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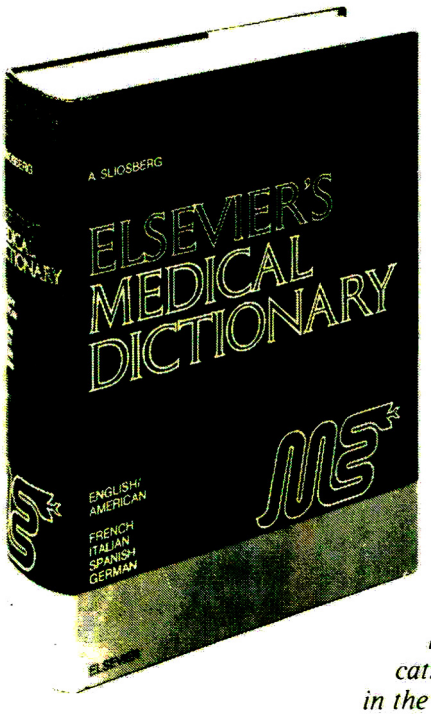

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CHROMBIO. 719

LONGITUDINAL URINARY EXCRETION OF SOME "TRACE" ACIDS IN A HUMAN MALE

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(Received July 17th, 1980)

SUMMARY

Conjugated and unconjugated urinary levels of phenylacetic acid (PAA), *m*-hydroxyphenylacetic acid (*m*-HPA) and *p*-hydroxyphenylacetic acid have been determined for 24-h urine samples obtained from a single healthy male over a 28-day period. Gas chromatographic—electron-capture and mass spectrometric—integrated ion current techniques incorporating appropriate internal standards were used. The average urinary excretion values obtained were (in mg/24 h): PAA unconjugated 0.67, conjugated 96.6; *m*-HPA unconjugated 7.3, conjugated < 0.1; *p*-HPA unconjugated 22.4, conjugated < 1.2. Following the ingestion of appropriate deuterated amino acid precursors the expected urinary deuterated trace acids were identified and quantitated; in the case of deuterated phenylethylamine, *m*-HPA and *p*-HPA as well as PAA were identified and quantitated. This is the first evidence of phenylethylamine hydroxylation in the human. The longitudinal excretion of the trace acids was compared with that of the trace amines.

INTRODUCTION

Quantitative determinations of the levels of urinary phenylacetic acid (PAA), *m*-hydroxyphenylacetic acid (*m*-HPA) and *p*-hydroxyphenylacetic acid (*p*-HPA), the so-called "trace" acids, in the normal human have been reported [1—3]. These acids are the principal metabolites of the trace amines β-phenylethylamine (PE) and *m*- and *p*-tyramine (*m*-, *p*-TA) which have been claimed to be abnormally excreted in persons suffering from certain neurological and psychiatric disorders (see ref. 4 for a review).

Although the acid levels in the urines of large numbers of different control individuals have been determined [1—3], there has not been an investigation of the daily variations in these trace acids in the urine of a particular individual as has been the case for the trace amines [5]. In this paper, we describe the procedures and the results of the analysis of free and conjugated PAA, *m*-HPA

and *p*-HPA in the urines of a human male (the same one as referred to in ref. 5) over a period of twenty-eight days (16 concurrent days in 1976 and 12 concurrent days in 1977). On certain days likely precursors of these acids (labelled with deuterium) were ingested and the urines analyzed for labelled acids.

Identification and quantitation of the acids was achieved by gas chromatography with electron-capture detection (GC—ECD), with periodic confirmation of the results by the gas chromatography—mass spectrometry—integrated ion current (GC—MS—IIC) technique.

MATERIALS AND METHODS

HPLC-grade solvents were purchased from Caledon Labs. (Georgetown, Canada), heptafluorobutyric anhydride (HFBA) and pentafluorobenzyl bromide (PFB-Br) from Pierce (Rockford, IL, U.S.A.). *m*- and *p*-hydroxyphenylacetic acids (Aldrich, Milwaukee, WI, U.S.A.) were deuterated by exchange reactions. Three exchanges involving heating 500 mg acid and 5 ml of 9% deuterium chloride in deuterium oxide at 110°C for 24 h yielded labelled acids containing no unlabelled acid, as determined by mass spectrometry. The major component in the *p*-HPA product contained four deuterium atoms (91%), two on the ring and two on the chain; in the *m*-isomer the major component contained five deuterium atoms (65%), three on the ring and two on the chain. Phenylacetic acid-α,α-D_2 was prepared by refluxing phenylacetonitrile in a 30% solution of NaOD in D_2O for 6 h. Incorporation was 98.3% D_2 and 1.7% D_1.

Twenty-eight 24-h urine samples starting with the first voiding each day were collected in May 1976 and 1977 in polyethylene bottles containing 10 ml conc. HCl. After recording the volume and pH, 250-ml aliquots of each urine were stored at —16°C until required for analysis. Precursors labelled with deuterium were ingested on day 2 (DL-phenylalanine-α,β-D_2, 3 g), day 6 (DL-tyrosine-D_2, 3 g), and days 13 and 24 (β-phenylethylamine-β,β-D_2), 200 mg each day.

At the time of analysis the samples were thawed and thoroughly mixed, 1-ml aliquots were removed and to each aliquot was added the appropriate internal standard homologue: *p*-hydroxyphenylpropionic acid (*p*-HPP) (27 μg) for GC—ECD analyses of *m*-HPA and *p*-HPA, and phenylpropionic acid (PPA) (70 μg) for analyses of PAA; similar quantities of deuterated *m*- and *p*-hydroxyphenylacetic acids were added for GC—MS—IIC analyses of *m*-HPA and *p*-HPA, and PAA-D_2 for analyses of PAA. The samples were saturated with NaCl (about 0.5 g) and extracted with ethyl acetate (3 × 2 ml); the extracts were then concentrated to 100—200 μl and transferred to 1-ml Reacti-vials (Pierce). Samples being analysed for PAA were treated at this stage with 50 μl triethylamine in order to form triethylamine salts of the acids and thus reduce losses due to evaporation. All samples were evaporated to dryness in a stream of nitrogen. Traces of water were removed by the addition of 100 μl benzene followed by evaporation to dryness in a stream of nitrogen.

For the quantitation of *m*-HPA and *p*-HPA, 150 μl of 15% HCl in methanol was then added to the samples. After 30 min at room temperature, the methanol was evaporated in a stream of nitrogen at 55°C, and the residue redried following the addition of benzene. To the residue was added 150 μl hexane

and 50 μl HFBA and this was then heated at 65°C for 1 h in a heating block. After cooling, 100 μl of phosphate buffer (pH 6.0) was added and the mixture vigorously shaken. The organic layer was withdrawn with a micropipette, the aqueous layer was then washed once with 100 μl hexane which after removal and combination with the original hexane layer was diluted to 500 μl with hexane.

For the quantitation of PAA, 200 μl of a benzene solution of PFB-Br (3.0%) and 18-crown-6 (3.0%) and a few milligrams of anhydrous potassium fluoride were added to the extract residue and heated at 50°C for 1 h [6]. The benzene solution was washed (2 × 200 μl) with water, evaporated in a stream of nitrogen almost to dryness and then dissolved in 0.5 ml hexane for GC—MS—IIC analysis or in 4.0 ml hexane for GC—ECD analysis.

For the GC quantitation, 0.5 μl (containing about 5 ng PAA or 50 ng *p*-HPA) was injected onto a GSC-SP2100 support-coated open-tube (SCOT) capillary column, 31 m in length, mounted in a Hewlett-Packard 5710A gas chromatograph fitted with an electron-capture detector (ECD). The flow-rate of carrier gas (5% methane in argon) through the column was 24 cm/sec. For the analysis of *m*-HPA and *p*-HPA as their HFB-methyl derivatives, the chromatograph conditions were: initial oven temperature 140°C isothermal for 2 min, then 4°C/min until the internal standard had been eluted (about 14 min), and finally 8°C/min to 260°C in order to purge the column of other uninteresting compounds. The injector temperature was 200°C and the detector temperature, 250°C. For the analysis of PAA as its PFB derivative, the chromatograph conditions were: initial oven temperature 195°C isothermal for 2 min, then 4°C/min to 260°C with baking at this temperature for 16 min. The injector temperature was 250°C and the detector temperature, 300°C.

For GC—MS quantitation, 1—5 μl of the hexane solution was transferred by means of a syringe to a solids injector, the solvent was allowed to evaporate, and the sample then injected onto a GSC-SP2250 SCOT column (57 m) mounted in a Hewlett-Packard 5710 gas chromatograph equipped with a flame ionization detector and coupled by means of a S.G.E. jet separator to an A.E.I. MS 902S mass spectrometer. The mass spectrometer was focused on the exact masses of the molecular ions of the derivatives of unlabelled acid and deuterated acid; as the sample was eluted from the column into the mass spectrometer source, the signal from each was recorded alternately as the instrument switched from one mass to the other. This procedure, the so-called integrated ion current (IIC) technique, has been described previously for the quantitation of trace amines [7]. The above described procedures are summarized in Fig. 1. Blanks were determined by carrying 1 ml of 2 *N* HCl through the entire procedure. Conjugated urinary acid levels were determined in an identical manner after hydrolysis of 1 ml urine for 60 min at 100°C following the addition of 0.5 ml conc. HCl. The conjugated acid values were calculated by subtracting the unconjugated value from that of the hydrolyzed urine.

Known amounts (10 ng to 100 μg) of authentic acids were also carrie through the above procedures in order to calculate correction factors; these take into account differential extraction, derivatization, and detector response of the acids and internal standards. Both procedures yield linear calibration curves at least in the range 10^{-8}—10^{-4} g.

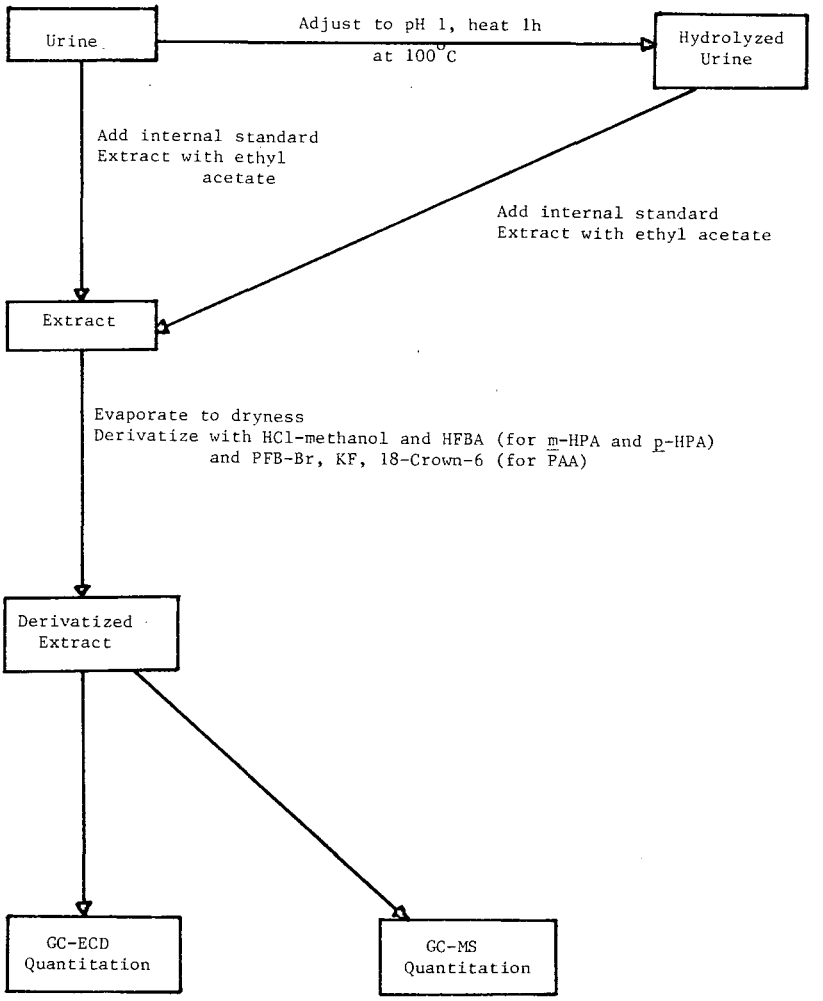

Fig. 1. Schematic outline for the isolation and quantitation of some trace acids.

