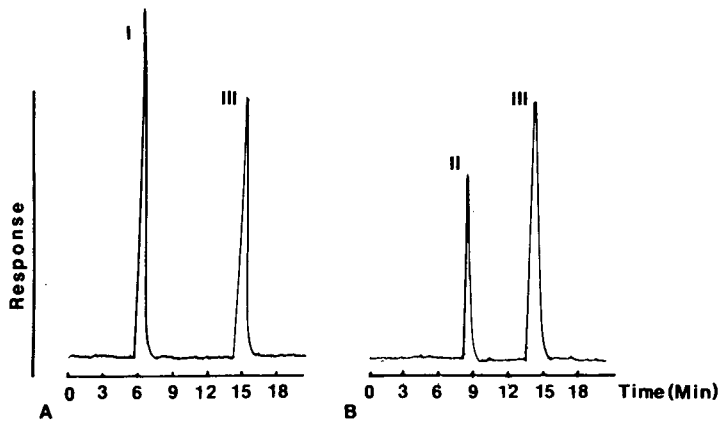

Fig. 1. Representative chromatograms obtained from direct HPLC 20-μl injections of (A) 10 μg/ml aqueous chloramphenicol; (B) 10 μg/ml aqueous chloramphenicol-3-monosuccinate. Peaks: I, chloramphenicol; II, chloramphenicol-3-monosuccinate; III, ethylparaben (internal standard).

conditions, chloramphenicol, its 3-monosuccinate ester, and ethylparaben (internal standard) have retention times of 6, 9, and 15 min, respectively. Retention times were unchanged with the samples of chloramphenicol and chloramphenicol-3-monosuccinate extracted from plasma.

The major metabolites, the 3-glucuronide and deacylated form of chloramphenicol [7] are more polar than the parent drug. Under the reversed-phase separation conditions, they would be eluted early from the column and not interfere with the drug peak [2,4].

The precision of this method was determined by assaying samples of known concentrations of chloramphenicol and its 3-monosuccinate ester. The results of this study, which involved extraction of the drug from plasma, are given in Table I. Peak height ratios (peak height drug/peak height internal standard) were used for quantitation of drug concentration. From comparison of chloramphenicol and chloramphenicol-3-monosuccinate peak heights obtained from direct injection of aqueous solutions and from samples carried through the assay procedure, extraction efficiency was estimated as 51% (coefficient of variation 10%, $n = 5$) for chloramphenicol and 45% (coefficient of variation 8%, $n = 5$) for the 3-monosuccinate ester.

It was desired to demonstrate that citric acid—phosphate pH 3.0 buffer used in the sample preparation did not cause hydrolysis of chloramphenicol-3-monosuccinate and at the same time determine the stability of the ester to 0.8 *M* Tris pH 10.4 buffer. A constant peak height ratio obtained over a 2-h period

TABLE I

PRECISION OF EXTRACTION PROCEDURE AND ANALYSIS OF CHLORAMPHENICOL AND CHLORAMPHENICOL-3-MONOSUCCINATE FROM PLASMA

$n = 6$.

Concentration (μg/ml)	Peak height ratio (mean)	Standard deviation	Coefficient of variation (%)
Chloramphenicol			
2.5	0.304	0.013	4.1
5.0	0.601	0.032	5.3
10.0	1.237	0.021	1.7
25.0	3.218	0.081	2.5
40.0	5.241	0.227	4.3
Chloramphenicol-3-monosuccinate			
2.5	0.177	0.019	10.5
5.0	0.344	0.018	5.2
10.0	0.655	0.006	0.9
25.0	1.718	0.054	3.1
40.0	2.881	0.119	4.1

for chloramphenicol-3-monosuccinate buffered with citric acid—phosphate pH 3.0 buffer substantiated the stability of the drug in this system (Fig. 2).

In the 0.8 *M* Tris pH 10.4 buffer system, hydrolysis of the ester to chloramphenicol occurred slowly; a third peak observed with a retention time of 7.5 min was clearly related to drug concentration (Fig. 3). Sandmann and coworkers [8,9] found that the anion of the 3-monosuccinate ester in aqueous solution at near neutral pH formed an equilibrium mixture with a form which Sandmann identified as a rearranged cyclic hemi-"ortho"-succinate ester of the

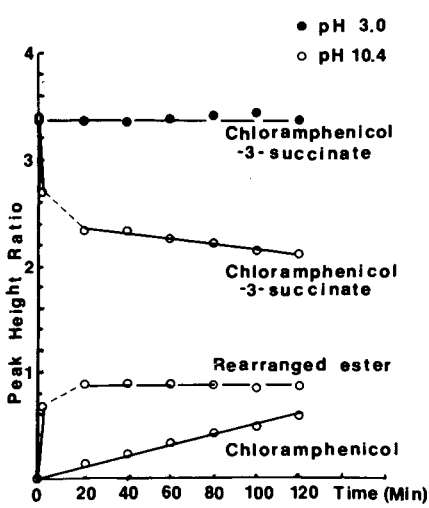

Fig. 2. Peak height ratio (drug species/internal standard) vs. time of direct HPLC injections following addition of citric acid—phosphate pH 3.0 buffer or 0.8 *M* Tris pH 10.4 buffer to aqueous chloramphenicol-3-monosuccinate.

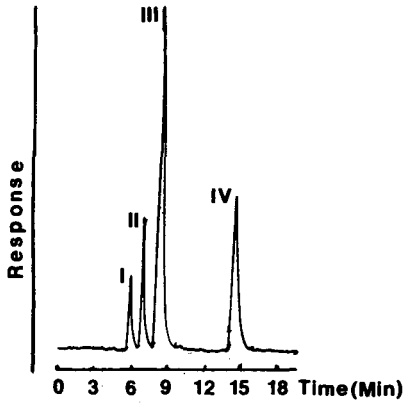

Fig. 3. Chromatogram obtained from direct HPLC injection of aqueous chloramphenicol-3-monosuccinate to which 0.8 *M* Tris pH 10.4 buffer had been added 100 min prior to injection. Peaks: I, chloramphenicol; II, rearranged ester; III, chloramphenicol-3-monosuccinate; IV, ethylparaben (internal standard).

linear 3-monosuccinate ester of chloramphenicol.

The HPLC results of the alkaline buffered solution of chloramphenicol-3-monosuccinate over a 2-h period are represented in Fig. 2. This clearly demonstrates a dramatic decrease in the concentration of chloramphenicol-3-monosuccinate when the solution is buffered at pH 10.4. A 20% rearrangement of this ester occurred immediately; after 20 min, rearrangement to the extent of 30% was observed. Hydrolysis of the esters to chloramphenicol occurred only to the extent of 2% within 20 min. Assuming that the rearranged ester has an absorptivity similar to that for the linear ester, it may be deduced from the peak height ratios for the esters that only 5% of the esters hydrolyzed to chloramphenicol after 1 h exposure to 0.8 *M* Tris pH 10.4 buffer. Confirmation of the hydrolysis was made by calculation using a calibration curve for chloramphenicol.

When chloramphenicol-3-monosuccinate was left in contact with pH 10.4 buffer for 144 h, the rearranged ester followed a first-order decline which paralleled the disappearance of the peak corresponding to the linear ester after approximately 24 h.

In conclusion, this methodology can be used to quantitate chloramphenicol and the monosuccinate ester(s) of chloramphenicol in plasma. Chloramphenicol-3-monosuccinate is stable to hydrolysis for a minimum of 2 h when buffered with a citric acid–phosphate pH 3.0 buffer. No internal rearrangement of the linear ester occurs with this buffer. Although the use of 0.8 *M* Tris pH 10.4 buffer causes rapid rearrangement of the linear 3-monosuccinate ester of chloramphenicol, hydrolysis of ester to chloramphenicol occurs slowly. Consequently, unless this buffer remains in contact with the ester(s) for prolonged periods, it is unlikely that any ester present in the plasma will be erroneously reported as chloramphenicol.

REFERENCES

1 A.J. Glazko, W.A. Dill, A.W. Kinkel, J.R. Goulet, W.J. Holloway and R.A. Buchanan, Clin. Pharmacol. Ther., 21 (1977) 104.
2 J.R. Koup, B. Brodsky, A. Lau and T.R. Beam, Jr., Antimicrob. Ag. Chemother., 14 (1978) 439.
3 G.W. Peng, M.A.F. Gadalla and W.L. Chiou, J. Pharm. Sci., 67 (1978) 1036.
4 R.L. Thies and L.J. Fischer, Clin. Chem., 24 (1978) 778.
5 J. Crechiolo and R.E. Hill, J. Chromatogr., 162 (1979) 480.
6 J.M. Wal, J.C. Peleran and G. Bories, J. Chromatogr., 145 (1978) 502.
7 A. Glazko, in G.L. Hobby (Editor), Proc. 6th Intersci. Conf. Antimicrob. Ag. Chemother., Philadelphia, PA, October 26—28, 1966, American Society for Microbiology, Ann. Arbor, MI, 1967, p. 655.
8 B. Sandmann, D. Szulczewski, J. Windheuser and T. Higuchi, J. Pharm. Sci., 59 (1970) 427.
9 B.J. Sandmann, Ph.D. dissertation, University of Wisconsin, Madison, WI, 1969.

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Biomedical Applications

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Note

High-performance liquid chromatographic method for isolation of tritiated digoxin and metabolites in urine

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High-performance liquid chromatography (HPLC) with UV detection has been used successfully to isolate and quantitate digoxin, digoxigenin and its mono- and bis-digitoxosides [1, 2], but not with the sensitivity necessary for assay of therapeutic concentrations in biological fluids. To achieve this level of sensitivity, it has been necessary to combine HPLC and radioimmunoassay [3, 4]. The use of tritiated digoxin as a tracer gives improved specificity and sensitivity compared with UV detection and permits isolation and quantitation of [^{3}H]digoxin-12α and its metabolites digoxigenin and the mono- and bis-digitoxosides, each in combination with their dihydro metabolite if present. We have also found three additional peaks which remain of unknown composition. Our knowledge of the biotransformation of digoxin in man remains incomplete or controversial and improved methods for its study are necessary.

EXPERIMENTAL

Materials

The HPLC system used was Constametric II (Laboratory Data Control Division of Milton Roy, Riviera Beach, FL, U.S.A.) with a Partisil 10 ODS 25 cm × 4.6 mm I.D. reversed-phase column and a 5 cm × 2.1 mm guard column packed with Co-Pell ODS (Whatman, Clifton, NJ, U.S.A.). The injection port was Model 7105 from Rheodyne (Berkeley, CA, U.S.A.). The flow-rate was 2.0 ml/min and elution was carried out initially with 35% methanol and then with 45% methanol at a pressure of 2000 p.s.i. (138 bar). The solvent change was

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made after the digoxigenin-mono-digitoxoside peak started to descend. The UV detector used to monitor peaks at 220 nm was a modified Beckman Model 25 spectrophotometer (Beckman, Palo Alto, CA, U.S.A.). The chart speed was 0.2 in./min and the span 0.25 A. Eluted fractions of 0.5 ml were collected with an LKB Produkter fraction collector Model 7000 (Bromma, Sweden). [^{3}H]Digoxin and metabolites in 0.5-ml fractions were counted in Riafluor (New England Nuclear, Boston, MA, U.S.A.) using a Beckman liquid scintillation spectrometer, Model LS-330, with a counting error of <2%.

Spectranalyzed methylene chloride and methanol were purchased from Fisher Scientific (Montreal, Canada) and the methylene chloride later double distilled. The water was purified by reverse osmosis and a Milli-Pore A filter system. The mixtures of methanol and water used for elution were degassed and filtered under vacuum before application.

[^{3}H]Digoxin-12α (Lot No. 690-186) with a specific activity of 16 mCi/mg was purchased from New England Nuclear. Digoxigenin-bis-digitoxoside, digoxigenin-mono-digitoxoside and digoxigenin were purchased from Boehringer Mannheim (G.F.R.) (Lots 61016, 7681 and 154182 respectively), and dihydrodigoxin was a gift from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). The above four compounds were tritiated by New England Nuclear by catalytic exchange. Specific activities were 1.16 mCi/mg for [^{3}H]-digoxigenin-bis-digitoxoside, 1.01 mCi/mg for [^{3}H]digoxigenin-mono-digitoxoside, 2.3 mCi/mg for [^{3}H]digoxigenin and 4.4 mCi/mg for dihydrodigoxin. Dihydrodigoxigenin and [^{3}H]dihydrodigoxigenin were prepared by hydrolysis of dihydrodigoxin and [^{3}H]dihydrodigoxin in acid. The purity of all compounds except for the dihydro derivatives, was achieved and verified before use by thin-layer chromatography and Sephadex LH-20 chromatography [5]. Thin-layer chromatography was used for the purification of the dihydro compounds [6]. Keto-digoxigenin and epidigoxigenin were also purchased from Boehringer.