The presence and amount of formed deuterated acids following the ingestion of the deuterated precursors was determined mass spectrometrically. First, the IIC area ratio for the masses of the derivatives of the D_2-acid and non-labelled acid (designated D_2/H ratio) was determined on authentic non-deuterated acid. This provides the isotopic contribution of non-deuterated acid to the signal for the deuterated acid. The D_2/H ratio was then determined for derivatized samples isolated from the urine extracts. After subtraction of the isotopic contribution from the non-deuterated acid it is possible to calculate the absolute amount of deuterated acid present since the total amount of acid is known from the GC analysis and the ratio of deuterated to non-deuterated acid is known from the GC—MS—IIC quantitation. In the case in which phenylalanine-α,β-D_2 was the precursor, the product acids contain only one deuterium atom, so the ratio measured was D_1/H.

RESULTS

The urinary excretion profiles as determined by GC—ECD for conjugated PAA and unconjugated *m*-HPA and *p*-HPA along with earlier conjugated amine values are shown in Figs. 2, 3 and 4. [The unconjugated amine values (see

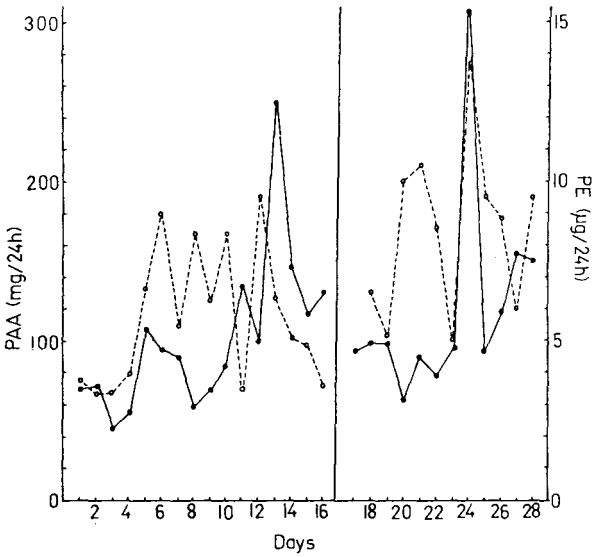

Fig. 2. Urinary excretion of conjugated phenylacetic acid (•——•) and conjugated phenylethylamine (○ — — — ○) in a human male.

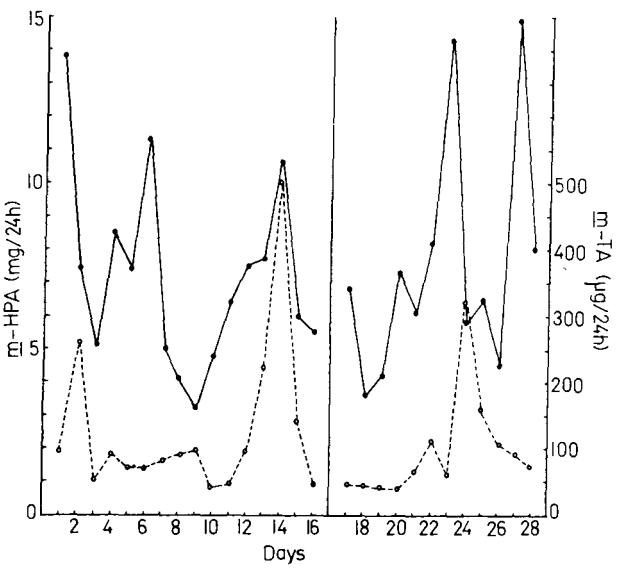

Fig. 3. Urinary excretion of unconjugated *m*-hydroxyphenylacetic acid (•——•) and conjugated *m*-tyramine (○ — — — ○) in a human male.

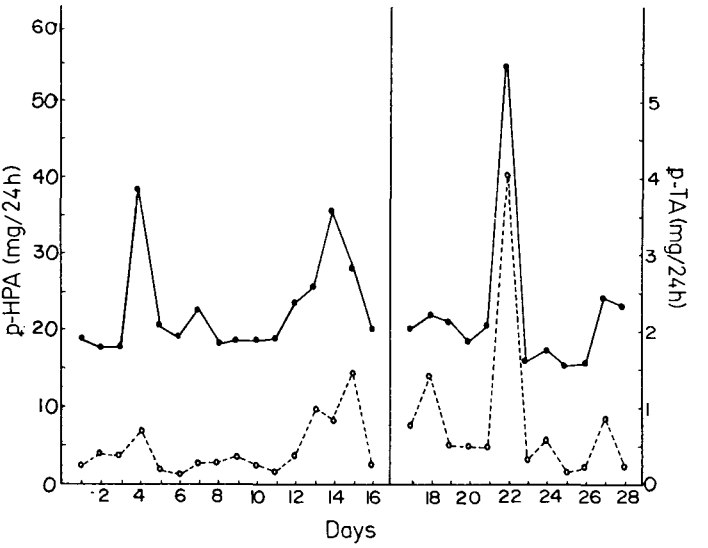

Fig. 4. Urinary excretion of unconjugated *p*-hydroxyphenylacetic acid (•——•) and conjugated *p*-tyramine (○ — — — ○) in a human male.

Figs. 2—4 in ref. 5) were not plotted because they show relatively little variation from day to day.] Unconjugated PAA in this particular individual constituted less than 1% of total PAA and so has not been included in the figure. The average unconjugated PAA level was 0.67 mg/24 h compared to 96.6 mg/24 h conjugated PAA. This subject normally excreted insignificant amounts of conjugated *m*-HPA or conjugated *p*-HPA except on day 1 in this series when 19% of the total *m*-HPA and 20% of the total *p*-HPA was conjugated. The mean values ± standard error of the means, and the ranges of these urinary trace acids are summarized in Table I.

TABLE I

URINARY EXCRETION OF PHENYLACETIC ACID AND *m*- AND *p*-HYDROXYPHENYLACETIC ACIDS IN A HUMAN MALE

Values using the GC—ECD technique represent daily excretion over the 28-day period (mean ± S.E.M.) of the indicated acid except in the first line where days 13 and 24 (i.e., the days of ingestion of phenylethylamine) are omitted.

Acid	Amount (mg/24 h)	Range (mg/24 h)
Phenylacetic (conjugated)	96.4 ± 5.6	45.5—155.6
Phenylacetic (conjugated)	110.0 ± 10.6	45.5—306.8
m-Hydroxyphenylacetic (unconjugated)	7.3 ± 0.6	3.2— 14.9
p-Hydroxyphenylacetic (unconjugated)	22.4 ± 1.5	15.3— 54.0

The presence and amounts of deuterated acids formed from the ingested deuterated precursors are listed in Table II.

When standard solutions of the acids were carried through the procedures,

TABLE II

URINARY DEUTERATED TRACE ACIDS ARISING FROM LABELLED PRECURSORS

— = not present; nd = not determined. Values obtained using GC—MS—IIC procedure. Figures in parentheses represent percentage of labelled acid as proportion of total acid excreted.

Precursors	PAA (mg) (conjugated)	*m*-HPA (mg) (unconjugated)	*p*-HPA (mg) (unconjugated)
Phenylalanine-D_2 (day 2)	2.5 (3.5)	—	—
p-Tyrosine-D_2 (day 6)	—	—	2.7 (14.5), day 6 4.6 (20.6), day 7 0.9 ((5.0), day 8
Phenylethylamine-D_2 (day 13)	31.9 (12.8)	0.031 (0.4)	0.026 (0.1)
Phenylethylamine-D_2 (day 24)	36.5 (11.9)	nd	nd

the results obtained indicated that correction factors needed to be applied in some cases. With respect to the GC—ECD analyses of PAA for example, the correction factor was 0.658; this is presumably in part at least because PPA is not a perfect internal standard and as such does not extract, derivatize or activate the electron-capture detector in exactly the same way as PAA. In the GC—MS analyses, the correction factor was higher at 0.825. We presume that the reason for this being less than 1.0 relates to the fact that during hydrolysis in strong acid some back-exchange of the deuterated internal standard occurs.

DISCUSSION

From Figs. 2, 3 and 4, and Table I, it can be seen that in this particular individual there is a considerable variation in the daily excretion of conjugated PAA and unconjugated *m*-HPA and *p*-HPA. The excretion of unconjugated *p*-HPA appears to correlate quite well with conjugated *p*-TA (see also ref. 5) but much less so between unconjugated *m*-HPA and *m*-TA. There appears to be little, if any, correlation between the excretion of conjugated PE and conjugated PAA.

The ingestion of deuterated phenylalanine and *p*-tyrosine does not appear to have exerted any particularly significant effect on the total urinary excretion of *m*-HPA, *p*-HPA or PAA; the ingestion of PE-D_2, on the other hand, influenced markedly the output of PAA on days 13 and 24. In this latter case, however, it is interesting to note that although the conjugated urinary PAA output increased from an average of about 100 mg/day before the ingestion of 200 mg phenylethylamine-D_2 to 250 mg/day on day 13 and 306 mg/day on day 24 (when the PE-D_2 was consumed), only 12% of that urinary PAA was deuterated. This means that only 30—40 mg of the 250 or 306 mg was labelled; consequently more than 200 mg of extra unlabelled conjugated PAA was ex-

creted on days 13 and 24. This is more than double the daily average and seems to point to the induction of much greater than normal amounts of urinary PAA being formed when PE had been ingested.

A complicating factor in determining the actual amount of urinary deuterated conjugated acids present in the urine was the back-exchange between deuterium and hydrogen atoms which occurred when the acidified urine was heated at 100°C for 60 min. In standard solution, 17.5% of the D_2-acid was lost under these conditions [90% of this became D_1-acid and 10% unlabelled (i.e. hydrogen) acid]. For shorter hydrolysis times, this loss is reduced but in these cases the hydrolysis is incomplete.

Although the ingestion of deuterated amino acid precursors did not significantly increase the total urinary excretion of the trace acids, these acids labelled with deuterium were identified and quantitated in the urine in many cases (see Table II). After eating 3 g of phenylalanine-D_2 on day 2 for example, the excreted conjugated PAA was 3.5% labelled, although no labelled *m*-HPA or *p*-HPA was identified. Ingestion of labelled *p*-tyrosine on day 6 produced labelled unconjugated *p*-HPA on days 6, 7 and 8 amounting to 14.5%, 20.6% and 5.0% respectively of the total unconjugated *p*-HPA excretion (see Table II). Ingestion of PE-D_2, as stated earlier, produced substantial quantities of labelled conjugated PAA, but in addition small but easily measurable amounts of labelled *m*-HPA and *p*-HPA. From this it is permissible to conclude that ring hydroxylation of PE occurs in man yielding *m*- and *p*-TA which are then oxidatively deaminated to *m*-HPA and *p*-HPA as is the case in the rat [8]. It is interesting to note, as has been noted in an earlier publication [2], that there can be quite a considerable lag (up to 3 days in some cases) between the ingestion of a precursor and its subsequent appearance, as a metabolite, in the urine.

A comparison of the values obtained in this longitudinal study on a single individual with those from earlier studies in which many different individuals were assessed gives good agreement. Thus Martin et al. [1] obtained a value of 137.4 ± 15.8 mg/24 h for total urinary PAA using a mass fragmentographic method and Sandler et al. [3] reported 119.8 ± 15.0 mg/24 h using a gas chromatographic procedure. The average value obtained in this study was 97.4 mg/24 h, as determined by GC—ECD and confirmed by GC—MS—IIC (see Table III for a comparison of the two procedures). In the case of *m*-HPA the value reported here (7.3 mg/24 h) agrees quite well with that listed by Sandler et al. [3] (5.8 mg/24 h) and for *p*-HPA, our value (22.4 mg/24 h) similarly agrees closely with that listed by Sandler et al. [3] (21.3 mg/24 h) although it is lower than that listed by Boulton [2] in an earlier study using a fluorimetric procedure.

Finally there is the question of the name "trace" in reference to these particular amines and their acid metabolites. Whilst it is a fact that in mammalian tissues PE, *m*-TA and *p*-TA are found in very small amounts [9] this is not so in some invertebrates [10]. It is also not so in mammalian tissues in the presence of certain drugs such as monoamine oxidase inhibitors [11]. In the case of the acids PAA, *m*-HPA and *p*-HPA it is again a fact that in mammalian brain they are found in small amounts [12—14], in urine however they are found in substantial amounts approximately equivalent to the acid

TABLE III

COMPARISON OF RESULTS OBTAINED BY GC—ECD AND GC—MS—IIC IN SELECTED URINE SAMPLES

See text for details.

Acid (mg/24 h)	Day	GC—ECD	GC—MS—IIC
PAA	1	70.7	58.3
	2	74.7	85.2
	16	132.5	119.9
	21	89.7	73.4
	26	117.1	116.4
m-HPA	2	7.4	8.1
	3	5.1	4.0
	22	8.2	7.4
p-HPA	2	17.5	15.2
	3	17.5	13.3
	22	54.0	50.6

metabolites of the putative neurotransmitters (homovanillic acid, dihydroxyphenylacetic acid, 5-hydroxyindole acetic acid, etc.) and this indicates that in the body the so-called trace amines are in a state of rapid and dynamic turnover [4, 9, 15]. This suggests that they play an important role in metabolism and may perhaps be involved in the modulation or propagation of nervous impulses as has been proposed [4, 9].

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CHROMBIO. 735

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETECTION OF CIRCULATING PLASTICIZERS IN SURGICAL PATIENTS

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SUMMARY

Gas chromatographic and gas chromatographic—mass spectrometric analytical techniques were employed to quantitate and confirm levels of circulating organic plasticizers in critically ill surgical patients. Two plasticizers, dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP), have been identified. DEHP can be found in many plastic medical devices. The DEHP levels were significant soon after transfusion or in the presence of renal dysfunction. The source of DBP is not clear at present and requires further study. The prevention of this contamination and the toxicity of these plasticizers should be investigated to ensure the safe use of plastic medical devices.

INTRODUCTION

We have employed gas chromatography (GC) and gas chromatographic—mass spectrometric (GC—MS) analytical techniques to confirm and quantify the levels of circulating plasticizers in selected surgical patients requiring intravenous therapy. This study was prompted by the identification of dibutyl phthalate (DBP) in the lipid fraction of the serum of one of our surgical patients maintained on continuous therapy for 3.5 months and the subsequent

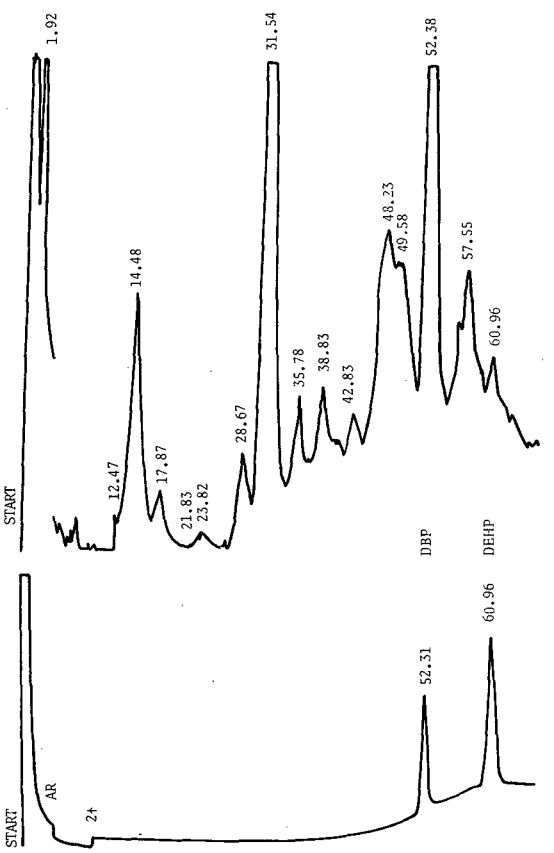

Fig. 1. A gas chromatogram of standard solutions of dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) is shown at the bottom. The gas chromatogram at the top is the fatty acid ester fraction of our patient in whom plasticizers were detected.

observation of the possible presence of DBP and another plasticizer, di-(2-ethylhexyl) phthalate (DEHP), in other patients (Fig. 1).

The use of plastic devices in medical care has increased tremendously. Surgical practice requires exposure of the patients to multiple uses of such materials; in many instances exposure is via the parenteral route and for extended periods of time. Various investigators have demonstrated plastic contamination of biological material and the ability of fluids contained in plastic containers used for parenteral therapy to leach out the plasticizer used in the manufacture of the containers [1—9]. Ono et al. [10] detected measurable levels of the plasticizer DEHP in peripheral blood samples of hemodialysed patients immediately after dialysis therapy. Hillman et al. [11] used GC—MS to identify and measure the same plasticizer in autopsy tissues (heart and small intestine) of infants who had had umbilical catheters inserted and had received varying amounts of blood products. Aronson et al. [12] reported that DEHP significantly decreased spontaneous heart rate, coronary flow and isometric tension but elevated diastolic tension in isolated perfused heart. In addition, significant concentration changes were noted for tissue glycogen, ATP, creatine phosphate, etc.

EXPERIMENTAL

Patient selection

Four critically ill patients in the Surgical Intensive Care Unit and two other subjects were selected for study. They were selected with respect to the duration of parenteral therapy, volume of blood and blood products infused, and the presence of renal dysfunction. One subject was selected as a control.

Control subject

The newest (less than two months) member of the Surgery Department's secretarial staff volunteered for the study. She had no previous history of intravenous infusions or contact with any medical environment and had minimal exposure to the surgical research facility's environment.

Preparation of sample

Peripheral venous blood samples were drawn using glass syringes and aluminum-coated needles and immediately transferred to rubber-stoppered glass tubes. The serum was separated from the clotted red cells and then frozen prior to analysis. Aliquots (1 ml) of the serum were extracted with either (A) 2.5 ml of Dole's mixture (2-propanol—*n*-heptane—sulphuric acid, 40:10:1, v/v) [13], or (B) 10 ml of absolute alcohol and 10 ml of *n*-heptane to denature the proteins and separate the lipids [10]. The remainder of the serum was then refrozen. The heptane layer containing the lipid fraction was separated, washed with water and then evaporated to dryness under a stream of nitrogen. The residue was redissolved in benzene and the fatty acids methylated at 60°C using BF_3—methanolic solution kits (Applied Science Labs., State College, PA, U.S.A.). The benzene layer, containing the lipids, was separated by centrifugation, dried over anhydrous sodium sulphate and evaporated to dryness under nitrogen. The methylated lipids were redissolved in 100 μl of acetone. The plasticizers were quantified by GC and their presence confirmed by GC—MS.

Gas chromatographic and gas chromatographic—mass spectrometric analysis

Aliquots of the processed extracts were analysed on a dual-column Hewlett-Packard 5830A gas chromatograph (with an auto sampler and data system) and flame ionization detectors using a 1.83 m × 2 mm I.D. glass column packed with 10% Silar-10C on Gas-Chrom Q (100—120 mesh) (Applied Science Labs.). The compounds were identified by comparing their retention times and mass spectra with those of authentic samples of DBP and DEHP (Applied Science Labs.). The column oven was held at 145°C for 25 min after injection and then programmed to 225°C at 2.5°C/min, and finally held at 225°C for 45 min. These conditions were used because the methyl esters of fatty acids are resolved at lower temperatures, and the phthalate esters are resolved at higher temperatures.

The mass spectra were obtained with a DuPont 21-492 double-focusing mass spectrometer coupled to a Varian 1400 gas chromatograph. Electron-impact ionization was used and the mass spectrometer was operated at a resolution of 1000, an ionization potential of 120 V and an accelerating voltage of 1800 V. Data were collected with a VG 2040 data system. GC peaks were identified by plots of total ion current or of selected ion currents. The GC column and GC conditions described above were used in some experiments. In other experiments, which were undertaken to analyse only the plasticizer, the GC oven was operated isothermally at 200°C.

RESULTS

GC and GC—MS proved suitable for examining the presence of plasticizers in blood extracts. The background levels in the ethanol-extracted samples were too high to be of value, and only the Dole's solution extract proved suitable for evaluation. DBP and DEHP were identified by their retention times and mass spectra in which prominent ions were observed at *m/e* 149 and *m/e* 169 for DEHP and at *m/e* 149 for DBP. Fig. 2 shows a plot of selected ion currents of a typical GC—MS run of DBP and DEHP. In the 200°C isothermal GC run an extra GC peak eluted between DBP and DEHP. To identify this unknown com-

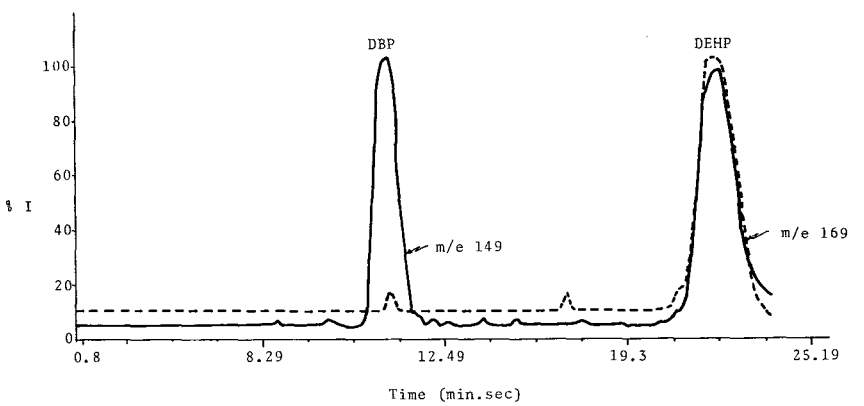

Fig. 2. The plot of *m/e* 149 and *m/e* 169 ion currents in a GC—MS analysis of a standard solution of dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP).

pound we used the Cornell University Probability Based Matching System [14]. The compound was identified as cholesta-3,5-diene with a confidence index K = 96 and K of 11. It is possible that cholesta-3,5-diene was formed by dehydration of cholesterol, and further experiments are under way to investigate this. Complete identification of the chromatograms was not attempted and the results of fatty acids will be the subject of another communication.

The levels of plasticizers in the water blank and the serum samples extracted by Dole's solution are summarized in Table I and compared to the duration of intravenous therapy, the quantity of blood transfused, and the time between the sampling and blood transfusion. The distilled-water blank did not contain DBP but did contain some DEHP. Similar DEHP contamination of water has been reported by Ishida et al. [9]. The control subject and patient No. 1, who received only 2000 ml of intravenous fluids 37 days before sampling, have almost the highest levels of DBP and relatively low levels of DEHP. Since these two subjects had received little or no surgical treatment near the time of sampling, the plasticizer found in their blood could have come from their normal environment and not from medical devices. Patient No. 2, who received the largest volume of crystalloid replacement over 43 days of complete intravenous replacement, showed relatively small levels of DBP and DEHP. Patient No. 3 had the highest levels of DBP and DEHP when sampled after having received three units of blood within 24 h in the diuretic phase of renal failure, but showed clearance 15 days later when renal function had improved and no further transfusions were required. Patient No. 4, who received the largest volume of transfused blood, 17 units, still showed some evidence of DBP and DEHP 21 days after having received the blood in spite of adequate urinary function. Patient No. 5 with oliguria showed a high level of DEHP.

TABLE I

SERUM PLASTICIZER LEVELS MEASURED IN SURGICAL PATIENTS

Sample*	Duration of intravenous therapy (days)	Volume of blood transfused (liters)	Interval between transfusion and sampling (days)	DBP (μg/ml)	DEHP (μg/ml)
Distilled water	—	—	—	0	(0—4)**
Control subject	—	—	—	37	3
Patient 1	12 (hours)	—	—	35	8
Patient 2	43	2	3	0.2	4
Patient 3	9	3	24	41	14
Patient 3	24	3	15	17	7
Patient 4	45	17	21	0.3	3
Patient 5	8	5	7	0	6

*Patient 1 was sampled 37 days after receiving intravenous therapy. Patient 2 required total parenteral nutrition therapy. Patients 3 and 5 had an element of renal failure. Patient 5 required subtotal gastrectomy for hemorrhage and 17 units of blood replacement.