Procedures

Urine voided by healthy volunteers was used immediately for studies which involved assessment of recovery. As part of a study of the influence of gastric acidity on the biotransformation of digoxin [7], six volunteers were given 150 μCi [^{3}H]digoxin-12α and 250 μg unlabelled digoxin (Lanoxin, Burroughs Wellcome) down a naso-gastric tube, firstly with stimulation of acid secretion by a pentagastrin infusion, and secondly without such stimulation. Urine was collected serially for assessment of endogenously formed metabolites and frozen at −25° C until assayed. Some results on the specimens collected 0–5 h after drug administration are reported; other results will be reported in detail elsewhere. All urine specimens were centrifuged and the supernatant extracted three times with methylene chloride. The volume of urine extracted was calculated to contain approximately 30,000 cpm (usually about 5 ml) and the volume of methylene chloride used was four times the urine volume. Aliquots of urine before and after extraction, as well as methylene chloride extracts were counted and recoveries calculated. [^{3}H]toluene was used as an internal standard to correct for quenching. Extracts were taken to dryness under vacuum, reconstituted in 100 μl of methanol and the entire volume injected

into the injection port of the chromatograph. Glassware was treated with Siliclad (Clay Adams, New York, NY, U.S.A.). The state of the column was checked daily before assays were performed by monitoring the UV peaks produced by application of a mixture of standards of digoxin, digoxigenin and its mono- and bis-digitoxosides in methanol.

These studies were approved by an ethics committee and volunteers gave informed consent.

RESULTS AND DISCUSSION

UV detection of digoxin and metabolites

Aliquots (5 μg each) of digoxigenin, its mono- and bis-digitoxosides and digoxin in combination in 20 μl methanol were applied to the column and peaks recorded with the UV detector. Mean retention times for 14 runs with standard deviations were 5.2 ± 0.2 min for digoxigenin, 9.5 ± 0.4 min for the mono-digitoxoside of digoxigenin, 16.7 ± 0.5 min for the bis-digitoxoside of digoxigenin and 21.9 ± 0.7 min for digoxin. Variations of up to 5 min occurred with different columns. Keto-digoxigenin and epi-digoxigenin had the same retention time as digoxigenin, based on UV detection.

When the four drugs were dissolved in methanol and 20 μl of the combination injected, the limit of sensitivity of this HPLC system using UV detection considering peak heights and 2:1 signal-to-noise ratio, was 278 ng for digoxin, 50 ng for digoxigenin, 132 ng for its bis-digitoxoside and 150 ng for the mono-digitoxoside. Digoxin, for a given weight applied, gave the least UV absorbance with a peak height about one sixth of that obtained for the same amount of digoxigenin. These limits in sensitivity preclude use of this method for detection of digoxin and its metabolites in urine when therapeutic doses are used. Furthermore, considerable UV absorbing material is extracted from urine with methylene chloride giving a large peak from 0—5 min which obscures two peaks found after administration of [^{3}H]digoxin-12α and overlaps with the digoxigenin peak (Fig. 1). It has therefore been necessary to use tritiated digoxin to obtain amplification and improved separation.

Detection using ^{3}H-labelled digoxin and metabolites

[^{3}H]Digoxin-12α, [^{3}H]digoxigenin and its ^{3}H-mono- and bis-digitoxosides were added to urine in amounts comparable to that found in urine during the 24 h after ingestion of 150 μCi [^{3}H]digoxin-12α. A 5-μg amount of each compound in the unlabelled form was also added to each specimen before extraction. The retention times for peaks determined by measurement of radioactivity were about 1 min later than peaks found by UV detection due to the tubing leading to the fraction collector. Fig. 2 illustrates the excellent separation of the four compounds with radioactivity falling to baseline between peaks.

Results of recovery studies for digoxin, digoxigenin and its mono- and bis-digitoxosides, performed on three occasions, are shown in Table I. The digoxin concentration was 0.4 pmole/ml (0.3 ng/ml). Relative recoveries of the individual compounds were within 1% of the relative percentages added. The absolute recoveries of radioactivity for all four compounds ranged from

TABLE I

TRIPLICATE RECOVERIES OF TRITIATED DIGOXIN AND METABOLITES ADDED TO URINE

Extraction from 5 ml urine.

	pmole/ml added to urine	Relative radioactivity added to urine (%)	Relative ratioactivity recovered (%)			Absolute radioactivity recovered (%)			Mean recovery (pmole/ml)	Standard deviation (pmole/ml)
			Experiment No.							
			1	2	3	1	2	3		
Digoxin	0.368	43	43	42	43	71	73	69	0.261	0.008
Bis-digitoxoside	1.71	10	11	11	10	73	78	71	1.27	0.06
Mono-digitoxoside	6.76	29	29	29	29	72	78	73	5.03	0.22
Digoxigenin	2.56	18	17	18	18	68	73	70	1.80	0.06
Total radioactivity recovered (%)						71	76	72		

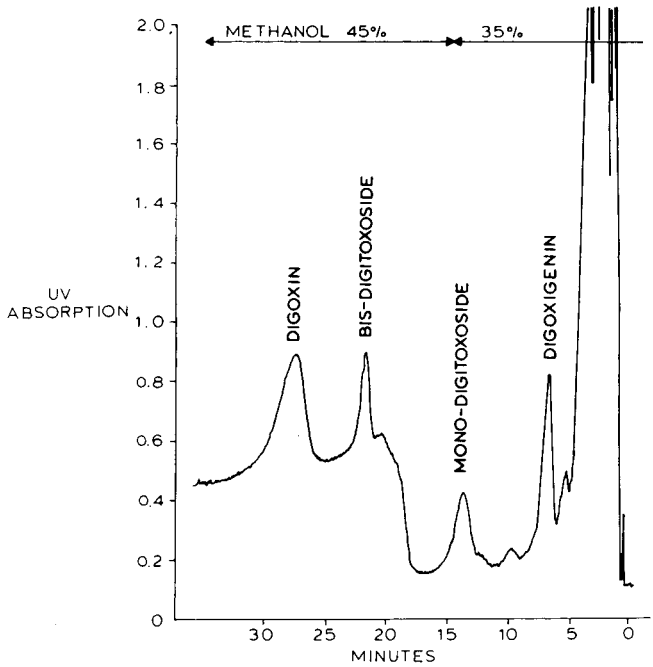

Fig. 1. UV tracing (220 nm) of eluate obtained after 5 μg each of digoxin, the mono- and bis-digitoxosides and digoxigenin, along with tritiated derivatives of each compound, were added to urine, extracted with methylene chloride, reconstituted in methanol and applied to a reversed-phase HPLC column. The solvent was changed from 35% to 45% methanol when the mono-digitoxoside UV peak started to descend. The high absorptivity recorded over the first 5 min is due to methylene chloride extractable material in urine unrelated to digoxin and metabolites.

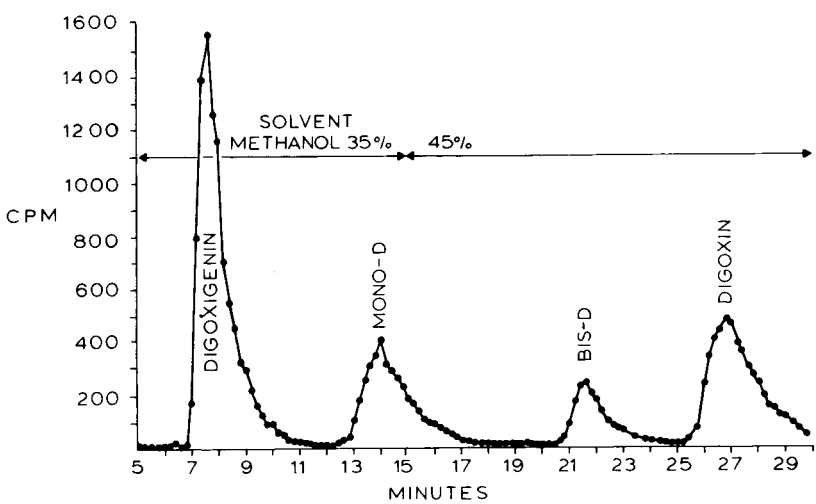

Fig. 2. Radioactivity profile in HPLC eluate obtained in the same study illustrated in Fig. 1 where tritiated and unlabelled digoxin, digoxigenin and its mono- and bis-digitoxosides were added to urine. The proportions of the four compounds found (digoxigenin 45%, mono-digitoxoside 18%, bis-digitoxoside 9% and digoxin 28%) were within 3% of those added to the urine.

68—78% (mean 73%), and differed by a maximum of 5% between the three runs for a given compound. The coefficients of variation for the four compounds ranged from 3—5%, considering the three recovery studies. There was no statistical difference between the recovery for digoxin and its three metabolites. Thus in the case of urine of unknown composition, when only a figure for recovery of total radioactivity can be determined, any error involved in making the assumption that recovery for all four compounds is the same will be small. Measured losses of radioactivity determined at each stage on three occasions averaged 3% during the extraction procedure, 8% on glassware (considering four transfers), 3% in the syringe, 3% in the injection port and on the column, and 8% in tubes used to collect fractions. Average total loss accounted for was 25%, compared with the average recovery of 73%.

Clear definition of peaks for urine extracts of the four tritiated compounds required maximum dpm of about twice the background of 50 dpm and a total dpm of 1000 when fractions were summed under peaks. These features provided sensitivity limits of about 40 pg for digoxin, 300 pg for digoxigenin and 650 pg for the mono- and bis-digitoxosides, considering a recovery of 70%. The limits of sensitivity for digoxin and metabolites in the studies involving urine collected after administration of [^{3}H]digoxin-12α, relate to the specific activity of [^{3}H]digoxin-12α of 16 mCi/mg and therefore were at the level of about 40 pg or less using 5 ml urine for extraction, or 8 pg/ml.

[^{3}H]Digoxin-12α and [^{3}H]digoxigenin-12α had the same retention times as their dihydro derivatives and it may be assumed that this also applies to dihydro metabolites of the mono- and bis-digitoxosides of digoxin. Dihydro metabolites were excreted in important amounts in some patients in two reports [8, 9] but only in small amounts in another [10].

Fig. 3 illustrates the radioactivity profile in the eluate obtained after application to the column of a methylene chloride extract of urine, reconstituted in

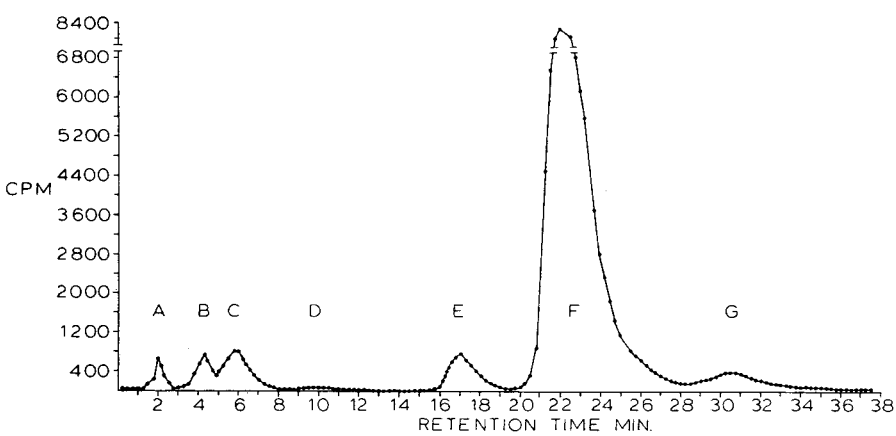

Fig. 3. Radioactivity profile obtained in reversed-phase HPLC column eluate after application of a reconstituted methylene chloride extract of urine collected from a volunteer 0—5 h after administration of 150 μCi [^{3}H]digoxin-12α. Most of the radioactivity (83%) is digoxin (peak F). The peaks C, D and E are digoxigenin and its mono- and bis-digitoxosides respectively. Peaks A, B and G are of unknown composition.

methanol. The urine was voided 0—5 h after administration of 150 μCi of [^{3}H]digoxin-12α without pentagastrin stimulation. The major peak, F, with 83% of the radioactivity is digoxin. Peaks C, D and E are digoxigenin and its mono- and bis-digitoxosides. The composition of peaks A, B and G is unknown. Peak A, at 2 min or 4 ml, starts to come off the column just after void volume: unstimulated it was 0—3% of radioactivity under the peaks, but averaged 5% after stimulation of gastric acid secretion with pentagastrin. Peak B, just preceding digoxigenin in the eluate, averaged 3% and 18% (maximum 31%) in the unstimulated and stimulated studies, respectively, and correlated with the amount of digoxigenin present. Peak G coming off the column after the digoxin peak, in contrast to peaks A and B, was present in greater amounts in the unstimulated series (mean 4%, maximum 9%) compared with the stimulated series (mean 1%) and correlated with the amount of digoxin present. The mean amounts of urine radioactivity extracted by methylene chloride were 46 and 85% for the stimulated and unstimulated series, respectively.

Although the primary objective of our method is the isolation and identification of metabolites of digoxin, it may also be used to quantitate digoxin, digoxigenin and its mono- and bis-digitoxosides, with the limitation that such values would include the dihydro metabolites if present. Quantitation of unlabelled digoxin and metabolites involves administration of labelled and unlabelled digoxin with known mass ratio and back calculation of mass in urine from the radioactivity under the HPLC peaks. The method assumes that the metabolism, excretion and recovery are similar for labelled and unlabelled digoxin.