**Range of the levels of the blanks from our laboratory.

DISCUSSION

Our attention was directed toward the confirmation and measurement of circulating plasticizer levels after identification of DBP in the lipid fraction of one of our patients. This patient had developed intestinal fistulas and complete wound disruption secondary to a colostomy for perforated diverticulitis. She required intravenous therapy throughout her 3.5-month course of treatment in the hospital because she was unable to tolerate an oral elemental diet. Total parenteral nutrition failed as she had a serum albumin of 1.5 g% and developed a large tracheoesophageal fistula secondary to tracheostomy damage. She was then selected for a complete nutritional evaluation, including analysis of serum unesterified fatty acids and amino acids. GC analysis of the fatty acids revealed an exceptionally large unidentified peak eluting after identifiable fatty acids. This sample was then examined by GC—MS and two components, *p*-nonylphenol and DBP, were identified in addition to the fatty acids. Subsequent GC revealed that there were really three GC peaks which could be resolved at an oven temperature of 200°C. The first of these peaks was identified as DBP by GC and GC—MS. The second peak, a very broad GC peak, was tentatively identified as cholesta-3,5-diene. The third GC peak was identified as DEHP. Unfortunately, further investigation of this patient was precluded by her death three days after the blood sample had been drawn; the blood sample was not drawn with a glass syringe so the results cannot be compared to the selected patients.

The phthalate ester plasticizers represent a large family of plasticizers which are approved by the Food and Drug Administration (Federal Register, 15 October, 1968, 33 F.R. 15281) for use in packaging materials for food intended for human consumption. The plastic resin (e.g. polyvinyl chloride) is combined with a plasticizer and stabilizer before it is finally manufactured into the item destined for medical use. The plasticizer DEHP is most commonly used in medical-grade plastic devices. Other investigators have measured this plasticizer in blood and tissues, which we have identified together with a second plasticizer, DBP, in the serum of five of the six subjects studied.

The use of plastics in various aspects of medical care continues to increase. Plastic devices are disposable and their use results in a savings of labor costs and avoids contamination between patients. The decrease in the costs makes their use more economically feasible. A survey of the parenteral solution devices used in our hospital revealed extensive use of plastic materials with potentially leachable plasticizers. Blood is stored in plastic bags, as are most of the crystalloid solutions; intravenous solutions are administered via plastic administration sets and catheters. The total parenteral nutrition amino acid solutions are, however, stored in glass containers. Patient No. 2, on parenteral fluids for 43 days, was primarily on these hyperalimentation fluids which may explain why the plasticizer was not detected in his blood. Our investigations of the solutions stored in plastic bags have revealed the presence of DEHP in stored blood, and both DEHP and DBP in the crystalloid solutions [15]. This may partially account for the presence of these plasticizers in our patients.

Teflon devices do not contain these plasticizers. Another possible source of DBP identified so far is the alcohol sponges used to wash skin; analysis of six

separate sponges revealed an average of 1.4 μg/ml if all of the DBP in the sponge contaminated the blood sample. The DBP levels are low in the hospitalized patients but highest in the subjects not hospitalized, suggesting contamination from environmental sources. Further studies of the source of the DBP are being pursued.

The circulating levels of DEHP were highest in patients soon after receiving blood transfusions and when there was associated renal dysfunction. Blood stored in plastic bags has high levels of plasticizers because the blood proteins are able to bind plasticizers. Once absorbed the plasticizers need to be metabolized by de-esterification to a water-soluble form which can be excreted in the urine. Absorbed plasticizers may also be deposited in tissues and may persist there for an unknown length of time. Surgical patients are exposed to contamination via the parenteral route. Special consideration may have to be given to reducing this contamination, especially for patients with renal dysfunction.

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DIFFERENTIAL PULSE AMPEROMETRIC DETECTION OF DRUGS IN PLASMA USING A DROPPING MERCURY ELECTRODE AS A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTOR

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SUMMARY

High-performance liquid chromatographic separation prior to reductive electrochemical determination at the dropping mercury electrode imparts specificity and sensitivity not attainable by conventional polarographic analysis of drugs and their metabolites. The utility of this novel approach is demonstrated by the analysis of chlordiazepoxide and its N-desmethyl metabolite in plasma which previously required thin-layer chromatographic separation prior to polarographic measurement. A mobile phase of methanol—isopropanol—0.0075 *M* acetate buffer, pH 3.5 (53:5:42), is used with the detector operated in the differential pulse mode at E_p = —0.820 V vs. Ag/AgCl. The response was linear (r = 0.998) in the concentration range of 0.05—2.0 μg/ml plasma for each component. The minimum detectability for each component under these conditions is 5.0 ng injected at a current range of 0.5 μA full scale. Techniques for oxygen removal and hydrodynamic considerations for the pumping system are presented.

INTRODUCTION

The utility of highly sensitive and specific high-performance liquid chromatographic (HPLC) assays using UV and fluorescence detection for the assay of drugs in body fluids is well documented [1—3]. Although spectrophotometric (UV) detectors are used in the majority of examples, electrochemical detectors are often required for improved sensitivity and specificity against endogenous substances and/or co-administered drugs. Electrochemical detectors operated in the oxidative mode using either glassy carbon or carbon paste electrodes have been used primarily for the analysis of phenolic and aromatic amine compounds of pharmaceutical and biological importance [4—6]. Recent publications have described the use of HPLC with oxidative amperometric (OA) detection for the analysis of perphenazine and fluphenazine [7], β-cetotetrine [8], 8-hydroxycarteolol [9], acetaminophen [10, 11], theophylline [12, 13],

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methyldopa [14], tocopherols [15], morphine [16], methyltetrahydrofolic acid [17], and mepindolol [18] in biological fluids. HPLC assays using reductive amperometric (RA) determination for drugs in biological fluids have been described for penicillamine using a mercury pool electrode [19] and for ubiquinones and phylloquinone using a glassy carbon electrode [15].

The use of a dropping mercury electrode (DME) as a RA detector for the analysis of drugs in body fluids has not yet been reported. The DME has the distinct advantage of presenting a continuously renewable fresh surface during chromatographic analysis and as such is not subject to "poisoning" as are the solid electrodes. The facile electrochemical reduction of the 4,5-azomethine group of the 1,4-benzodiazepines makes these compounds ideal for the evaluation of a DME—RA detector for the HPLC analysis of drugs in body fluids [20]. The HPLC—RA measurement of the 1,4-benzodiazepines in solution at a mercury pool [21] and at a mercury amalgamated gold disc electrode [22] has been described.

The present work describes a HPLC assay for chlordiazepoxide and its N-desmethyl metabolite in plasma which demonstrates the feasibility of using a DME as a RA detector for the assay of drugs in body fluids. Techniques for oxygen removal and hydrodynamic consideration of the pumping system are described.

EXPERIMENTAL

Column

The column used was a 30 cm × 3.9 mm I.D. stainless-steel prepacked reversed-phase column containing 10-μm μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.).

Instrumentation

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a Model 440 UV absorbance detector operated at 254 nm at an attenuation of 0.01 a.u.f.s. (Waters Assoc.). In series with the UV detector was a Model 310 liquid chromatographic detector controlled by a Model 174 polarographic analyzer [EG & G Princeton Applied Research Corp. (PARC), Princeton, NJ, U.S.A.]. The electrochemical detector was operated in the differential pulse mode at —0.820 V vs. Ag/AgCl (filling solution containing saturated AgCl and KCl) using a 100-mV pulse, large drop size (0.5 mg), 1 drop/sec rate, 1 sec low pass filter time constant and a current range of 1 μA. A stainless-steel tube 15 cm × 4.6 mm I.D. partially filled with mobile phase was placed between the UV and RA detector to act as a "pulse dampener". A Rheodyne Model 70-30 switching valve was placed after the pulse dampener to allow flushing of the HPLC column with methanol without contamination of the supporting electrolyte vessel of the EG & G-PARC Model 310. Chromatographic recording was made on a Model 626 dual-pen strip chart recorder with dual variable imputs (Leeds and Northrup, North Wales, PA, U.S.A.). The chart speed was 30 in./h using 10 mV for the UV detector and 5 V for the polarographic detector (effective current range of 0.5 μA) (see Fig. 1 for the instrumental schematic). Polarographic scans were performed using the EG & G-

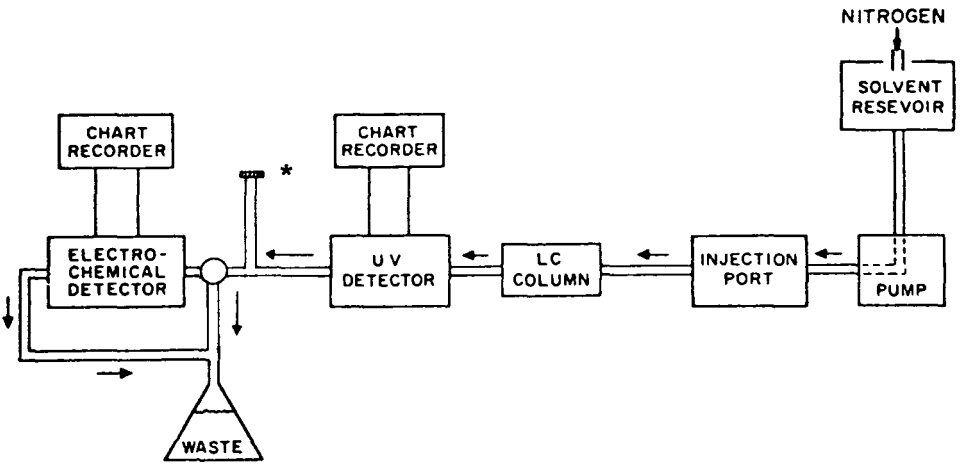

Fig. 1. Equipment used for HPLC (UV and RA) analysis. Asterisk indicates pulse dampener (see text).

PARC Model 174 with a Model 2200-3-3 Omnigraphic X-Y recorder (Houston Instruments, Bellaire, TX, U.S.A.). The X-axis was set at 100 mV/in. and the Y-axis at 1 V/in.

Chromatographic conditions

The isocratic mobile phase was a modification of the one described by Strojny et al. [23] and consisted of a mixture of methanol—isopropanol—0.0075 *M* acetate buffer pH 3.5 (53:5:42). The column head pressure was 1,000 p.s.i. (6.9 MPa) at a flow-rate of 0.9 ml/min. Under these conditions the retention time of N-desmethylchlordiazepoxide, chlordiazepoxide, and medazepam (the internal standard) were 9.8 (k' = 1.8), 12.3 (k' = 2.5), and 15.6 (k' = 3.5) min, respectively. Under the above assay conditions, 100 ng of chlordiazepoxide injected gave peaks which were 60% and 90% of full scale by UV and RA detection, respectively.

Standard solutions

Ten mg each of [I] chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{16}H_{14}N_3OCl$, mol. wt. = 299.71, m.p. = 236—236.5°C), [II] N-desmethylchlordiazepoxide (2-amino-7-chloro-5-phenyl-3H-1,4-benzodiazepine-4-oxide, $C_{15}H_{12}N_3OCl$, mol. wt. = 285.73, m.p. = 255—256°C), and [III] medazepam (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine, $C_{16}H_{15}N_2Cl$, mol. wt. = 270.76, m.p. = 95—97°C, used as an internal standard), were weighed into separate amberized 10-ml volumetric flasks. All solutions were made in methanol. These stock solutions (containing 1 mg/ml) are used to prepare six 10-ml working solutions (Nos. 1—6) containing 0, 0.5, 1.0, 2.0, 5.0, or 10 μg of [I] and [II] and 1.5 μg of [III] per ml of isopropanol. Aliquots (10 μl) of these working solutions (equivalent to 0, 5, 10, 20, 50, and 100 ng of [I] and [II] and 150 ng of [III]) are injected as the external standards for establishing the HPLC parameters using both UV and RA detection.

Reagents

All reagents were of analytical reagent grade purity and all inorganic reagents were made up in distilled deionized water. Solvents were purchased from Fisher Scientific (Springfield, NJ, U.S.A.) with the exception of diethyl ether (anhydrous, reagent grade) which was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), opened fresh and placed in a 1-l amberized glass-stoppered reagent bottle to which was added approximately 1 g of granular zinc. This prevented peroxide formation which would otherwise decompose low concentrations of [I] and [II] [23].