Other methods of determining digoxin and metabolites such as radioimmunoassay and more recently HPLC with radioimmunoassay [3, 4] are methods for quantitation. Radioimmunoassay by itself, because of lack of specificity of antibody, may measure metabolites to varying degrees along with digoxin [11]. When HPLC is used to isolate digoxin and metabolites before use of radioimmunoassay as reported [4], only digoxin, digoxigenin and its mono- and bis-digitoxosides can be determined. Our method has detected three new extractable metabolites. The HPLC-radioimmunoassay method appears to have a lower limit of sensitivity of about 200 pg/ml for plasma, compared with about 8 pg/ml for urine with our method and also a lesser degree of precision.

In conclusion, an HPLC method has been developed using a reversed-phase column for isolation and quantitation of tritiated digoxin, digoxigenin and its mono- and bis-digitoxosides in urine; if one or more of the dihydro metabolites of these four compounds are present, then the combination is measured. Sensitivity at the 40-pg level or better was achieved for digoxin, digoxigenin and its mono- and bis-digitoxosides after the administration of [^{3}H]digoxin-12α. Recoveries averaged 73%. After administration of [^{3}H]digoxin-12α to volunteers, three additional radioactivity peaks were found in methylene choride extracts of urine. UV detection in the system used provided insufficient sensitivity and specificity to assay these compounds in the amounts found in urine after therapeutic doses.

ACKNOWLEDGEMENT

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REFERENCES

1 M.C. Castle, J. Chromatogr., 115 (1975) 437.
2 W. Lindner and R.W. Frei, J. Chromatogr., 117 (1976) 81.
3 J.C.K. Loo, I.J. McGilveray and N. Jordan, Res. Commun. Chem. Pathol. Pharmacol., 17 (1977) 497.
4 H.A. Nelson, S.V. Lucas and T.P. Gibson, J. Chromatogr., 163 (1979) 169.
5 M.H. Gault, M. Ahmed, A.L. Symes and J. Vance, Clin. Biochem. 9 (1976) 46.
6 J.J. Sabatka, D.A. Brent, J. Murphy, J. Charles, J. Vance and M.H. Gault, J. Chromatogr., 125 (1976) 523.
7 H. Gault, J. Kalra, M. Ahmed, D. Kepkay and J. Barrowman, Clin. Pharmacol. Ther., 27 (1980) 16.
8 U. Peters, L.C. Falk and S.M. Kalman Arch. Int. Med., 138 (1978) 1074.
9 H. Greenwood, W. Snedden, R.P. Hayward and J. Landon, Clin. Chim. Acta, 62 (1975) 213.
10 M.H. Gault, D. Sugden, C. Maloney, M. Ahmed and M. Tweeddale, Clin. Pharmacol. Ther. 25 (1979) 499.
11 R.G. Stoll, M.S. Christensen, E. Sakmar and J.G. Wagner, Res. Commun. Chem. Pathol. Pharmacol., 4 (1972) 503.

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Biomedical Applications

CHROMBIO. 539

Note

Analysis of verapamil in plasma by liquid chromatography

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Verapamil hydrochloride [DL-2,8-bis(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile hydrochloride, Isoptin®], a drug which inhibits calcium flux across cell membranes, has been shown to have anti-anginal, anti-hypertensive, and anti-arrhythmic properties [1]. More recently, verapamil has been used as an alternative to propranolol in treating patients with hypertrophic cardiomyopathy [2—4]. Effective therapy would be greatly assisted by analysis of verapamil in plasma or whole blood. Known metabolites of verapamil, except for its N-demethyl derivative (D-591), are of much lower potency and of no clinical importance [5]. Verapamil has been analyzed previously by fluorometry [6], gas chromatography [7—10] and selected ion monitoring (mass fragmentography) [11]. Direct fluorometry may not be specific since some of the known metabolites (norverapamil, etc.) have similar spectra. Gas chromatography and mass spectral selected ion monitoring require internal standards, special detectors or both. In the case of the latter, the apparatus is complex and costly.

Recently, Harapat and Kates [12] reported a simple and sensitive liquid chromatographic procedure using a commercially available column and fluorometric detection for the analysis of verapamil in plasma and blood. Unfortunately, our attempt to reproduce the procedure using the recommended column and solvent system was unsuccessful; neither verapamil nor the internal standard (D-517) eluted within 3 h. Adjustment of the solvent system to 70% methanol—0.01 *M* 2-propanesulfonic acid sodium salt—0.15% glacial acetic acid did cause the compounds to elute in 16 min but resolution and peak shape were poor. A second column, generously provided by the same manufacturer, was no better in this regard, although excellent peak shapes were observed with the recommended non-basic test compounds, naphthalene and biphenyl. Accordingly, we sought alternative columns and conditions.

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MATERIALS AND METHODS

Materials

DL-Verapamil hydrochloride, its N-demethyl derivative (D-591), a methoxy analogue [2-(3,4,5-trimethoxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile hydrochloride, D-600], and a lower homologue [2,7-bis(3,4-dimethoxyphenyl)-5-methyl-2-isopropyl-5-azaheptanitrile hydrochloride, D-517] were all gifts from Knoll Pharmaceutical (Darmstadt, G.F.R.). The internal standards were stored in the mobile phase at 0°C. Acetic anhydride was purchased from Allied Chemical (Morristown, NJ, U.S.A.), solvents from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), tetramethylammonium chloride from Eastman Kodak (Rochester, NY, U.S.A.) and monobasic potassium phosphate from Mallinckrodt (St. Louis, MO, U.S.A.). Water was distilled and deionized by the Milli-Q System (18 megohm) (Millipore, Bedford, MA, U.S.A.).

Liquid chromatography

A Spectra-Physics Model 3500 liquid chromatograph and a Schoeffel Model FS 970 fluorometer were used for all analyses. The fluorometer was set at an excitation wavelength of 200 nm. The glass envelope of the phototube and a Corning 5970 filter (5.06 mm thick) allowed fluorescence (67% transmission) between 310 and 390 nm to be detected by the phototube. The detector range was varied between 0.2 and 0.5 μA full scale depending on concentration, and the time constant set at 6 sec. The fluorometer electronics were allowed to remain on constantly; only the ultraviolet source was turned off.

The chromatographic system finally found most useful was a LiChrosorb RP-18 (10 μm) Hibar II (EM Labs., Cincinnati, OH, U.S.A.) run at a flow-rate of 1.8 ml/min at 1210 p.s.i. inlet pressure. The isocratic mobile phase was 40% (v/v) acetonitrile—water, 0.01 *M* in tetramethylammonium chloride, 0.005 *M* in monobasic potassium phosphate and 0.074 *M* in phosphoric acid. The solution was degassed for at least 5 min and maintained under bubbling helium. The column was washed thoroughly with water and then with methanol at the end of each run. At the start of each run the column was washed with the mobile phase while the ultraviolet lamp of the fluorometer was allowed to stabilize.

All glassware was carefully cleaned with sulfuric—dichromate solution and washed extensively with distilled water, acetone and diethyl ether.

Hamilton microliter syringes were used for all quantitation.

Extraction procedure

The extraction procedure was essentially the same as that used by Harapat and Kates [12]: To 0.5 ml of plasma in a 15 × 125 mm glass test tube were added either 100 or 400 ng of D-600 internal standard depending on whether the verapamil was expected to be in the range of 10—100 or 100—500 ng/ml plasma. When the sample was found to be outside of the appropriate range the analysis was repeated, adjusting the quantity of internal standard. Twenty-five μl of 5 *N* sodium hydroxide were added, the tube agitated gently, 5 ml of diethyl ether added and the sample was vortex-mixed for 1 min. After a further minute of centrifuging, most of the top layer was carefully pipetted into a 120-

mm tube whose base had been elongated to accommodate ca. 50 μl. Acetic anhydride (25 μl) was then added to the diethyl ether to react with the dealkylated metabolites, the tube vortex-mixed for 15 sec and allowed to stand at room temperature for 5 min.

Fifty μl of 0.12 *M* sulfuric acid was then added and the tube vortex-mixed for 1 min and then centrifuged for 1 min in a receiver designed to protect the tube's coned tip. Depending upon the amount of verapamil expected, 6—25 μl of the aqueous layer were withdrawn with a syringe and injected into the chromatograph.

RESULTS AND DISCUSSION

An RP-18 (10 μm) Hibar II column (250 mm × 4.6 mm I.D.) (EM Labs.) with tetramethylammonium chloride—phosphate buffer—acetonitrile mobile phase [9, 13, 14], as suggested by Dr. G. Burce, finally provided excellent results with respect to separation of verapamil and either of two available internal standards. However, the separation between verapamil and its known major metabolite, N-demethylverapamil (D-591), while sufficient for qualitative identification (Fig. 1, before acetylation) interferes with quantitation of the parent compound. Treating the ethereal extract from plasma with acetic anhydride converts the metabolite to its N-acetyl derivative, ensuring that it will not be extracted with verapamil in the subsequent acid treatment (Fig. 1, after acetylation). The N-acetyl derivative elutes considerably later (18 min) with the same mobile phase and could be determined separately if desired. The detector

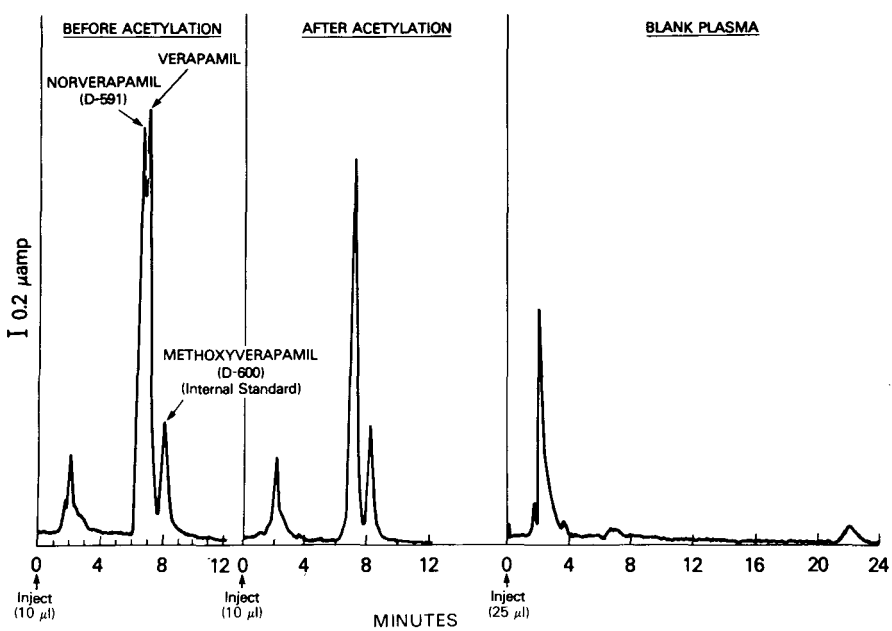

Fig. 1. Liquid chromatogram of 10-μl aliquots taken from 50 μl total extract of 500 μl of plasma containing 200 ng/ml of each component, showing effect of addition of acetic anhydride. Blank plasma, 25 μl of a similar extraction, is shown for reference.

response (peak area per mole) of N-acetyldemethylverapamil is approximately the same as demethylverapamil and verapamil under these conditions. Collection of the peak provided a sample for mass spectrometry by direct insertion probe. Important ions are observed at *m/z* 482 ($M^{+\cdot}$, 5), 289 [$M^{+\cdot}$–ketene–$(H_3CO)_2C_6H_4CH_2$, 52] and 164 [$(H_3CO)_2C_6H_4CH{=}CH_2$, 100].

Other primary and secondary amine metabolites of verapamil have been noted by McIlhenny [7] and Neugebauer [5]; these would likewise be removed by acetic anhydride. Tertiary amine metabolites [15] include 2-(3,4-dimethoxyphenyl)-5-dimethylamino-2-isopropylpentanitrile (metabolite XI), conjugates of a phenolic analogue of metabolite XI (metabolite XII) and an O-demethylverapamil [2-(4-hydroxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile (metabolite III)]. The first two are present in urine in trace quantities and because of their grossly different structures might be expected to separate easily from verapamil on chromatography. The latter accounts for 7% of the dose excreted in urine but since it is a conjugate it would not be extracted under the above conditions.

With most plasma samples we have encountered a very small conflicting peak eluting approximately at the same retention volume as verapamil (Fig. 1, blank plasma), but it has not yet exceeded a quantity equivalent to ca. 0.1 ng verapamil per ml plasma.

Standard curves were prepared using spiked plasma in two ranges, 0—100 ng/0.5 ml and 0—500 ng/0.5 ml. Excellent linearity was achieved in both cases (correlation coefficient=0.999) with the plots extrapolating through the origin.

Table I shows the coefficient of variation in replicate plasma analyses (n=6). Best results are obtained with peak height ratios of verapamil to D-600 between 0.8 and 3.2.

TABLE I

REPRODUCIBILITY AT A GIVEN PLASMA CONCENTRATION

n = 6.

Peak height ratio of verapamil to D-600 (mean)	C.V. (%)	Verapamil (ng/0.5 ml plasma)	Internal standard added (ng D-600)
0.37	3.1	10	100
0.86	1.7	100	400
1.59	1.6	50	100
2.14	1.2	250	400
3.16	1.4	500	400
3.66	3.6	100	100

The technique is currently being applied in these laboratories for the clinical analysis of verapamil in man at levels of 5—600 ng/ml, although the method has been found to be satisfactory at levels as low as 1 ng/ml. Preliminary clinical studies were performed in the cardiac catheterization laboratory to assess electrophysiologic responses to intravenous verapamil in eight patients with hypertrophic cardiomyopathy. Plasma verapamil levels for the group (mean ± S.E.M.) after 0.007 mg/kg/min, 0.014 mg/kg/min, and 0.021 mg/kg/min constant infusions, each preceded by 0.1 mg/kg intravenous bolus injections, were 144 ± 18

ng/ml (range 74–183 ng/ml), 319 ± 45 ng/ml (range 154—540 ng/ml), and 415 ± 13 ng/ml (range 164—560 ng/ml), respectively. Further studies, analyzing verapamil levels in patients on chronic oral therapy are currently in progress.

ACKNOWLEDGEMENT

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REFERENCES

1 B.N. Singh, G. Ellrodt and C.T. Peter, Drugs, 15 (1978) 169.
2 M. Kaltenbach, R. Hopf and M. Keller, Deut. Med. Wochenschr., 101 (1976) 1284.
3 D.R. Rosing, K.M. Kent, J.S. Borer, S.F. Seides, B.J. Maron and S.E. Epstein, Circulation, 60 (1979) 1201.
4 D.R. Rosing, K.M. Kent, B.J. Maron and S.E. Epstein, Circulation, 60 (1979) 1208.
5 G. Neugebauer, Cardiovas. Res., 12 (1978) 247.
6 R.G. McAllister and S.M. Howell, J. Pharm. Sci., 65 (1976) 431.
7 H.M. McIlhenny, J. Med. Chem., 14 (1971) 1178.
8 H.P. Gelbke, H.J. Schlicht and G. Schmidt, Arch. Toxicol., 37 (1977) 89.
9 R.G. McAllister, T.G. Tan and D.A. Bourne, J. Pharm. Sci., 68 (1979) 574.
10 H.G. Hege, personal communication, BASF-Pharmasporte, August 31, 1977.
11 B. Spiegelhalter and M. Eichelbaum, Arzneim.-Forsch., 27 (1977) 34.
12 S.R. Harapat and R.E. Kates, J. Chromatogr., 170 (1979) 385.
13 H.F. Proelss, H.J. Lohmann and D.G. Miles, Clin. Chem., 24 (1978) 121.
14 Liquid Chromatography at Work No. 87, Varian Instrument Division, Palo Alto, CA.
15 M. Eichelbaum, M. Ende, G. Remberg, M. Schomerus and H.J. Dengler, Drug Metab. Disp., 7 (1979) 145.

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Biomedical Applications

CHROMBIO. 540

Note

Determination of benoxaprofen in plasma and urine by liquid chromatography

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Benoxaprofen [2-(4-chlorophenyl)-α-methyl-5-benoxazoleacetic acid] (I) is a substance reported to possess anti-inflammatory, analgetic and antipyretic properties in animal tests [1, 2]. Three methods for its determination in biological fluids have been published [3]. The most simple one is based on extraction and measurements in a UV spectrophotometer. The other two methods utilize gas chromatography of the benoxaprofen methyl ester derivative which is detected by flame ionization detector or electron-capture detector. The present liquid chromatographic method is almost as simple to perform as the spectrophotometric method and offers a specificity and sensitivity in the same order as that of the more complicated gas chromatographic—electron-capture detection method [3].

MATERIALS AND METHODS

Standards

Benoxaprofen (I) and 2-(3,5-dichlorophenyl)-α-methyl-S-benzoxazoleacetic acid (II) used as internal standard were kindly donated by Lilly (Hants, Great Britain). Their structures are shown in Fig. 1.

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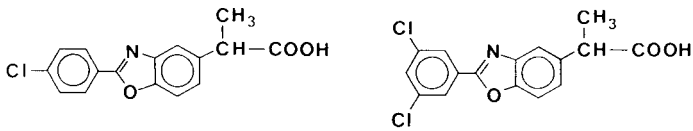


I II

Fig. 1. Structures of I, benoxaprofen [2-(4-chlorophenyl)-α-methyl-5-benoxazoleacetic acid] and II, internal standard [2-(3,5-dichlorophenyl)-α-methyl-5-benoxazoleacetic acid].

Liquid chromatography

The liquid chromatographic system used consisted of a M6000 pump, a U6K injector and a M440 filter UV detector (Waters Assoc., Milford, MA, U.S.A.). The column (0.15 m × 4.6 mm I.D., stainless steel) was slurry-packed with Spherisorb S5 ODS (particle size 5 μm). The mobile phase consisted of 0.01 *M* phosphate buffer (pH 6.5)—acetonitrile (65:35) operated at a flow-rate of 1.5 ml/min at room temperature (ca. 20—22°C). Since I had a UV maximum at 310 nm in the eluent the 313-nm filter was used in the detector.

Plasma assay

In a 10-ml screw-capped tube 100 μl plasma, 1 ml 0.1 *M* hydrochloric acid, 50 μl internal standard solution (20 μg/ml) and 4 ml diethyl ether were shaken for 5 min on a shake board. The tube was then centrifuged for 10 min at 500 *g* (Wifug X1). The diethyl ether phase was removed and transferred to a new tube (conical bottom) and the solvent was then evaporated by a stream of nitrogen. The residue was dissolved in 150 μl of the eluent mixture and after filtration through a pasteur pipette closed with fine glass-wool, 20 μl were injected in the chromatograph. When concentrations below ca. 300 ng/ml in plasma were measured 1 ml of plasma was used instead of 100 μl.

Urine assay (free I)

Urine (100 μl) was treated in the same way as the plasma sample described above.

Urine assay (free and conjugated I)

Urine (100 μl), 100 μl 0.2 *M* acetate buffer (pH 5) and about 1 mg of β-glucuronidase were incubated at 37°C overnight. One ml 0.1 *M* hydrochloric acid was added to the mixture and worked up as described above. The amount of internal standard used was adjusted so that it corresponded to the concentration of I in the urine samples.

RESULTS AND DISCUSSION

Extraction of I from an aqueous phase with diethyl ether or chloroform has been shown to yield about 98% in the organic phase [3]. Dichloromethane also gave a similar result. Diethyl ether was chosen for the extraction because it emulsified the plasma less.

The method has been described for 100-μl samples of plasma since this

volume may be easily collected from the finger tip in capillary tubes. The detection limit using this amount of plasma was 250 ng/ml. When analysing plasma containing benoxaprofen below this concentration the volume of the plasma sample was increased to 1 ml. This increase in sample volume did not, however, introduce any disturbing background peaks on the chromatogram. The detection limit of I was then about 25 ng/ml plasma.

Fig. 2 shows typical chromatograms obtained when analysing a plasma sample (100 μl) containing 5 μg/ml benoxaprofen and a blank plasma sample according to the method described above.

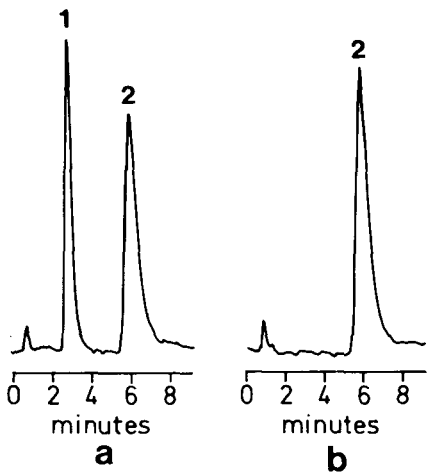
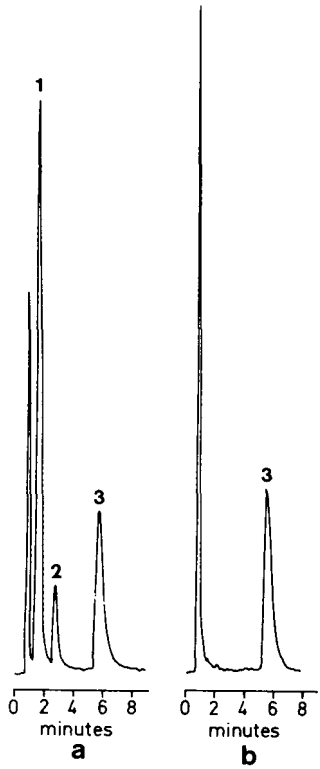


Fig. 2. Chromatograms of (a) plasma sample containing 5 μg/ml of benoxaprofen and (b) blank plasma sample treated according to the method described. Peaks: 1 = benoxaprofen; 2 = internal standard.

The reversed-phase column used in the method was eluted with acetonitrile—phosphate buffer (pH 6.5). At this pH benoxaprofen chromatographed in an ionized form. When the amount of acetonitrile in the eluent was 30% the retention time was 3.6 min. To obtain a similar retention time (3.3 min) with pH 3 phosphate buffer 80% of acetonitrile had to be present in the eluent mixture. Such a high acetonitrile concentration may cause problems with precipitation of salts from the buffer.

When urine samples were extracted in order to determine free I, a conjugate of I was also extracted to some extent under the conditions used. The peak corresponding to the conjugate can be seen in the chromatogram of Fig. 3a. This peak disappeared if the method for determination of free and conjugated I was followed, which involved incubation with β-glucuronidase prior to extraction. It is important to keep the pH of the eluent buffer at 6.5 since an increase in the pH reduces resolution between I and its conjugate and at pH 7 an overlap occurs.

Calibration graphs were constructed in the ranges 0.5—10 and 5—70 μg/ml plasma. The peak height ratios (I/internal standard) were plotted against the concentration of I. The curves were linear and passed through the origin. To



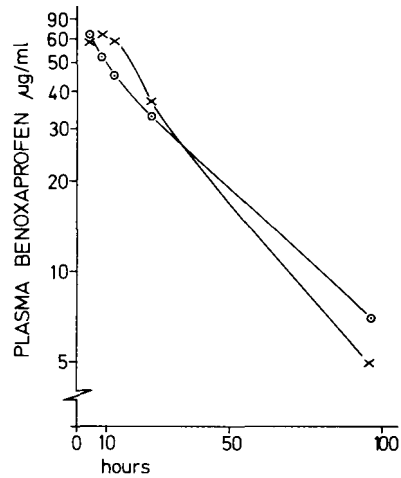


Fig. 3. Chromatograms of (a) urine sample containing 1.9 µg/ml of benoxaprofen and (b) blank urine sample treated according to the method described. Peaks: 1 = conjugate of benoxaprofen; 2 = benoxaprofen; 3 = internal standard.

Fig. 4. Plot of plasma concentration versus time from two subjects given 600 mg of benoxaprofen orally. Plasma concentrations of benoxaprofen in subject 1 (×) and in subject 2 (○).

determine the precision, I (4 µg/ml) was added to ten plasma samples and analysed by the above method. It was found to be 2.5%. The absolute recoveries of I from plasma and urine by the method were 90 and 92% respectively.

Two healthy subjects were each given 600 mg of benoxaprofen. The plasma concentration—time curves shown in Fig. 4 were constructed by analysing plasma samples from these subjects by the method described here. The result was in good agreement with earlier findings [3, 4].

REFERENCES

1 D.W. Dunwell, D. Evans, T.A. Hicks, C.H. Cashin and E.A. Kitchen, J. Med. Chem., 18 (1975) 53.
2 C.H. Cashin, W. Dawson and E.A. Kitchen, J. Pharm. Pharmacol., 29 (1977) 330.
3 D.H. Chatfield and T.J. Woodage, J. Chromatogr., 153 (1978) 101.
4 D.H. Chatfield and J.N. Green, Xenobiotica, 8 (1978) 133.

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Biomedical Applications

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Note

Determination of papaverine in plasma and urine by high-performance liquid chromatography

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Papaverine (6,7-dimethoxy-1-veratrylisoquinoline) is commonly used as a peripheral vasodilator. A number of analytical methods for the determination of papaverine in biological fluids such as plasma and urine have been described in the literature. The turbidimetric method [1], which involves phosphomolybdic acid, lacks in specificty for papaverine, whereas the differential spectrophotometric method [2] exhibits a specificity for papaverine, but unfortunately the limit of detection for papaverine is about 0.5 μg/ml. The gas chromatographic methods [3—5] for the determination of papaverine have the sensitivity necessary to detect low concentrations in plasma, however, these methods generally require large sample volumes and involve various extraction and purification steps. This leads to a considerable increase in the analysis time.

This paper reports a rapid and simple high-performance liquid chromatographic (HPLC) assay for papaverine in plasma and urine. The assay is quite specific for papaverine and requires a short sample preparation procedure prior to the chromatographic analysis.

EXPERIMENTAL

Reagents

Chloroform, isopropanol, methanol and sodium borate decahydrate were all reagent grade and purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Laudanosine, the internal standard, was obtained from Aldrich (Milwaukee, WI, U.S.A.). Papaverine was obtained from S.B. Penick & Co. (Lyndhurst, NJ, U.S.A.).