Assay and calculations

The assay is identical to that described by Strojny et al. [23] with the exception that a single diethyl ether extraction was used and that the residue for HPLC was reconstituted in isopropanol. Along with the samples, a 1.0-ml specimen of control plasma and six 1.0-ml specimens of control plasma containing 100 μl of the working solutions Nos. 1—6 (equivalent to 10, 50, 100, 200, 500, and 1000 ng of [I] and [II] and 1500 ng of [III] per ml of plasma) are processed. These standards are used to establish a linear least squares regression equation ($Y = mX + b$) for the quantitation of the unknowns using the peak height ratio of [I]/[III] or [II]/[III] vs. concentration of [I] or [II], respectively (see Tables I and II). The peak heights of [I] and [II] in these samples are used to calculate the percent recovery of the assay.

TABLE I

UV INTRA-ASSAY STATISTICS

Drug	Amount added (μg/ml)	Amount found (μg/ml) ± S.D.	N	% R.S.D.
Chlordiazepoxide	0.050	0.045 ± 0.001	2	2.22
	0.100	0.104 ± 0.005	3	4.81
	0.200	0.194 ± 0.005	2	2.58
	0.500	0.505 ± 0.003	3	5.94
	1.00	1.00 ± 0.005	3	0.50
	$Y = 0.7104\ X - 0.004743$;		$r = 0.9994$	
N-Desmethyl-chlordiazepoxide	0.050	0.056 ± 0.001	2	1.78
	0.100	0.100 ± 0.004	3	4.00
	0.200	0.199 ± 0.000	2	0.00
	0.500	0.494 ± 0.003	3	0.61
	1.00	1.00 ± 0.004	3	1.40
	$Y = 0.7206\ X - 0.008293$;		$r = 0.9998$	

RESULTS AND DISCUSSION

Selective HPLC detectors have the distinct advantage of being able to measure drugs and metabolites in body fluids in the presence of co-administered drugs and/or endogenous substances. This fact is well documented with exam-

TABLE II

RA INTRA-ASSAY STATISTICS

Drug	Amount added (μg/ml)	Amount found (μg/ml) ± S.D.	N	% R.S.D.
Chlordiazepoxide	0.050	0.048 ± 0.002	2	4.17
	0.100	0.103 ± 0.006	3	5.83
	0.200	0.192 ± 0.006	2	3.13
	0.500	0.505 ± 0.027	3	5.35
	1.00	1.00 ± 0.034	3	3.40
	$Y = 2.198\,X + 0.006028$;		$r = 0.9988$	
N-Desmethyl-chlordiazepoxide	0.050	0.052 ± 0.002	2	3.85
	0.100	0.102 ± 0.003	3	2.91
	0.200	0.198 ± 0.000	2	0.00
	0.500	0.496 ± 0.012	3	2.42
	1.00	1.00 ± 0.027	3	2.70
	$Y = 2.101\,X - 0.002681$;		$r = 0.9994$	

ples of selective detection using fluorescence [3] and OA detection [4—6]. The OA detectors which use either glassy carbon or carbon paste electrodes can typically be utilized in the range of +1.0 to —0.4 V vs. Ag/AgCl. In the negative region (0 to —0.4 V vs. Ag/AgCl) it is also possible to perform analysis on very easily reducible nitroso- and some nitro-containing compounds. However to extend reductions to the region of —1.0 V vs. Ag/AgCl, a mercury electrode is essential due to its high overpotential. The DME is the electrode of choice in the negative potential region because of its ability to present a fresh renewable surface for analysis. Contamination ("poisoning") of the electrode surface with corresponding decrease in sensitivity as it is seen with solid carbon or mercury amalgamated electrodes is eliminated with the DME. In addition, the long equilibration times (from minutes to hours, depending upon the value of the applied potential and purity of the solvent) to minimize background currents with the solid electrodes are also eliminated. The main objections to the use of a DME as a HPLC detector have been its awkwardness and the large dead volume in the measurement cell. The EG & G-PARC Model 310 is a compact device, which delivers the HPLC column effluent directly to the mercury drop, hence has an effective dead volume of less than 1 μl at the point of measurement [24].

In order to utilize the DME as a HPLC—RA detector for high sensitivity measurements, parameters such as the suitability of the mobile phase as a supporting electrolyte, deaeration, pump noise, stability of the reference electrode, and mode of amperometric measurement were examined in detail. In order to monitor the performance of the HPLC system (injector, pump, and column) a UV detector was placed prior to the RA detector in the system (see Fig. 1).

System design

The absolute sensitivity obtainable by RA detection for HPLC is limited by the background currents which result from the reducible impurities in the mobile phase, electrolytic decomposition of the mobile phase and the presence of oxygen. Although reagent grade chemicals are usually sufficiently pure for RA detection it was essential in this study to replace the phosphate buffer reported for HPLC with UV detection for [I] and [II] [23] with 0.0075 *M* acetate buffer to obtain a stable baseline. The applied potential (—0.820 V vs. Ag/AgCl) and the pH (3.5) were selected to minimize electrolytic decomposition of the mobile phase [25]. The removal of dissolved oxygen was problematic in that purging for 5 min with helium or nitrogen as required for UV detection was insufficient for RA detection. The reduction of baseline current due to oxygen removal required exhaustive deaeration of the mobile phase in the reservoir with continued vigorous infusion of an inert atmosphere of nitrogen or helium. In addition, all standard Teflon fluoroethylene polymer (FEP) lines leading from the reservoir to the pumping system were replaced with BEV-A-LINE V-HT tubing (Thermoplastic Scientific, Warren, NJ, U.S.A.) to prevent reabsorption of oxygen through these lines. This tubing, which is impervious to oxygen, consists of a cross-linked ethyl—vinyl acetate copolymer jacket which is lined with cross-linked polyethylene. Although the lining is not as chemically inert as Teflon FEP it is more than adequate for the solvents used in reversed-phase HPLC analysis. Glass or stainless-steel lines were found to be too rigid and PVC tubing non-chemically resistant to be used as transfer lines. With these precautions for oxygen removal, the background currents were reduced to allow measurement at 0.5 μA full scale. At this sensitivity, however, it was apparent that a low frequency cyclic noise of approximately 0.5 Hz was superimposed on the RA baseline (Fig. 2A). This noise, presumably caused by distortions of the double layer surrounding the mercury drop, results from pressure fluctuations (30—40 p.s.i.) during "piston-crossover" in the dual reciprocating pump. These pulsations could not be eliminated by use of the high sensitivity noise filter on the chromatographic pump. A low pass filter with a time constant of 3 sec on the polarograph was very effective in removing the short term noise from the RA signal (Fig. 2B). A dampening device, constructed from a 15-cm length of 4.6-mm stainless-steel tubing capped at one end and filled approximately half-way with either mobile phase or water placed in front of the RA detector (Fig. 1) was very effective in removing the long term cycling from the pumping system (Fig. 2C and D). The optimum conditions for analysis were found to be a 1-sec time constant and the dampener device just prior to the RA detector (Fig. 2E).

Mode of amperometric detection

The direct current (DC), pulse and differential pulse (DP) modes of amperometric detection were evaluated using the system described above with chlordiazepoxide as a test compound. The applied potentials were selected from the DC and differential pulse polarograms on the plateau region (E = —0.950 V vs. Ag/AgCl) and at the peak potential (E_p = —0.820 V vs. Ag/AgCl), respectively, for the reduction of the 4,5-azomethine in the mobile phase [26]. The DC mode of HPLC—RA detection was found to be approximately

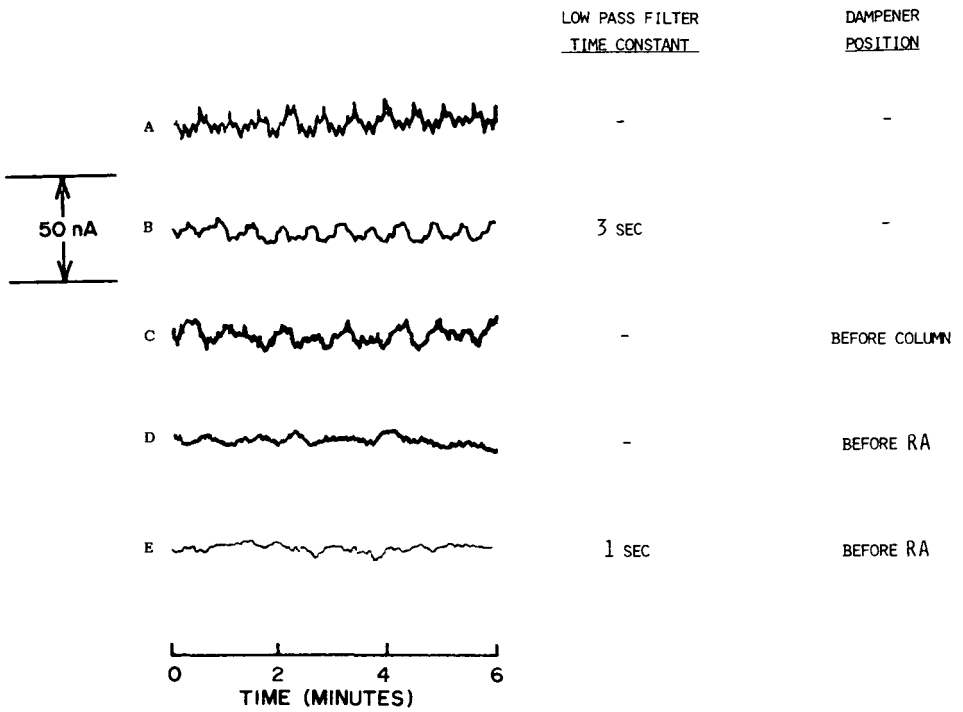

Fig. 2. Effect of filtering and dampening on baseline noise.

two orders of magnitude less sensitive than the DP mode. This result is in fair agreement with comparisons reported for the reduction of the 7-nitro functional group of nitrazepam, a 1,4-benzodiazepin-2-one, using the mercury pool HPLC—RA detector [21]. The sensitivity of 5 ng using DP—RA detection with a DME obtained in this study far exceeds the 300-ng level reported using glassy carbon, carbon paste or the mercury pool electrode for the HPLC—RA measurement of chlordiazepoxide using either the DC or DP amperometric modes [21]. A recent report utilizing HPLC—RA has demonstrated a DC amperometric sensitivity of less than 20 ng chlordiazepoxide using an amalgamated gold disc electrode [22]. The pulse amperometric mode was found to be unsuitable due to high backgrounds and large signal-to-noise ratios which is in agreement with work using the mercury pool electrode for HPLC—RA detection [21].

The use of the DP amperometric mode for high sensitivity determinations places stringent requirements upon the stability of the reference electrode. The RA detector utilized is designed to deliver the HPLC column effluent directly to the mercury drop with minimal band spread prior to mixing with the supporting electrolyte in a vessel which contains the auxiliary and reference electrodes. This vessel is drained via an overflow port and thus allows continuous flow of column effluent. The reference electrode consists of a Ag/AgCl wire in a solution of saturated KCl/AgCl contained in a glass jacketed sleeve with a Vycor frit (attached via heat shrinkable Teflon tubing). With this

experimental set-up repeated injections of chlordiazepoxide over a period of several hours yielded diminished peak heights. This lack of reproducibility can not be attributed to poisoning of the working electrode surface as noted with solid electrodes, since a clean working mercury electrode surface was exposed every second. Experimentation demonstrated that this loss in sensitivity was caused by a drift in the reference electrode potential and a consequent detuning of the selected applied DP potential. This drift appears to result from an exchange of chloride ions between the filling solution in the reference electrode and the supporting electrolyte through the Vycor frit causing a change in reference potential of 10—15 mV and a loss of 25—50% of the reduction current signal. The initial approach to this problem was to maintain a very small ionic concentration of chloride (0.01 *M*) in the mobile phase to prevent this exchange. This worked quite well, but was later abandoned (under advice of the HPLC manufacturer) due to possible corrosive effects of the chloride ion on the stainless-steel tubing throughout the instrument. A second approach which also worked well was to replace the KCl/AgCl filling solution with saturated potassium nitrate and use 0.01 *M* potassium nitrate in the mobile phase. [This change in reference electrode filling solution shifts the peak for the reduction of the 4,5-azomethine for chlordiazepoxide approximately 80 mV more negative, i.e. from —0.820 V vs. Ag/AgCl to —0.900 V vs. Ag/AgCl (saturated KNO_3).] Finally, it was found that removal of the Vycor frit from the reference electrode jacket and its replacement with the asbestos fiber tip (using waterproof epoxy) of a Ag/AgCl micro reference electrode (Microelectrode, Londonderry, NH, U.S.A.), and the use of a filling solution of saturated AgCl and KCl completely eliminated the ionic exchange and yielded a constant reference potential.