Apparatus

A Milton Roy Mini Pump (Milton Roy, Laboratory Data Control Division,

Riviera Beach, FL, U.S.A.) was used to deliver the mobile phase to a high-pressure loop injector (Model No. 7120 injector, Rheodyne, Berkeley, CA, U.S.A.), fitted with a 20-μl loop and a C_8 reversed-phase column (25 cm × 4.6 mm I.D., 10 μm particle size, Brownlee Labs., Santa Clara, CA, U.S.A.). The chromatography was carried out at ambient temperature. A variable-wavelength UV—Vis detector (Vari-chrom, Varian Instrument, Palo Alto, CA, U.S.A.) was used to monitor the column effluent at 239 nm. The output from the detector was connnected to a 1-mV potentiometric recorder (Linear Instrument, Irvine, CA, U.S.A.). The HPLC mobile phase was methanol—0.015 *M* sodium borate, pH 8.5 (58:42). This was pumped through the HPLC system at a rate of 2.7 ml/min and the resulting pressure was approximately $1.517 \cdot 10^7$ Pa.

Procedure

The plasma or urine sample (1.0 ml) was pipetted into a 16 × 125 mm culture tube and mixed with 0.3 ml of laudanosine solution (0.2 mg/l), followed by the addition of 10 ml of a mixture of chloroform—isopranol (95:5). After sealing the tube with a PTFE-lined screw cap, it was vortexed for 2 min and centrifuged for 30 min. The supernatant aqueous layer was aspirated and 8 ml of the organic phase were transferred to a clean tube. The organic phase was evaporated with an air stream at room temperature and the evaporated residue was reconstituted with 100 μl of methanol. A 20-μl aliquot of this solution was injected. The standard curves were developed by spiking blank plasma or urine samples with known amounts of papaverine to give concentrations from 0.0— 10.0 μg/ml.

Calculation

A standard curve was developed for each series of analyses by plotting the ratio of the height of papaverine peak to the height of laudanosine peak versus the concentration of papaverine. The concentrations of the unknown samples were subsequently determined from the standard curve.

RESULTS AND DISCUSSION

The chromatographic conditions for the analysis of papaverine in plasma and urine were selected after appropriate preliminary investigations with a number of different mobile phases. It was observed that the elution of papaverine from the HPLC column was considerably influenced by the pH and methanol concentration of the mobile phase.

The chromatograms resulting from the analysis of a blank plasma sample and a plasma sample spiked with a known amount of papaverine are shown in Fig. 1. Fig. 2 presents the chromatograms from blank and spiked urine samples. Under the chromatographic conditions selected for this assay, papaverine eluted from the HPLC column with a retention time of 5.0 min while laudanosine, the internal standard, eluted at 9.5 min. No endogenous components with a retention time similar to either papaverine or laudanosine were observed during the analyses of the plasma or urine samples. The specificity of the assay was tested by spiking the plasma and urine samples with theophylline, caffeine, theobromine, tetracycline, oxytetracycline, gentamycin,

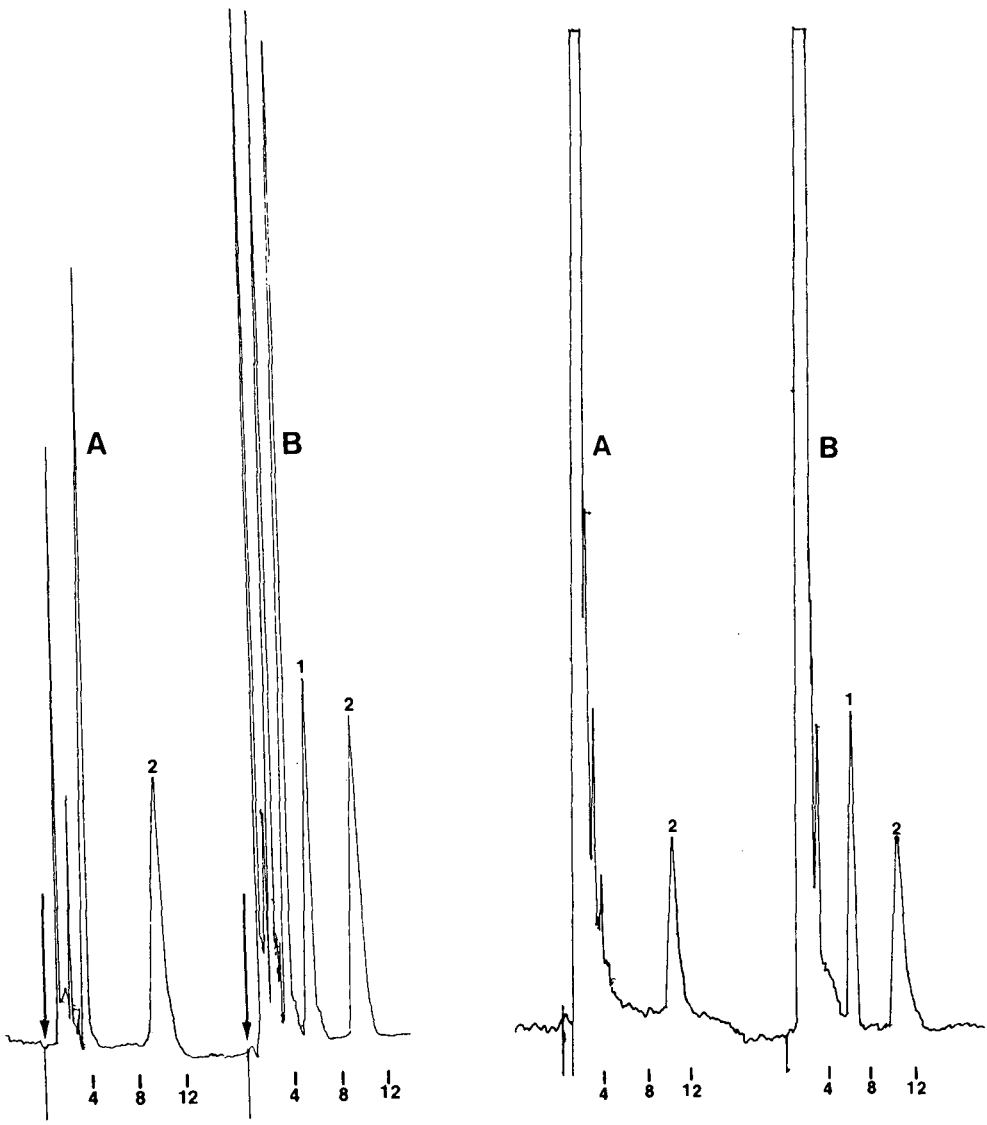

Fig. 1. Chromatograms of blank plasma (A) and plasma spiked with 0.5 μg/ml papaverine (B). Peaks: papaverine (1) and laudanosine (2).

Fig. 2. Chromatograms of blank urine (A) and urine spiked with 0.5 μg/ml papaverine (B). Peaks: papaverine (1) and laudanosine (2).

chlorothiazide and hydrochlorothiazide. These samples were carried through the entire assay procedure and none of these drugs were found to interfere with the analysis of papaverine.

The standard curve for papaverine from the plasma and urine samples was linear over the concentration range studied (Table I). The linearity of the standard curve was studied over a concentration range of 0.05–1.0 μg/ml of

TABLE I

STANDARD CURVE DATA FOR PAPAVERINE

$n = 5$.

Sample	Conc. of papaverine (μg/ml)	Ratio*	Equation
Plasma	0.00	0.00	$Y = 2.058X + 0.01$
	0.05	0.1858	$r = 0.997$
	0.10	0.3927	
	0.50	1.2058	
	1.00	2.1120	
Urine	0.00	0.00	$Y = 1.567X + 0.02$
	0.05	0.0973	$r = 0.999$
	0.10	0.1989	
	0.50	0.8253	
	1.00	1.5758	

*Ratio of papaverine peak height to laudanosine peak height.

papaverine, both in plasma and urine, and was found to be linear with correlation coefficients for linear regression of 0.997 and 0.999, respectively. The maximum sensitivity for papaverine detection was 25 ng/ml of plasma and urine, since at this concentration the signal-to-noise ratio was about 4 or 5 to 1.

The efficiency of the extraction step was checked by extracting known concentrations of papaverine in replicates of five, from plasma and urine samples. The recovery of papaverine from plasma samples ranged from 78.6–87.2% with a mean recovery of 84.3%, whereas the recovery from urine samples ranged from 78.57—104.46% with a mean recovery of 88.2%.

The precision and reproducibility of the assay procedure was determined by analyzing papaverine at three concentration levels (0.1, 1.0, 10.0 μg/ml), in triplicate. In plasma, at these levels, the coefficients of variation were found to be 6.16, 1.93 and 7.5% while the coefficients of variation at the same levels from the urine samples were 3.39, 1.13 and 0.8% (Table II).

TABLE II

REPRODUCIBILITY DATA FOR PAPAVERINE

Sample	Conc. of papaverine (μg/ml)	Ratio* (mean ± S.D.)	C.V. (%)
Plasma	0.10	0.4250 ± 0.0262	6.16
	1.00	1.9593 ± 0.0378	1.93
	10.0	45.583 ± 3.42	7.5
Urine	0.10	0.5152 ± 0.017	3.39
	1.00	4.0747 ± 0.0463	1.13
	10.0	39.3900 ± 0.345	0.8

*Ratio of papaverine peak height to laudanosine peak height.

The method described is the first HPLC-method which is rapid, simple and with sufficient sensitivity for the quantitative determination of papaverine in plasma and urine. The lower limit of detection of papaverine in plasma and urine is 25 ng/ml with excellent reproducibility. The method is specific for papaverine and no interference from other drugs was observed.

ACKNOWLEDGEMENTS

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REFERENCES

1 S.R. Elek and H.C. Bergman, J. Appl. Physiol., 6 (1953) 168.
2 J. Axelrod, R. Shofer, J.K. Inscoe, W.M. King and A. Sjoerdsma, J. Pharmacol. Exp. Ther., 124 (1958) 9.
3 D.E. Guttman, H.B. Kostenbauder and G.R. Wilkinson, J. Pharm. Sci. 63 (1974) 1625.
4 E. Missini and A. Marzo, Biochem. Biol., 4 (1973) 3.
5 J. de Graeve, J. van Cantfort and J. Gielen, J. Chromatogr. 133 (1977) 153.

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CHROMBIO. 550

Note

Rapid high-performance liquid chromatographic method for the quantitative determination of diflunisal in plasma

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Diflunisal [5-(2′4′-difluorophenyl)salicylic acid] is a new ring-substituted salicylic acid derivative with promising analgesic and anti-inflammatory properties which make the drug useful for the treatment of osteoarthritis [1, 2]. Recent studies [3—5] indicate that the drug exhibits a dose-dependent pharmacokinetic profile. As compared to other salicylates diflunisal has a long plasma half-life time which, similarly to salicylic acid itself [6], increases with increasing dose. This rather long $t_{1/2}$ is especially relevant since it permits a dosage regimen with only one or two daily doses. On the other hand the capacity limited elimination rate implicates the necessity of monitoring plasma drug levels for the establishment of optimal dosage regimens.

For the assay of drug levels in the course of a clinical pharmacological study comparing the therapeutic efficacies of diflunisal and of naproxen, a rapid and specific assay method for these drugs was needed.

In this report a description of a high-performance liquid chromatographic (HPLC) method for the determination of diflunisal is given, which combines a short-time analysis with ease of handling. The procedure is based upon almost quantitative extraction of the diflunisal from plasma followed by reversed-phase liquid chromatography with UV spectrophotometric detection. As an internal standard the analgesic naproxen (6-methoxy-α-methyl-2-naphthaleneacetic acid) is used. This method has several advantages in comparison with published procedures involving spectrofluorometry [4] and has been already applied in preliminary pharmacokinetic studies.

MATERIALS AND METHODS

Materials

Diflunisal, a pure substance for reference purposes, was kindly provided by Merck, Sharp and Dohme (Brussels, Belgium) and naproxen by Syntex (Palo Alto, CA, U.S.A.).

The column-filling material, LiChrosorb RP-8 (5 μm) was obtained from Merck (Darmstadt, G.F.R.) and the tetramethylammonium hydrogen sulphate, used as a counter-ion, from Fluka (Buchs, Switzerland). All other reagents were analytical grade products from Merck. The diethyl ether was distilled shortly before use.

Stock solutions of diflunisal and naproxen were prepared by dissolving 25 mg of either drug in 100 ml of methanol.

Apparatus

A Hewlett-Packard 1084B high-pressure liquid chromatograph equipped with an autosampler and a HP 79850 LC terminal was used (Hewlett-Packard, Waldbronn, G.F.R.). The column (stainless steel, 15 cm × 0.46 cm I.D.) was packed with LiChrosorb RP-8 (5 μm) and eluted with a methanol—water mixture (50:50), containing 0.01 *M* tetramethylammonium hydrogen sulphate and Tris [tris(hydroxymethyl)aminomethane]. The apparatus was operated at a column temperature of 32°C and a flow-rate of 1.4 ml/min. The column pressure was $26 \cdot 10^3$ KPa. The eluting compounds were detected and measured at 254 nm.

Extraction

Plasma (0.5 ml) containing diflunisal, is pipetted into a test tube provided with a screw-cap. Naproxen (50 μg) in methanolic solution (0.2 ml) is added. After thorough mixing on a whirlmixer 5 ml of a diethyl ether—*n*-hexane (50:50) mixture and 0.7 ml of a 1.5 *N* hydrochloric acid solution are added. The closed tube is mechanically shaken for 30 min and then centrifuged for 15 min at 1500 *g*. The organic layer is transferred into another test tube and evaporated to dryness at 30°C under a gentle stream of dry, filtered air. The residue is taken up into 1 ml of methanol of which 10 μl are injected into the liquid chromatograph.