Application

Based upon the reported HPLC—UV assay for chlordiazepoxide, its N-desmethyl, demoxepam and nordiazepam metabolites [23], this study aimed to compare the sensitivity and specificity of the UV and RA HPLC detectors. However, upon examination of the HPLC—DP—RA behavior of 500 ng (20-μl injections) of each of these compounds (see Fig. 3), it was noted that the response of the molecules with the 1,4-benzodiazepin-2-one structure (demoxepam, nordiazepam, and diazepam) was considerably reduced when compared to the 1,4-benzodiazepine structure (chlordiazepoxide and N-desmethylchlordiazepoxide) throughout the entire range of potential applied, viz. —0.775 to —0.875 V vs. Ag/AgCl. This is in direct contrast to the DP polarographic activity which shows approximately equal sensitivity at the respective E_p value for the five compounds [20, 26]. This behavior appears to be a kinetic phenomenon which is peculiar to hydrodynamic voltammetry (amperometry in a flowing system) and will be a subject of future investigations. Due to the loss of sensitivity for demoxepam and N-desmethyldiazepam (approximately one order of magnitude), these compounds cannot be measured in plasma samples following therapeutic drug administration. The internal standard medazepam, a 1,4-benzodiazepine, showed equivalent sensitivity to chlordiazepoxide over this potential range. Chromatograms of control plasma and control plasma containing 100 ng/ml chlordiazepoxide and its N-desmethyl metabolite using

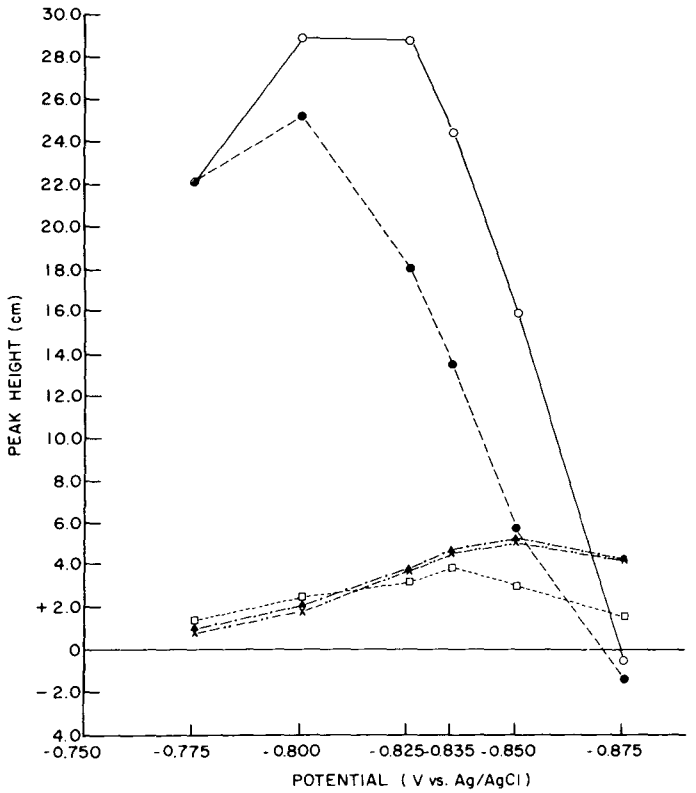

Fig. 3. Amperometric responses of 500 ng (20 μl injected) of 1,4-benzodiazepines and 1,4-benzodiazepin-2-ones in the mobile phase. ○, Chlordiazepoxide; ●, N-desmethylchlordiazepoxide; X, demoxepam; ▲, nordiazepam; □, diazepam. Current range: 5 μA full scale.

HPLC with UV and RA detection are shown in Figs. 4 and 5, respectively. The presence of a large negative peak ($t_R \approx 7.5$ min) is due to the injection of a small amount of oxygen and is always noted at this high attenuation. Deaeration of the sample does not effectively remove this HPLC peak which has also been reported using the mercury amalgamated gold electrode [22].

The HPLC assay was statistically evaluated over the concentration range of 0.05—2.0 μg/ml for [I] and [II] using both UV and RA detection and showed excellent linearity [correlation coefficient (r) $\geqslant$ 0.99] and precision [average percent relative standard deviation (% R.S.D.) $\approx$ 2—4%], for both techniques (see Tables I and II). The assay can be performed in less than one-half the analysis time required using thin-layer chromatographic separation, elution, and polarographic quantitation of the respective compounds [26].

The clinical utility of the HPLC—RA assay was demonstrated by the analysis of [I] and [II] in a series of plasma samples (0.5—60 h) taken from a subject who received Limbitrol® (product containing 10 mg of chlordiazepoxide and 25 mg of amitriptyline) twice daily. Equivalent data were obtained for these samples using UV and RA detectors in series (r = 0.999 and 0.988 for [I] and

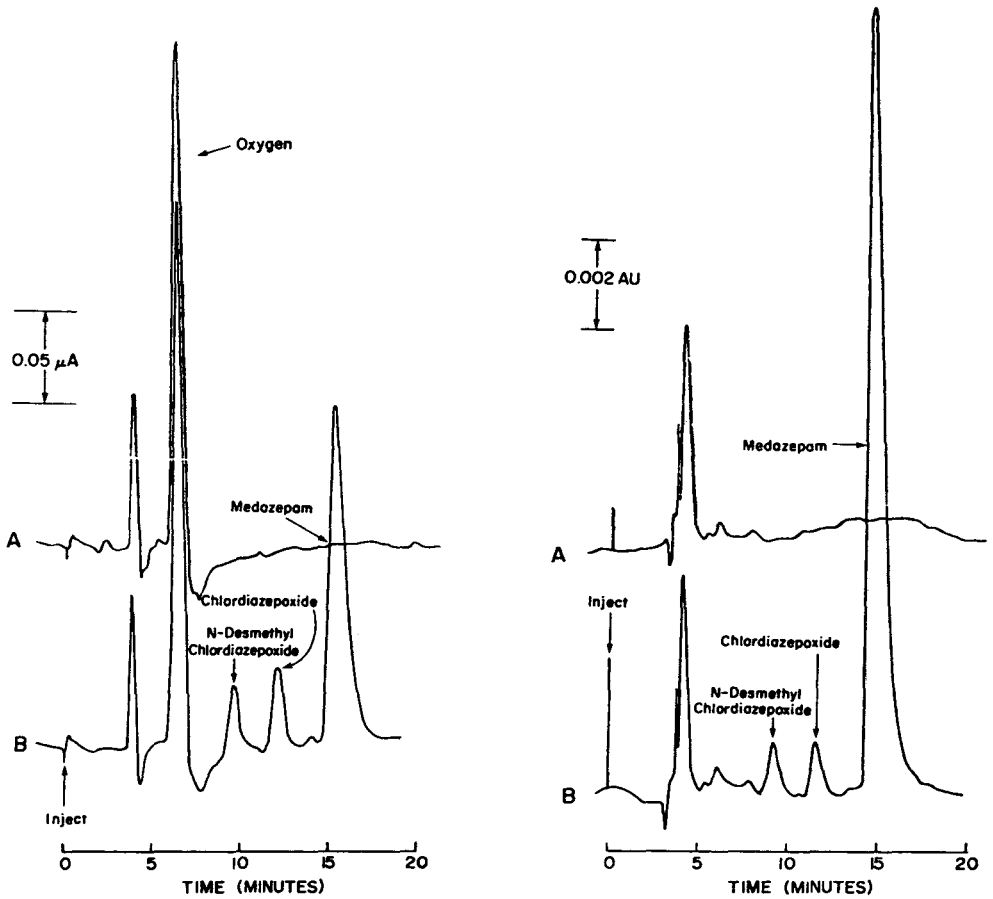

Fig. 4. HPLC chromatogram with RA detection (E_p = —0.820 V vs. Ag/AgCl). A, Control plasma; B, control plasma containing 100 ng/ml chlordiazepoxide and N-desmethylchlordiazepoxide with 1.5 μg/ml medazepam (internal standard).

Fig. 5. HPLC chromatogram with UV detection (λ = 254 nm). A, Control plasma; B, control plasma containing 100 ng/ml chlordiazepoxide and N-desmethylchlordiazepoxide with 1.5 μg/ml medazepam (internal standard).

[II], respectively), see Table III. The sensitivity limit for both detectors is 50 ng/ml for [I] and [II]. Neither the UV nor RA detector showed interference from amitriptyline or its N-desmethyl metabolite which were below the limit of detection for both detectors.

CONCLUSIONS

Reductive electrochemical detection using DP amperometry at the DME with HPLC is a useful technique for the determination of electroactive drugs and their metabolites in biological fluids.

HPLC system requirements for high sensitivity RA detection such as oxygen

TABLE III

PLASMA CONCENTRATIONS OF CHLORDIAZEPOXIDE AND N-DESMETHYLCHLORDIAZEPOXIDE IN MAN FOLLOWING ORAL ADMINISTRATION OF LIMBITROL* TWICE DAILY AS DETERMINED BY BOTH UV AND RA ASSAYS

Time (h)	Chlordiazepoxide		N-Desmethylchlordiazepoxide	
	UV	RA	UV	RA
0.5	1.30	1.30	0.046	0.056
1	1.04	1.04	0.065	0.120
1.5	1.01	1.00	0.055	0.049
2	1.10	1.11	0.074	0.086
3	1.05	1.07	0.090	0.097
4	0.92	0.94	0.14	0.12
6	0.55	0.59	0.43	0.45
8	0.63	0.63	0.17	0.17
10	0.53	0.56	0.21	0.21
12	0.53	0.49	0.23	0.23
16	0.36	0.39	0.31	0.30
24	0.28	0.31	0.36	0.39
30	0.17	0.19	0.36	0.37
36	0.091	0.11	0.30	0.33
48	0.040	0.053	0.17	0.21
60	N.M.**	N.M.	0.11	0.10
	$r = 0.9989$		$r = 0.9877$	

*See text.

**N.M. = < 0.050 μg/ml for RA and < 0.040 μg/ml for UV.

removal, a stable reference electrode and pulse dampening were optimized for the performance of this detector.

Applications of RA detection with simultaneous UV analysis showed both techniques to be of equal sensitivity and precision with excellent correlation for the analysis of plasma samples containing chlordiazepoxide and its N-desmethyl metabolite.

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ANALYSIS OF SOME TRYPTOPHAN AND PHENYLALANINE METABOLITES IN URINE BY A STRAIGHT-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE

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SUMMARY

A high-performance liquid column chromatographic technique is reported for the analysis of some tryptophan and phenylalanine acid metabolites in the urine. An acidified and NaCl-saturated urine sample is loaded on to a C_{18}-bonded silica microcolumn. After washing the microcolumn with clean and deionized water, the metabolites of interest are selectively extracted by successive elutions with organic solvents of variable polarity. Acids are eluted first and the neutral compounds with the next fraction. Basic compounds and other neutral substances of higher polarities were eliminated during the washing procedure.

The chromatography was performed in the straight-phase isocratic elution mode utilizing 5-μm silica-gel columns loaded with a triethanolammonium perchlorate—perchloric acid aqueous solution. The separations achieved have permitted the application of the chromatographic technique to the analysis of urinary metabolites with acceptable accuracy.

INTRODUCTION

Interest in techniques for the separation, identification and quantitation of tryptophan and phenylalanine acid metabolites stems from specific requirements in clinical diagnosis. The analysis of hydroxyindole acid metabolites and some mandelic acid derivatives in urine is not only important for the diagnosis of carcinoid malignancies and catecholamine-secreting tumors [1—4], but it also serves as a monitoring system in the course of treatment of these diseases.

Furthermore, study of the vast number of affections in which abnormalities in the tryptophan excretory pattern have been noticed [5, 6], but in which no real insight into the relationship of cause and effect has yet been achieved, requires the introduction of new analytical methods in addition to the existing paper chromatographic [7], thin-layer chromatographic (TLC) [8—12], gas

chromatographic [13] and electrophoretic [14] techniques, which have proved to be sometimes time-consuming, relatively insensitive and low in accuracy for the quantitative estimation of the compounds of interest.

For these reasons a large number of studies have recently been undertaken on the application of high-performance liquid chromatography (HPLC) to the analysis of biogenic amines and related metabolites [15—20]. Relative simplicity, good reproducibility as well as high efficiency are the practical advantages of HPLC over the techniques mentioned above and ion-exchange chromatography; moreover, HPLC does not require any chemical processing of the samples before analysis.

The reversed-phase mode of HPLC, when it is operated under gradient-elution conditions, affords excellent simultaneous separations of a large number of aromatic acids [17] so that urine extracts can be effectively assayed for their phenylalanine and tryptophan acid metabolites.

On the other hand, normal-phase ion-pair HPLC has been found to give rapid and fair separations of some biogenic amines and their metabolites [18]. Such a procedure, despite the reduced number of compounds separated, indeed has the advantage of being simpler as it is performed isocratically. However, the ion-pair partition mode requires a sample preparation step (i.e. ion-pair formation in an organic phase of the compounds being tested before injection on to the chromatographic columns), which may complicate the application of this technique to the assay of physiological materials, where direct injection of underivatized and if possible not pretreated samples is to be preferred.

In the present paper we describe a normal-phase HPLC method that allows the isocratic separation of a relatively high number of metabolites, and its application to the analysis of clinically important urinary acid metabolites of tryptophan and phenylalanine.

EXPERIMENTAL

Materials

For chromatographic purposes use was made of the following analytical-grade organic solvents, without any further purification: *n*-heptane, isobutanol and 2-propanol. Ethyl acetate and methanol of highest purity grade (ACS UvaSol certified grade from Carlo Erba, Milan, Italy) were used for the extraction procedures. Urine extraction was accomplished on Sep-Pak^{tm} C_{18}-bonded silica cartridges (Waters Assoc., Milford, MA, U.S.A.).

Distilled and deionized water was used. Triethanolammonium perchlorate ($TETOLA^+ClO_4^-$) was formed by neutralizing an aqueous perchloric acid solution of a given molarity by an equal amount of triethanolamine.

The standard compounds listed in Table I were purchased from Sigma (St. Louis, MO, U.S.A.).

Instrumentation

Chromatography was performed on a Hewlett-Packard liquid chromatograph Model 1010A, equipped with an UV absorption detector (Model HP 1032A) at two interchangeable fixed wavelengths (280 and 254 nm), a linear potentio-

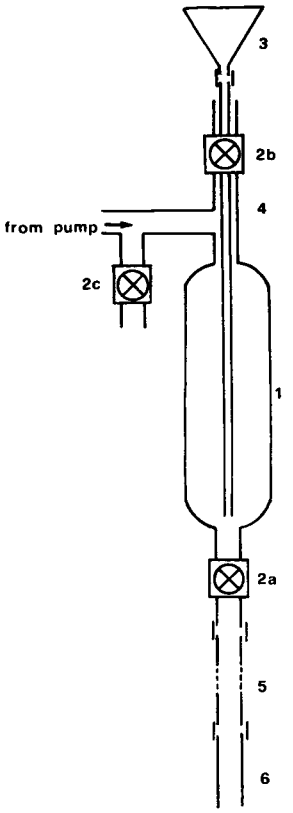

Fig. 1. Diagram of the packing apparatus. 1 = slurry reservoir; 2a, 2b, 2c = two-way high-pressure valves (Whitey); 3 = glass funnel; 4 = PTFE tube; 5 = precolumn; 6 = column.

metric recorder (Model HP 7127A) and with a high-pressure on-line injection block.

Throughout the investigation 24.75 × 0.4 cm I.D. stainless-steel columns, packed with LiChrosorb silica-gel particles (5 μm) were used. The column was packed using the equipment represented in Fig. 1. Pressure was applied to the packing system by one of the liquid chromatograph pumps, operating at the maximum feed capacity (about 240 bars).

Packing procedures

Columns were wet-packed using the slurry method with isopropanol—stationary phase (5:3, v/v) as dispersing solvent. The stationary phase consisted of an aqueous solution of 0.5 *M* $TETOLA^+ClO_4^-$—0.3 *M* $HClO_4$.

A 1.8-g amount of LiChrosorb SI-100-5 (E. Merck, Darmstadt, G.F.R.) is dispersed by mechanical agitation in the isopropanol—stationary phase mixture (10 ml). The opalescent suspension formed is then heated in a water-bath at 50—60° C for 3—4 h to remove any gas residue, and cooled at ambient temperature. Using a pasteur pipette the suspension is rapidly delivered into the column and previously connected precolumn; then the precolumn is assembled on to the reservoir via the two-way ballvalve 2a (Fig. 1). By means of a glass

funnel and a flexible PTFE tube attached to the funnel stem, 75 ml of the same solvent as that contained in the solvent reservoir (ethyl acetate) are gently poured in.

The funnel is now completely withdrawn. With valve 2b open and valves 2a and 2c closed, the pump is started at its maximum pressure feed. When the first drop of ethyl acetate has emerged through valve 2b, this valve is shut and valve 2a opened. Now the system is allowed to run until 120—150 ml of liquid have passed through the column.

Chromatographic procedure

All chromatographic experiments were performed at ambient temperature, in the isocratic elution mode. The mobile phase was *n*-heptane—isobutanol—2-propanol (10:10:30, v/v/v) saturated with 2.5—3.0 ml of stationary phase.

Equilibration of the chromatographic system with the mobile phase was carried out by allowing 150—200 ml of eluent to pass through the column at a flow-rate of 0.60—0.75 ml/min.

The standard compounds were prepared by dissolving weighed amounts in 1 ml of either *n*-butanol—dichloromethane—6 *N* HCl (5:5:0.1) or 2-propanol—diethyl ether—0.15 *M* HCl (4.5:4.5:1.0), depending on the solubility of the substances. Five microlitres of these solutions, containing 0.05—2.0 μg of each solute, were injected into the column. The standard solutions had to be prepared fresh before injection because most of the compounds are easily degraded in organic solvents.

Sample preparation

Urine samples were obtained from ten of our laboratory technicians and students, from five children admitted to the Pediatric Department for diseases not correlated with any of the tryptophan or the phenylalanine metabolic anomalies, from one patient affected with neuroblastoma, from two cases of phenylketonuria, and from one patient affected with a hypochromic microcytic anemia. The specimens were in every case 24-h urines collected under controlled nutritional conditions and kept frozen after being acidified to about pH 2 with hydrochloric acid.

Isolation of aromatic acids and neutral compounds

Two C_{18}-bonded silica cartridges were prepared for each urine sample to be extracted by inserting the longer end into the tip of a 10-ml disposable plastic syringe barrel (after removal of plunger).

The C_{18} microcolumns are then pre-wetted by allowing to pass successively through the cartridges 2.5 ml of isopropanol and 5 ml of clean distilled and deionized water.

Then 5 ml of urine, previously acidified to pH 2 and saturated with NaCl, are poured into the syringe barrel of one of the two cartridges and, after the liquid has passed completely through the microcolumn, it is washed with 3.0 ml of water and the effluents are discarded.

Then 5 ml of a glycine—NaOH buffer (pH 9.0) [21] are allowed to pass through the cartridge and the effluent collected in a 10-ml test-tube and care-

fully acidified to pH 1.5—2.0 with 125 μl of 3 *N* HCl. The acidified solution is then poured into the second cartridge and the effluent discarded. Now the column is washed with 3.0 ml of clean water and finally with 0.5 ml of ethyl acetate.

Acid metabolites are now extracted by eluting with 2.5 ml of ethyl acetate—methanol (1:1). The eluate is collected and evaporated to dryness under a stream of dry nitrogen under mild temperature conditions (50—60°C); the residue is redissolved in 100—500 μl of ethyl acetate—2-propanol (1:1) and constitutes fraction A.

The residual neutral substances on the first microcolumn are now extracted by 2.0 ml of ethyl acetate—methanol (1:1) of which the first 0.5 ml are discarded, while the successive effluent liquid, collected and evaporated as above, constitutes fraction B.

Another fraction (C) may still be obtained by subjecting the first microcolumn to further elution with methanol.

The fractions A, B and, eventually, C are kept frozen until they are subjected to chromatographic analysis.

In all cases, the passage of the liquids through the cartridges may be accelerated by gently pushing the syringe plunger. Any air bubbles that may be formed inside the narrow bore between the syringe and the cartridge must be removed by a pasteur pipette.

RESULTS

The chromatographic parameters of twenty tryptophan and phenylalanine metabolites are reported in Table I, where a wide range of k' values is displayed that permits the separation of a group of indole and catechol derivatives (Fig. 2). The number of compounds separated is even higher when urine extracts were subjected to analysis (Figs. 3—7).

In some urine extracts identification of the peaks corresponding to homovanillic acid (HVA), vanilmandelic acid (VMA), 5-hydroxyindole-3-acetic acid (5HIAA) and 5-methoxyindole-3-acetic acid (5MIAA) was tentatively carried out by co-chromatography with standard compounds once the effective presence of the considered metabolites has been ascertained by specific detection reagents on TLC [22].

For quantitative purposes, plots of peak heights versus amount of injected sample were performed for 5HIAA, indole-3-acetic acid (IAA), HVA, VMA and tryptophan (Try). They were found to be linear over the range of the quantities tested: 0.15—1.5 μg/μl for 5 HIAA and IAA; 0.45—3.5 μg/μl for HVA, VMA and Try.

The reproducibility of the retention times of the compounds tested over repeated injections of reference solutions and urine extracts (ten and five times, respectively) gave relative standard deviations of 1.3%. The standard deviation for peak height was 1.1%.

The efficiency of the present extraction procedure was tested for IAA, HVA and VMA. The recovery was approximately 98%. Similarly, the selectivity was found to be fairly high (see Table II).

TABLE I

CHROMATOGRAPHIC PARAMETERS AND SENSITIVITY LIMITS OF SOME TRYPTOPHAN AND PHENYLALANINE METABOLITES

Solvent system: *n*-heptane—2-propanol—isobutanol + 2.50 ml of stationary phase. Flow-rate: 0.71 ml/min. t_0 = 2 min 21 sec.

Substance	Abbreviation	t_R	k'*	Sensitivity limit (μg/ml)**
3-Hydroxyethylindole***	3HEI	7 min 22 sec	2.13	40
5-Hydroxyindole***	5HI	9 min 42 sec	3.13	40
Indole-3-acetamide***	IAM	23 min 02 sec	8.80	60
Indole-3-acetic acid	IAA	7 min 14 sec	2.08	60
Indole-3-acrylic acid	IAcrA	5 min 21 sec	1.27	35
Indole-3-glyoxylic acid	IGA	8 min 34 sec	2.64	40
Indole-3-lactic acid	ILA	12 min 21 sec	4.25	85
5-Hydroxyindole-3-acetic acid	5HIAA	22 min 48 sec	8.70	60
5-Methoxyindole-3-acetic acid	5MIAA	10 min 12 sec	3.34	60
Tryptophan	Try	27 min 48 sec	10.83	60
5-Hydroxytryptophan	5HTry	50 min 10 sec	20.34	
Anthranilic acid	An	8 min 54 sec	2.78	30
3-Hydroxyanthranilic acid	3Han	12 min 17 sec	4.15	100
Xanthurenic acid	XA	12 min 45 sec	4.30	25
Kynurenic acid	KA	18 min 30 sec	6.87	70
Vanillic acid	VA	5 min 41 sec	1.42	20
Homovanillic acid	HVA	9 min 32 sec	3.05	90
Vanilmandelic acid	VMA	21 min 50 sec	8.29	150
3,4-Dihydroxycinnamic acid	DHCA	6 min 44 sec	1.86	20
3-Methoxy-4-hydroxycinnamic acid	MHCA	6 min 44 sec	1.86	20

*$k' = \frac{t_R(\text{sec}) - t_0(\text{sec})}{t_0(\text{sec})}$

**μg of compound in 1 ml of unconcentrated urine.

***Compounds extracted in fraction B. Remaining compounds are extracted in fraction A.

TABLE II

EXTRACTION SELECTIVITY OF THE C_{18}-BONDED SILICA CARTRIDGES

Five millilitres of an acidified (pH ≈ 2) aqeuous solution containing indoleacetamide (5.2 μg), indole-3-ethanol (5.2 μg), indole-3-lactic acid (6.2 μg) and homovanillic acid (6.2 μg) were extracted as described in the Experimental section, and the amounts of these substances in the resulting fractions A and B determined. Standard errors (S.E.) have been calculated from the means of six repeated determinations.