Calibration and recovery

The procedure is calibrated by programming the LC terminal in the internal standard. Various known amounts of diflunisal and a fixed amount of naproxen (50 mg/l) are added to blank plasma. These samples are analysed as outlined above and calibration graphs are obtained by plotting the concentration of diflunisal calculated by the terminal (from integration of the signal) against the concentration added, assuming a linear relation between the surfaces under the curves of the different diflunisal concentrations and the 100 mg/l concentration as a reference.

The overall recovery of the procedure is determined by comparison of the values obtained after extraction with values obtained after direct injection of standard solutions.

RESULTS AND DISCUSSION

Under the conditions employed no interferences by endogenous plasma constituents occurred in the chromatogram. For example, Fig. 1 shows a chromatogram obtained from blank plasma as well as one from plasma containing diflunisal and naproxen. A whole run is completed within 10 min, the retention times of naproxen and diflunisal being about 2.5 and 4.2 min respectively.

Linear calibration curves were obtained over the concentration range of 5—100 mg/l of diflunisal in plasma. The limit of detection of diflunisal with the method described is substantially below 5 mg/l, but in order to obtain reproducible, quantitative results at such low concentrations a separate calibration has to be performed. The deviation from linearity in the very low concentration range is caused by a slight tailing of the diflunisal peak which leads to a certain loss of signal in the automatic integration procedure. In the low concentration level (9.936 mg/l) the detected concentration was 9.06 mg/l with an absolute error —0.88 mg/l, the relative error - 8.85%. In the high concentration level 99.36 mg/l the detected concentration was 98.60 mg/l with an absolute error of —0.76 mg/l, the relative error —0.76%. On the other hand, for practical purposes it is not necessary to extend the diflunisal assay to such low concentrations since therapeutic plasma levels probably exceed the 10 mg/l concentration.

The overall recovery of the extraction of diflunisal from the plasma in this procedure is almost complete. Over the entire concentration range studied it is 96% on average, without any systematic changes.

Upon repeated measurement (10 assays) of two samples, one in the low con-

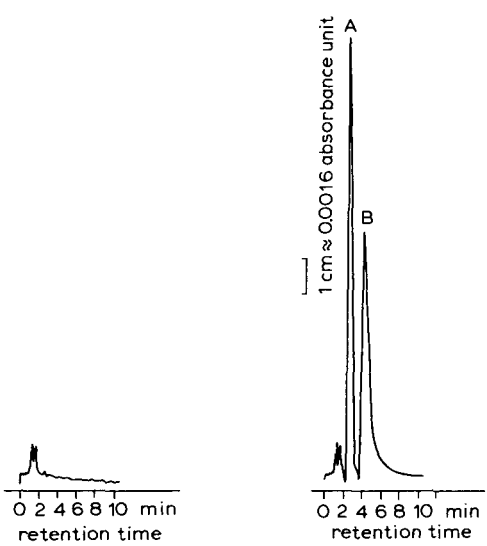

Fig. 1. Chromatogram of (left) blank plasma and (right) plasma containing diflunisal (50 mg/l) and naproxen (100 mg/l). Retention times: naproxen (A) 2.55 min, diflunisal (B) 4.11 min.

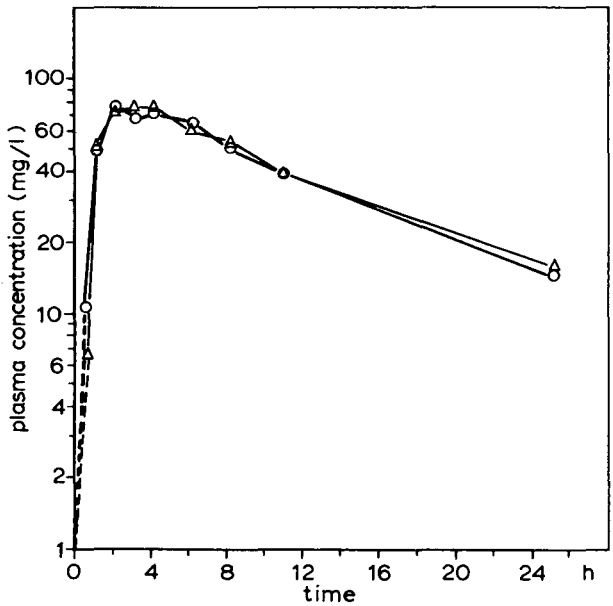

Fig. 2. Plasma curves obtained after oral administration of 500 mg diflunisal to a female volunteer, 21 years old, height 172 cm and weight 58.9 kg. △ = 2 tablets of 250 mg; ○ = 2 tablets in capsules.

centration level (9.936 mg/l) and one in the high concentration level (99.36 mg/l), a mean concentration was calculated of 9.06 mg/l with a standard deviation of 0.14, and 98.60 ± 1.66 mg/l, respectively. The relative standard deviations were 1.46% and for the high concentration 1.68%, so again no systematic changes occurred over the entire concentration range observed, with respect to the reproducibility of the method.

Currently the method is in use for the measurement of the diflunisal concentration in plasma of patients chronically medicated with the drug in the setting of a clinical trial. In connection with this trial, a preliminary study of some pharmacokinetic characteristics of diflunisal in volunteers after ingestion of a 500-mg dose either as tablets or as capsules, has been established with this procedure. The results are shown in Fig. 2. Obviously diflunisal is well absorbed, has a small apparent volume of distribution (less than 10 l) and a plasma halflife time about 10 h at the dose level applied. The method reported here is very suitable for pharmacokinetic studies and drug level monitoring of diflunisal in man.

REFERENCES

1 A. Andrew, B. Rodda, L. Verhaest and C. van Winzum, Brit. J. Clin. Pharmacol., 4, Suppl. 1 (1977) 45S—52S.
2 C.G. Van Arman, E.A. Risley and V.J. Lotti, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 36 (1977) 1037, nr. 4034.

3 K.F. Tempero, V.J. Cirillo and S.L. Steelman, Brit. J. Clin. Pharmacol., 4, Suppl. 1 (1977) 31S—36S.
4 D.J. Tocco, G.O. Breault, A.G. Zacchei, S.L. Steelman and C.V. Perrier, Drug Metab. Disp., 3 (1975) 453—466.
5 F.W.J. Gribnau, in preparation.
6 G. Levy, T. Tsuchiga and L.P. Amsel, Clin. Pharmacol. Ther., 13 (1972) 259—268.

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Note

Rapid determination of renal contrast media in biological fluids by means of high-performance liquid chromatography

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Several iodinated organic acids are very frequently used for intravenous urography. The reason for their usefulness is the fact that they are rapidly excreted by the kidneys. Some of them are excreted solely by glomerular filtration but most of them are also secreted via a carrier-mediated mechanism in the proximal tubules.

In the course of a fundamental study on the kinetics of urinary excretion of a number of agents, secreted by the renal tubules of the mammalian kidney, we needed a rapid, sensitive and quantitative method for the detection of some contrast media in plasma and urine. Several existing methods [1, 2] are laborious, lack the required specificity or need the use of radioactive materials which imposes special handling and safety measures.

The procedure described in this paper enables the quantitative detection of renal contrast media in a simple specific way, by means of high-performance liquid chromatography (HPLC). Although developed for iodopyracet (I) and iodamide (II) (Fig. 1), the procedure is applicable to some other contrast media as well, e.g. metrizoic acid, diatrizoic acid, iothalamic acid and acetrizoic acid.

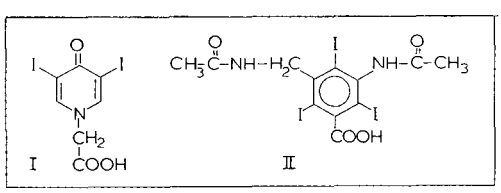

Fig. 1. Structural formulae of iodopyracet (I) and iodamide (II).

EXPERIMENTAL

Materials

Iodopyracet and iodamide were obtained from Dagra (Diemen, The Netherlands). LiChrosorb RP-8 (5 μm), tetrabutylammonium hydrogen sulphate and Tris [tris(hydroxymethyl)aminomethane] were obtained from Merck (Darmstadt, G.F.R.). All other reagents used were of analytical grade.

Apparatus

The equipment used was a Waters Assoc. (Milford, MA, U.S.A.) high-pressure liquid chromatograph consisting of a M 6000A pump, a 46K universal injector and a M440 absorbance detector. I was detected at 280 nm, whilst II was detected at 254 nm.

HPLC conditions

A stainless-steel column (10 × 0.46 cm I.D.) was packed with LiChrosorb RP-8 (5 μm). Chromatography was performed using a mixture of 15% water and 85% methanol containing 0.01 *M* tetrabutylammonium hydrogen sulphate and 0.01 *M* Tris. The elution rate was 1.5 ml/min. A typical chromatogram obtained from a plasma sample is shown in Fig. 2.

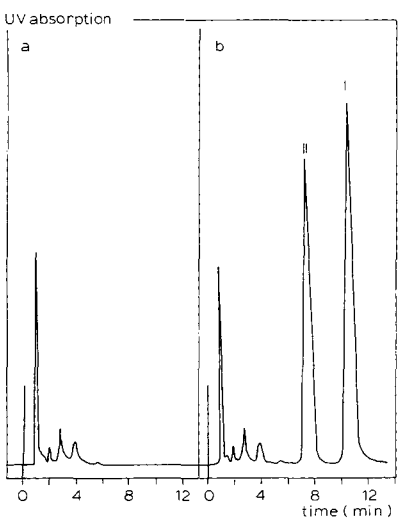

Fig. 2. Chromatograms of (a) blank plasma and (b) plasma containing iodopyracet (I) and iodamide (II).

Assay procedure

For the determination of I, II was used as an internal standard, whilst I was used as an internal standard for the determination of II. Standard solutions of the appropriate salts were prepared in water. Samples of plasma (1 ml) were transferred into a test tube and spiked with 100 μl of internal standard solution

(500 μg/ml). Next, 5 ml of methanol were added to precipitate the plasma proteins. After centrifugation at 1500 *g* for 20 min the supernatant was transferred into another tube and evaporated to dryness under a stream of dry filtered air in a water bath at 65°C. The residue was dissolved in 300 μl of water—methanol (10:90, v/v). After standing for 10 min, 10 μl of the clear supernatant were injected directly into the liquid chromatograph.

Urine samples were treated in a different way. After diluting 100 μl of the urine 10 times with mobile phase and spiking it with 100 μl of internal standard solution (500 μg/ml), 10 μl of the resulting solution were injected into the liquid chromatograph.

Calibration procedure

Samples of blank plasma (1 ml) or urine (0.1 ml) were spiked with various known amounts of the compound to be determined. After treating the calibration samples as outlined above and injecting them into the liquid chromatograph peak height ratios of compound to internal standard were plotted against the amount of compound added.

RESULTS AND DISCUSSION

Under the conditions employed no interfering substances from blank plasma appear in the chromatogram (Fig. 2). This holds true for urine as well.

The procedure described can also be used for the assay of other contrast media, such as metrizoic acid, diatrizoic acid, iothalamic acid and acetrizoic acid. For this last agent it is necessary to change the mobile phase to contain more methanol and less water.

The method involves no extraction, but the deproteinization of plasma should be performed carefully in order to obtain clear solutions for injection. The residue after the evaporation of methanol is not completely free of protein. Therefore it is taken up in the water—methanol (10:90) mixture in which the rest of the proteins settles in about 10 min. During protein precipitation, evaporation and redissolution no significant dissipation of analysed substances occurred. Pure methanol should not be used for this redissolution, since injection of the pure methanolic solution causes asymmetric peaks of the contrast media. Also a mixture with a higher water content is not useful since the protein remains emulsified.

Calibration graphs are linear up to concentrations of at least 100 μg/ml. For iodopyracet the linearity was checked further and appeared to prevail up to 1000 μg/ml. The limit of detection of the various compounds is about 0.2 μg/ml with the procedure described. By small modifications (less dilution) this limit may still be decreased. Over the whole range studied the standard deviations of the plasma and urine determinations were 2—4%.

The method reported here was successfully applied to pharmacokinetic studies aimed at describing kinetic drug interactions. As an example of a pilot study for these investigations Fig. 3 shows a plasma and an urinary excretion curve obtained after application of an intravenous dose of iodopyracet to a male beagle dog. The plasma curve shows three distinct phases, indicating the occurrence of a three-compartmental model. Iodopyracet is recovered almost

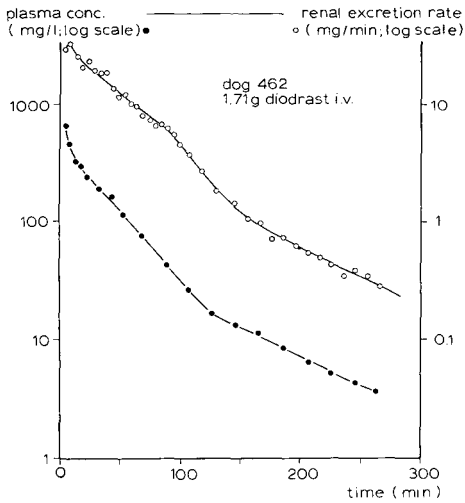

Fig. 3. Plasma and urinary excretion curves obtained after intravenous administration of iodopyracet in the form of its diethanolamine salt to a male beagle dog. Urinary excretion rates are shown at the midpoints of urine collecting periods.

completely unchanged from urine. By careful analysis of plasma and urine kinetics it is possible to obtain insight into the mechanisms of renal excretion and into the nature of possible drug interactions at the level of tubular secretion.

REFERENCES

1 J.F. Rodrigues de Miranda, J. Pharm. Pharmacol., 19 (1967) 161—166.
2 P.G. Welling, M.R. Dobrinska and P.O. Madsen, J. Clin. Pharmacol., 16 (1976) 142—148.

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Note

Direct determination of valproate in serum by isotachophoresis

Comparison with a gas chromatographic method

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Sodium valproate is known as a useful anticonvulsant drug in primary generalized epilepsy [1]. The determination of the anticonvulsant in serum is of importance for the correct treatment of epileptic patients, especially in establishing the pharmacotherapy. Optimal therapeutic serum concentrations are known to be ca. 60 μg/ml.

Several gas chromatographic (GC) procedures have been described [2—9], each with its own advantages and limitations. A disadvantage, common to all GC procedures, is the treatment of the sample prior to chromatography. Dependent on the specific procedure, solvent extraction, derivatisation and evaporation have to be used.

Isotachophoresis [10] is an electrophoretic method that can provide both qualitative and quantitative results on ionic solutes in a relatively short analysis time. The method requires no sample pretreatment and only minute amounts of sample are necessary for an accurate determination. Since valproate is an ionic solute and its therapeutic concentration level is just below the millimolar range, it is possible to determine it directly by analytical isotachophoresis.

MATERIALS AND METHODS

All chemicals were of analytical grade or additionally purified by conven-

tional methods. Sodium valproate was obtained from Labaz (Maassluis, The Netherlands). Test and reference sera were obtained from a hospital pharmacy (Apotheek Haagse Ziekenhuizen, Den Haag, The Netherlands). In addition to valproic acid the test sera contained phenobarbital, phenytoin, ethosuximide, primidone, clonazepam, carbamazepine and 10,11-epoxycarbamazepine.

Serum samples were taken from venous blood; after clotting and centrifugation the sera were stored at −20°C until use.

Gas chromatography

For the GC determinations a Packard-Becker 421 chromatograph was used. Separations were performed in a glass column, packed with 5% FFAP on Chromosorb W HP (Free Fatty Acid Phase, Chrompack, Middelburg, The Netherlands). The injection temperature was 160°C, the oven temperature was kept isothermal at 150°C. Nitrogen was the carrier gas, and flame ionization detection at 175°C was used. Cyclohexanecarbonic acid was used as the internal standard.

Isotachophoresis

For the isotachophoretic separations the coupled column system developed by Everaerts et al. [11] was used. The inner diameter of the preseparation compartment was 0.8 mm. At a leading ion concentration of 0.01 *M* an electrical driving current of 377 μA was used. The valproate zone was trapped in the analytical column, which had an inner diameter of 0.2 mm. The electrical driving current in the analytical column was 10 μA. The electrolyte systems and other operational conditions are given in Table I.

The constant electrical driving current was taken from a modified Brandenburg power supply (Thornton Heath, Great Britain). The voltages varied between 1 and 15 kV. Serum samples were injected directly, using a microliter syringe. Separated zones were detected by measuring the electrical conductance as well as the UV absorption at 254 nm.

TABLE I

ELECTROLYTE SYSTEMS AND OPERATIONAL CONDITIONS

	Preseparation compartment	Separation compartment	Terminating compartment
Anion	Chloride	Chloride	MES*
Concentration (*M*)	0.01	0.005	0.005
Counter constituent	EACA**	EACA	Tris***
pH	5.00	5.00	5.00
Additive	0.3% HEC†	0.3% HEC	
Current density (A/cm²)	0.075	0.0318	

*MES = 2-(N-morpholino) ethanesulfonic acid.
**EACA = ϵ-aminocaproic acid.
***Tris = tris(hydroxymethyl) aminomethane.
†HEC = hydroxyethylcellulose.

RESULTS AND DISCUSSION

One of the major advantages of isotachophoresis is that ionic solutes can often be analyzed without sample pretreatment. Therapeutic levels of valproate in serum, however, differ from the physiological chloride concentration by at least two orders of magnitude. Due to this unfavourable sampling ratio, the electrolyte system will have a rather low separation efficiency [12]. Hence, for a reliable determination, a large column volume must be available, resulting in a large analysis time. Most of these problems can be solved using a coupled column system [11]. This system not only allows the use of a high sample load, but also the use of different electrolytes (see Table I). For the determination of valproate the concentration of the leading ion in the preseparation compartment was 0.01 *M*. At a high driving current serum samples were separated in approximately 6 min. The swamping amount of chloride was allowed to pass the bifurcation with the analytical column. The valproate zone was trapped in the analytical column, which contained the leading ion at a concentration of 0.005 *M*. Fig. 1 shows a representative result when 3 μl of a patient serum were injected. The total analysis time was less than 15 min. Since trapping was started 2 sec before the valproate zone reached the bifurcation point, some other solutes were also analyzed. The valproate zone, however, can easily be localized in both the UV trace (Fig. 1c) and the conductimetric trace (Fig. 1a). From the separation in Fig. 1 it follows that uric acid could have been used as the terminating ion, instead of morpholino-ethanesulfonic acid. As a result a

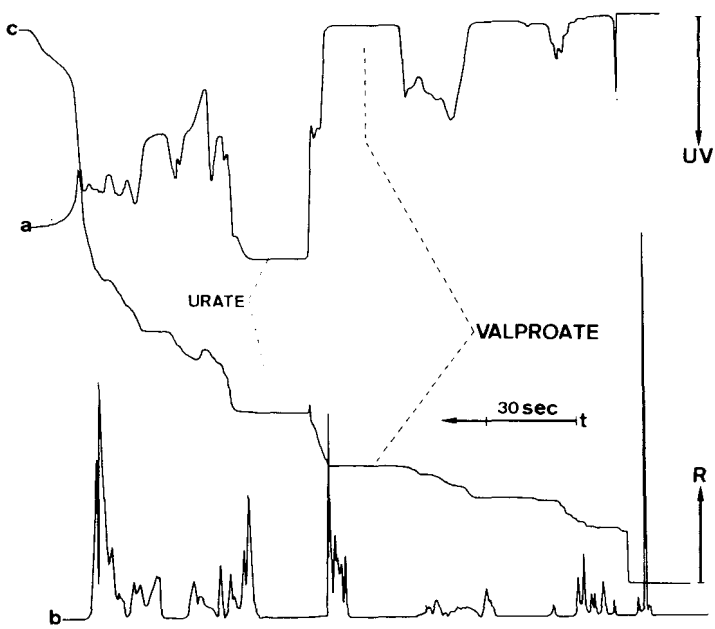

Fig. 1. Isotachophoretic separation of a patient serum. UV = UV absorption at 254 nm; R = increasing resistance; t = increasing time. Injected volume: 3.0 μl of serum. a, Conductimetric trace; b, differentiated conductimetric trace; c, UV trace.

lower end-voltage would have been obtained allowing further optimization of the analysis time. For the quantitative analyses, the characteristics of the calibration line, i.e. zone length versus amount of valproic acid, were determined. The calibration points were measured with standard valproate solutions in water and in serum. Additionally several test sera, containing various other drugs, were analyzed (Fig. 2). A good linear relation was found with a correlation coefficient of 0.99914 (n = 26). The response was found to be 6.12 ng/sec and the mean error per point was 3.8 ng in the detection range 50—500 ng. Re-

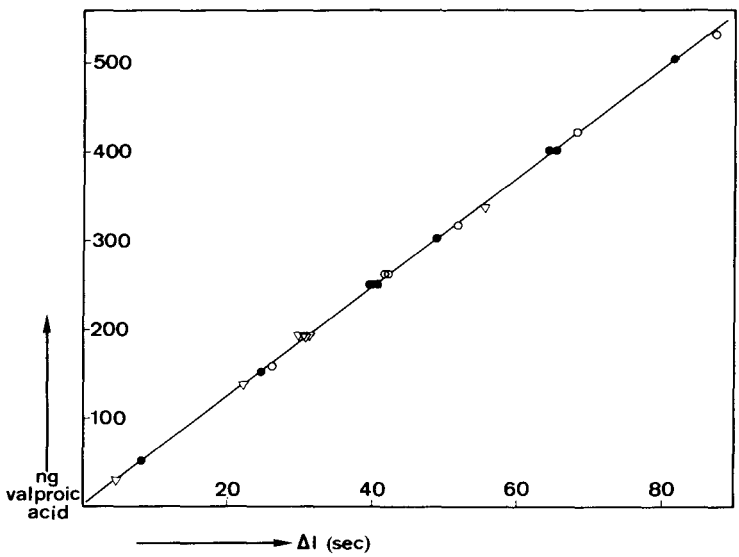

Fig. 2. Calibration graph for isotachophoretic valproic acid determinations. (•) Standard solutions in water; (○) standard solutions in serum; (▽) test sera.

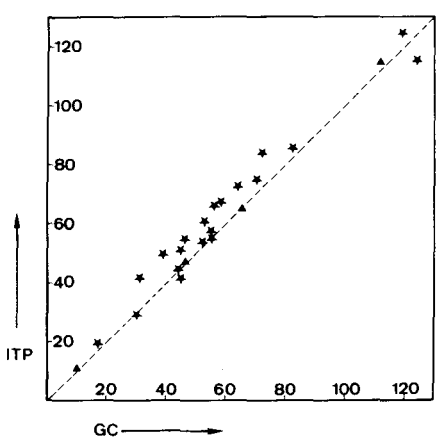

Fig. 3. Comparison of gas chromatographic and isotachophoretic results. Abscissa: μg/ml valproic acid by gas chromatography. Ordinate: μg/ml valproic acid by isotachophoresis. (★) Patient sera, (▲) test sera.

producibility was better than 2% and day-to-day variations were small. The additional other drugs did not interfere.

For the GC procedure 50 μl of the internal standard solution (500 μg/ml cyclohexanecarbonic acid in water) were mixed with 200 μl of serum. After the addition of 500 μl of carbon tetrachloride and 50 μl of 10% perchloric acid solution in water, the sample was mixed with a vortex-type mixer. The sample was then centrifuged, the upper layer was removed by suction and the interfacing layer of protein was lifted with a Pasteur pipette. A 5-μl sample of the organic layer was applied to the tip of the moving needle. After the evaporation of the organic solvent the sample was directly injected.

The GC procedure also yielded a good calibration curve, with a correlation coefficient of 0.99969. In the detection range of 2—20 ng the mean error per point was 0.1 ng. Reproducibility and day-to-day variations were better than 5%.

Using these calibration data, valproic acid concentration levels were determined in the sera of twenty patients. The isotachophoretic results were compared with the GC results. As can be seen from Fig. 3 there is a good correlation. The experimental slope deviates by only 1% from the ideal value of unity. A positive intercept of 4.9 ng of valproic acid, however, is present. The group mean was 62.1 μg/ml for the isotachophoretic determinations and 57.8 μg/ml for the GC determinations. The origin of the systematic deviation is still under investigation. The results of four different test sera (calf serum) were in good agreement with the expected values for both methods.

ACKNOWLEDGEMENTS

The authors are indebted to the analytical staff of the Kempenhaege Epileptic Institute (Heeze, The Netherlands) for the accurate GC determinations.

REFERENCES

1 R. Pinder, R. Brogden, T. Speight and G. Avery, Drugs, 13 (1977) 81.
2 C. Jakobs, M. Bojash and F. Hanefeld, J. Chromatogr., 146 (1978) 494.
3 S. Willox and S.E. Foote, J. Chromatogr., 151 (1978) 67.
4 D.J. Berry and L.A. Clarke, J. Chromatogr., 156 (1978) 301.
5 J.-C. Libeer, G. Scharpé, P. Schepens and R. Verkerk, J. Chromatogr., 160 (1978) 285.
6 O. Gyllenhaul and A. Albinsson, J. Chromatogr., 161 (1978) 343.
7 G. Chabert, P. Bondon, M. Revol and J.C. Plasse, in A. Frigerio and L. Renoz (Editors), Recent Developments in Chromatography and Electrophoresis, Elsevier, Amsterdam, Oxford, New York, 1979, p. 123.
8 J.-C.P. Libeer, P.J. Schepens and F.V. Lakiere, in A. Frigerio and L. Renoz (Editors), Recent Developments in Chromatography and Electrophoresis, Elsevier, Amsterdam, Oxford, New York, 1979, p. 163.
9 I. Dijkhuis and E. Vervloet, Pharm. Weekbl., 109 (1974) 42.
10 F.M. Everaerts, J.L. Beckers, and Th.P.E.M. Verheggen, Isotachophoresis: Theory, Instrumentation and Applications, Elsevier, Amsterdam, Oxford, New York, 1976.
11 F.M. Everaerts, Th.P.E.M. Verheggen and F.E.P. Mikkers, J. Chromatogr., 169 (1979) 21.
12 F.E.P. Mikkers, F.M. Everaerts and J.A.F. Peek, J. Chromatogr., 168 (1979) 293.

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Book Review

Recent developments in mass spectrometry in biochemistry and medicine, Vol. 1, edited by A. Frigerio, Plenum Press, New York, London, 1978, XII + 658 pp., price US$ 66.00, ISBN 0-306-31138-0.

This volume represents the proceedings of the 4th International Symposium on Mass Spectrometry in Biochemistry and Medicine held in June 1977 in Riva del Garda, Italy. It contains 52 articles, a list of approximately 300 participants of the symposium, an author index and a subject index. Unfortunately, the subject index is too short to be very useful. Since the book covers a broad spectrum of topics the reader will miss a classification and grouping of the articles under some principal sections.

There could be seven sections. More than one quarter of the papers deals with biotransformation, metabolism, pharmacokinetics, distribution or transfer of drugs in animals and humans. One fifth of the articles is concerned with the qualitative and quantitative analysis of various endogenous organic compounds (amines, amino acids, prostaglandins, organic acids in an inborn error of metabolism, direct quantitative mass spectrometric determination of amino acids, fatty acids and cholesterol). Another fifth of the papers deals with mass spectrometric fragmentation and structure determination, e.g. of diterpenoid alkaloids, sterols in red and brown algae, permethylated disaccharides, pterins and sialic acids. The last quarter covers four groups of papers: one group is dedicated to methods, techniques and instrumentation, another group describes environmental analyses (polychlorodibenzodioxins, TCDD, allylbenzenes), the next group is concerned with the mass spectrometric analysis of respiratory and blood gases with special emphasis on continuous monitoring using flexible catheters, and the last group deals with naturally occurring differences in ^{15}N abundance.

The experimental details given by the authors of the papers are generally good and are valuable information for the reader. The same holds true for the many mass spectra shown in the book, for the interpretations of the spectra and for the discussions of the fragmentations. The user of the book finds information on mass spectrometry applied in its various forms: direct sample insertion systems, combination with gas chromatography, single and multiple ion monitoring, electron impact and chemical ionization.

The book has been produced from camera-ready manuscripts and contains well presented figures and tables. It provides a valuable compilation of practical information and can be recommended to researchers especially in the fields of drug metabolism, metabolism of endogenous substances and to some extent to scientists engaged in environmental analyses.

Tübingen (G.F.R.) H.M. LIEBICH

Author Index

Subject Index

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

APPARATUS

N-1412

GEL FILTRATION HANDBOOK

Pharmacia Fine Chemicals have issued their completely revised handbook on gel filtration. In this book, "Gel Filtration in Theory and Practice", the company gives full details of the geld filtration media from Pharmacia with procedures for packing and running columns to obtain the best results.

N-1435

BROCHURE ON ELECTROPHORESIS EQUIPMENT

In the brochure "Equipment for Immunoelectrophoresis" the Danish manufacturer Holm-Nielsen gives a survey of the apparatus, methods and reagents in their program. The brochure describes the Power Pack II power supply, the electrophoresis chamber, ancillary equipment and methods, application and working procedures.

N-1405

GEL SCANNER ACCESSORY

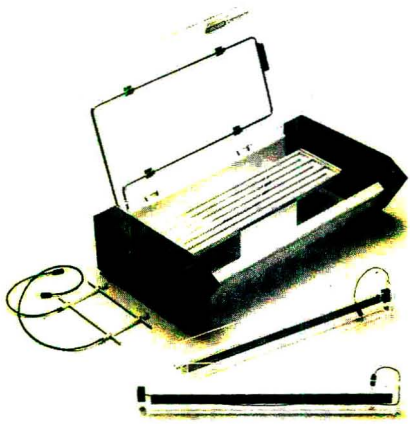
Varian Associates have introduced their gel scanner accessory for use with the Cary Models 210 and 219 UV-Vis spectrophotometers. The device provides for an automatic transport of gels across the instrument light beam to produce a record of absorbance (or transmittance) as a function of gel position. While intended primarily for tubular gels from electrophoresis separations, the new accessory can be used also to measure sheet film. Tubular gels up to 20 cm long can be handled either as free gels in boats or as intact gels in their electrophoresis tubes. The gel scanner accessory can be mounted inside the sample compartment of the Varian Cary 210 or 219.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1414

FLAT-BED ELECTROFOCUSING/ ELECTROPHORESIS CELL

The Model 1415 electrofocusing/electrophoresis cell from Bio-Rad Labs. is the basic unit in five systems for high-resolution analytical and preparative electrofocusing, immunoelectrophoresis, zone electrophoresis and preparative DNA separation. The cell has a large, rectangular cooling platform, a condensation control coil that keeps moisture off lid and gels and dual electrode jacks for running two electrofocusing experiments at once. The platinum electrofocusing electrodes are detachable, adjustable and are available in two sizes for maximum flexibility in experimental design.



N-1399

AMINO ACID ANALYZER

A complete analysis of a protein hydrolysate is performed in approximately 1 hour with the Model 3A29 amino acid analyzer introduced by Carlo Erba. Physiological fluids take less than 3 hours. The instrument uses one column and up to five buffer solutions to run a complete analysis. To achieve a good reproducibility the pH and the ionic strength are increased in steps. A separation of all components without the use of expensive resins is obtained by using an improved column packing technique and a reduction of the dead volume of the analyzer. The Carlo Erba 3A29 is equipped with Milton Roy pumps and with a colorimeter detecting at 440 and 570 nm and constantly referring to 690 nm. With the available autosampler the new Carlo Erba amino acid analyzer can be operated as a fully automatic, microprocessor-controlled analysis system.

CHEMICALS

N-1438

PHARMACIA PUBLICATIONS

From Pharmacia Fine Chemicals we received a number of publications on their products and the application. The 9th and 10th 1979 issues of the periodical "Separation News" contain articles on new Pharmacia products for affinity chromatography, electrophoresis, isoelectric focusing, the new synthesis of SP-Sephadex C-25 and C-50, immunoadsorbents for cell separation, Sephacryl™ S-300 Superfine in 6 *M* guanidine–HCl. With these "Separation News" issues we received the 1979 index for the periodical. Nearly 1000 literature references are collected in a new Pharmacia booklet "Literature References 1979". Finally the new Pharmacia 1980 catalog gives a full description of the company's products. New in the catalog are a group-specific adsorbent for affinity chromatography, Heparin–Sepharose® CL-6B, and Silane A174, a polyacrylamide–glass plate adhesion promotor for use in isoelectric focusing.

N-1395

HANDBOOK OF LIPIDS AND SELECTED CARBOHYDRATES

Supelco has released the 5th edition of their Handbook of Lipids and Selected Carbohydrates. The 104-page handbook presents lipids, carbohydrates and derivatives, mixtures and kits for gas chromatography and thin-layer chromatography, aflatoxins and mycotoxins, and clinical materials. The handbook also contains the physical and chemical properties and chemical structures of fatty acids, esters, glycerides, phospholipids, glycolipids, tocopherols, steroids, bile acids, selected carbohydrates, glycosphingolipids, and clinical materials.

N-1431

AGAROSE GEL KITS FOR ELECTROPHORESIS

Two agarose gel kits from Beckman Instruments are designed for use with the Beckman Microzone® electrophoresis systems. Maxizone™ gel is preslotted for up to eight sample inoculations to handle large workloads quickly. Because Maxizone gels are rehydratable and are said to have a shelf life of 18 months, they can be cut and the unused pieces stored. The Minizone™ gel has the same gel characteristics as the Maxizone gel. Its smooth and uniform surface provides the capability to perform many electrophoresis techniques; it is said to be very good for immunoelectrophoresis applications.

N-1424

HEPARIN–SEPHAROSE CL-6B

Pharmacia Fine Chemicals supplies Heparin–Sepharose® CL-6B for affinity chromatography of a wide range of proteins including coagulation proteins, other plasma proteins, lipases, steroid receptors, protein synthesis factors and enzymes which bind to nucleic acids. The adsorbent can be sterilized by autoclaving. The cross-linked matrix, Sepharose CL, withstands dissociating conditions.

PROCEDURES

N-1455

DATA SHEET ON GLA ANALYSIS

An applications data sheet from Beckman Instruments describes the amino acid analysis of γ-carboxyglumatic acid (Gla). Gla is an unusual amino acid that has been identified as a constituent of proteins involved with blood coagulation. It is also present in bone protein, in kidney protein and in placenta protein. The data sheet is written by Dr. Peter Hauschka of Children's Hospital (Boston, MA) and Harvard University School of Dental Medicine, and Edward Fong of Beckman Instruments. The sheet provides sample chromatograms and operating parameters for the Beckman Model 121M amino acid analyzer, as well as many references.

N-1392

SCIENCE TOOLS, VOL. 26 (1979) NO. 2

Science Tools is the LKB Instrument journal, published a few times a year and containing scientific publications and news on the articles in the LKB sales program. This 2nd 1979 issue of Science Tools has articles on "ultrogel polyacrylamide–agarose gel as a matrix for biospecific sorbent of carboxylic proteinases" by Borovikova, Lavrenova and Stepanov; "a standard technique for the comparison of tapeworm soluble proteins by thin layer isoelectric focusing in polyacrylamide gels" by Kumuratilake and Thompson; "properties of an anti-scratch-layer-free X-ray film for the autoradiographic registration of tritium" by Ehn and Larsson; and "technical aspects of the bioluminescent firefly luciferase assay of ATP". Further, the issue contains the Ampholine Electrofocusing News, and informations on LKB's integrated column chromatography system.

N-1445

SCIENCE TOOLS, VOL. 26 (1979) NO. 4

The 4th 1979 issue contains articles on applications of isoelectric focusing in the clinical and medical fields, on Ampholine® electrofocusing and on some new products of the company.

NEW BOOKS

Progress in medicinal chemistry 16, edited by G.P. Ellis and G.B. West, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, X + 292 pp., price Dfl. 130.00, US$ 63.50, ISBN 0-7204-0667-6.

Atlas of the three-dimensional structure of drugs, by J.P. Tollenaere, H. Moereels and L.A. Raymaekers, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, XII + 322 pp., price Dfl. 75.00, US$ 36.50, ISBN 0-444-80145-6.

Radioimmunology 1979 (Proc. 4th Int. Symp., Lyon, April 19–21, 1979), edited by Ch.A. Bizollon, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, XII + 308 pp., price Dfl. 99.00, US$ 48.25, ISBN 0-444-80154-5.

Chemical porphyria in man, edited by J.J.T.W.A. Strik and J.H. Koeman, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, XIV + 236 pp., price Dfl. 85.00, US$ 41.50, ISBN 0-444-80159-6.

Human plasma proteins, by J.W. Keyser, Wiley, New York, Chichester, 1979, *ca.* 304 pp., price *ca.* US$ 38.50, £ 14.00, ISBN 0-471-27598-0.

Metals in biochemistry, by P.M. Harrison and R. Hoare, Chapman & Hall, London, 1980, 80 pp., price ca. £ 2.25, ISBN 0-412-13160-9.

Radioimmunoassay of drugs and hormones in cardiovascular medicine (Proc. Int. Symp. Applications of Radioimmunoassay to Cardiovascular Medicine and Int. Theoretical and Practical Course on Radioimmunoassay of Drugs and Hormones, Gardone Riviera and Brescia, May 29–June 1, 1979), edited by A. Albertini, M. Da Prada and B.A. Peskar, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, X + 364 pp., price Dfl. 115.00, US$ 56.00, ISBN 0-444-80176-6.

Tumour markers: impact and prospects (Workshop on Tumour Markers: Fundamental Aspects and Clinical Evaluation, Lunteren, December 12–14, 1978), edited by E. Boelsma and Ph. Rümke, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, 346 pp., price Dfl. 110.00, US$ 53.75, ISBN 0-444-80133-2.

Cell electrophoresis: clinical application and methodology (Proc. 1st Int. Symp., Bristol, June 12–15, 1979), edited by A.W. Preece and D. Sabolović, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, XVI + 496 pp., price Dfl. 115.00, US$ 56.00, ISBN 0-7204-0674-9.

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

MEETING

FIFTH INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY

The fifth International Symposium on Column Liquid Chromatography will be held in Avignon (France) from May 11–15, 1981. The program will comprise discussion papers as well as poster papers dealing with all areas of column liquid chromatography and related techniques. Lectures will be held in English. A poster session will be organized daily, followed by a discussion session on the same topic. The symposium will be held in the Centre de Congrès, Palais des Papes, Avignon.

Notification of discussion papers and poster communications submitted for consideration should reach the organizing committee not later than November 14, 1980, together with a 1-page, 300-word abstract. A scientific committee will review all papers and decide upon acceptance by December 15, 1980.

The abstracts of the discussion papers and poster communications will be reproduced in a booklet distributed to those attending the meeting. The papers will be published in a special issue of the Journal of Chromatography. Authors will be requested to hand their manuscripts to the Editor at the meeting.

In conjunction with the symposium, an exhibition of liquid chromatography instruments and accessories is planned.

Further details concerning the symposium can be obtained from the organizer, Professor G. Guiochon, Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, Route de Saclay, 91128 Palaiseau Cedex, France.

PUBLICATION SCHEDULE FOR 1980

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

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	The publication schedule for further issues will be published later.			
Chromatographic Reviews			184/1	184/2					184/3				
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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