Substance	Fraction A			Fraction B		
	μg	± S.E.	%	μg	± S.E.	%
Indole-3-ethanol	0.052	0.008	1.02	5.096	0.009	98.8
Indoleacetamide	0.425	0.062	8.34	4.60	0.071	90.2
Indole-3-lactic acid	6.07	0.035	98.00			
Homovanillic acid	6.10	0.035	98.00			

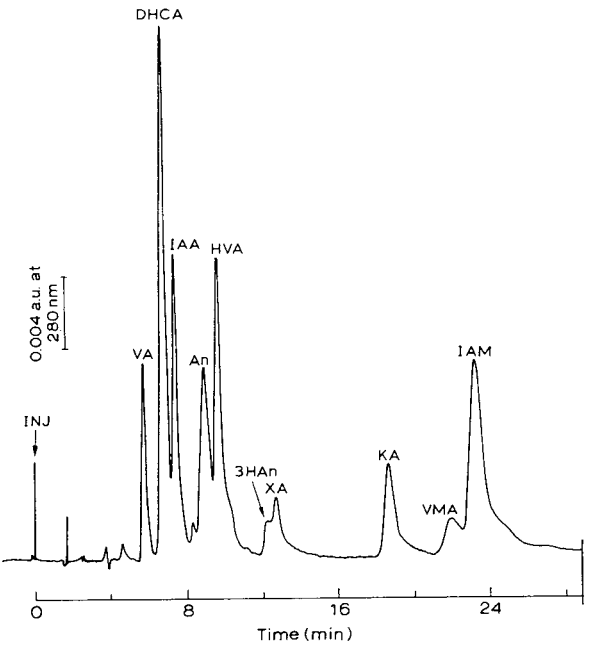

Fig. 2. Chromatogram of a standard mixture containing some indole and catechol derivatives. Volume injected was 2.50 μl, in which 0.08—0.5 μg of each compound was dissolved. Column: LiChrosorb SI 100 (Merck); the silica gel was loaded with a triethanol-ammonium perchlorate—perchloric acid solution (see text). Mobile phase: *n*-heptane—2-propanol—isobutanol (60:30:10) saturated with the stationary phase. Flow-rate: 0.71 ml/min. Temperature, ambient (18—22° C). Chart-speed: 0.25 in./min. For abbreviations see Table I.

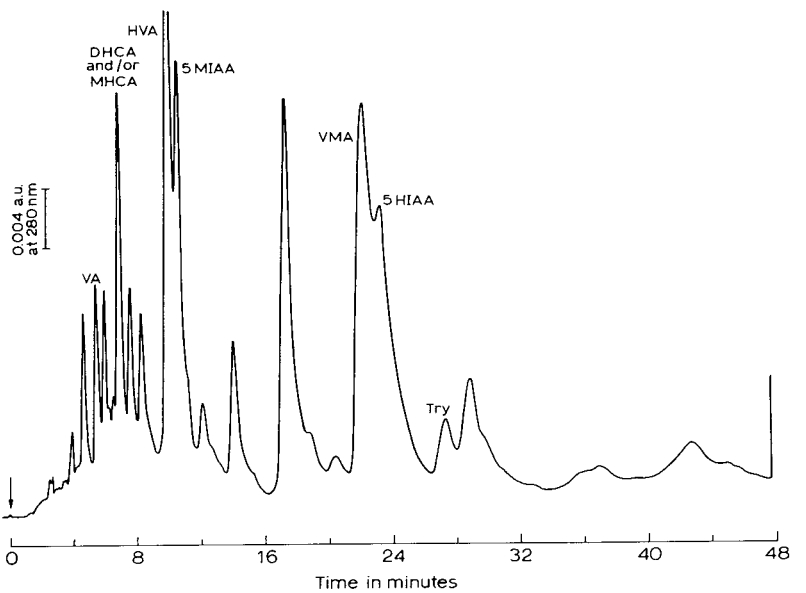

Fig. 3. Chromatogram of fraction A of the extract of a urine sample from a patient with neuroblastoma. Volume of extract injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

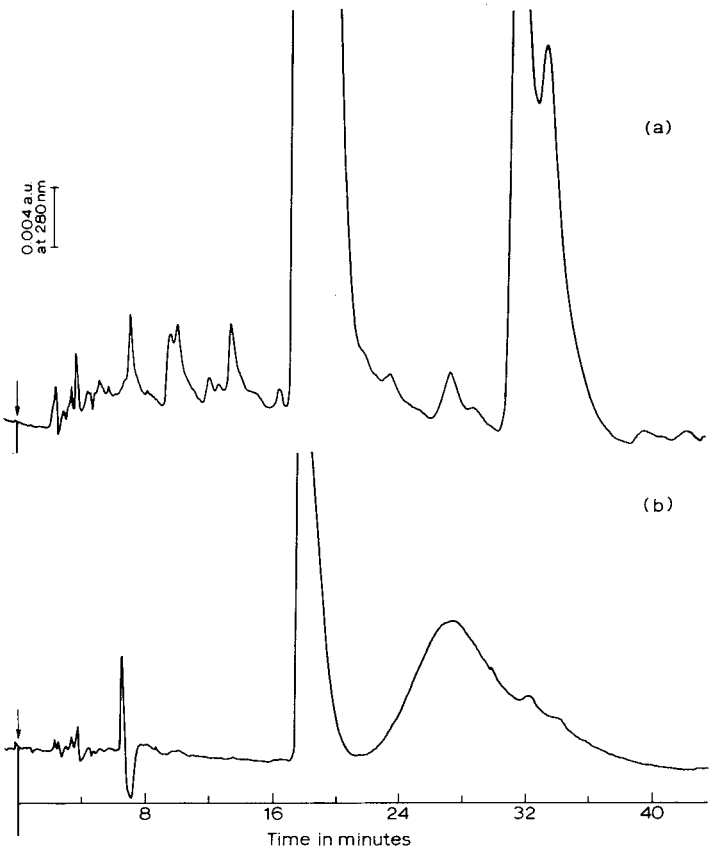

Fig. 4. Chromatograms of fraction B (a) and fraction C (b) of the extract of the same urine sample as in Fig. 3. Volume injected: 2.5 μl. Chromatographic conditions as in Fig. 2.

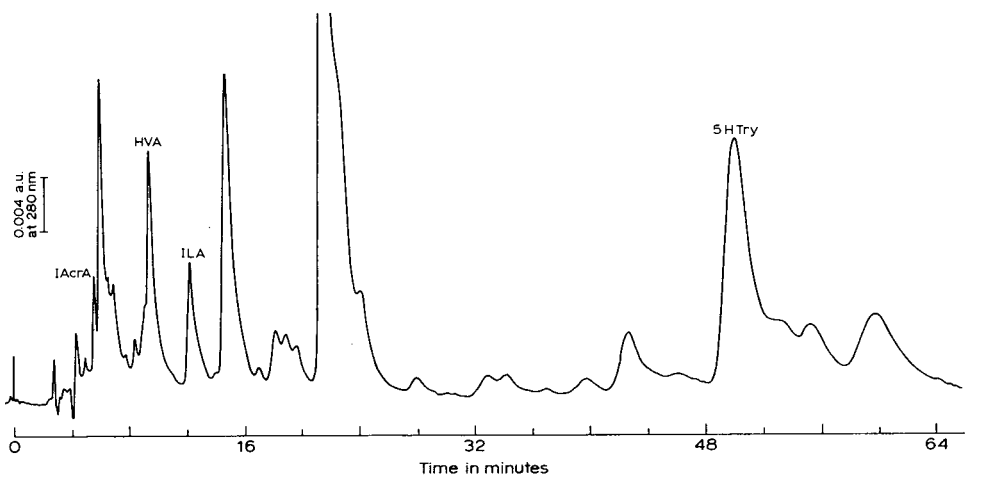

Fig. 5. Chromatogram of the extract (fraction A) of a urine sample from a case of phenylketonuria. Volume injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

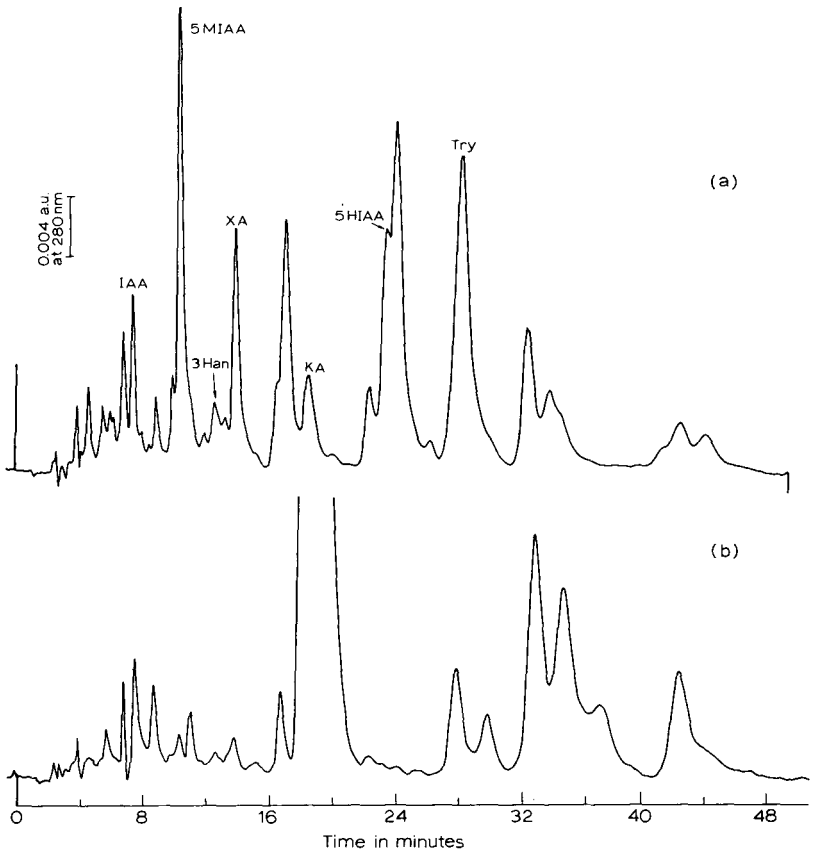

Fig. 6. Chromatograms of fraction A (a) and fraction B (b) of the extract of a urine sample from a patient affected with hypochromic microcytic anemia. A 24-h urine sample was collected after loading the patient with tryptophan. Volume injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

DISCUSSION

The present chromatographic system was selected from several we tested in a preliminary study (unpublished results) because it proved to be fully adaptable for the separation of (a) the major tryptophan metabolites of the kynurenine and serotonin pathways, (b) homovanillic acid, vanilmandelic acid and other related compounds, and (c) 5HIAA, 5MIAA, HVA and VMA. In fact, this kind of separation should enable one to examine the pathological conditions which involve the acid metabolites of tryptophan and phenylalanine.

We have tested the adaptability of this chromatographic technique to the solution of practical problems in clinical pathology, subjecting to analysis real urine samples from healthy adult subjects as well as from infants affected with various diseases. The results obtained (Figs. 3—6) show that the technique is capable of resolving a discrete number of compounds with acceptable efficiency and selectivity even without gradient elution, so that it can be profitably used for testing the aromatic constituents of the urine.

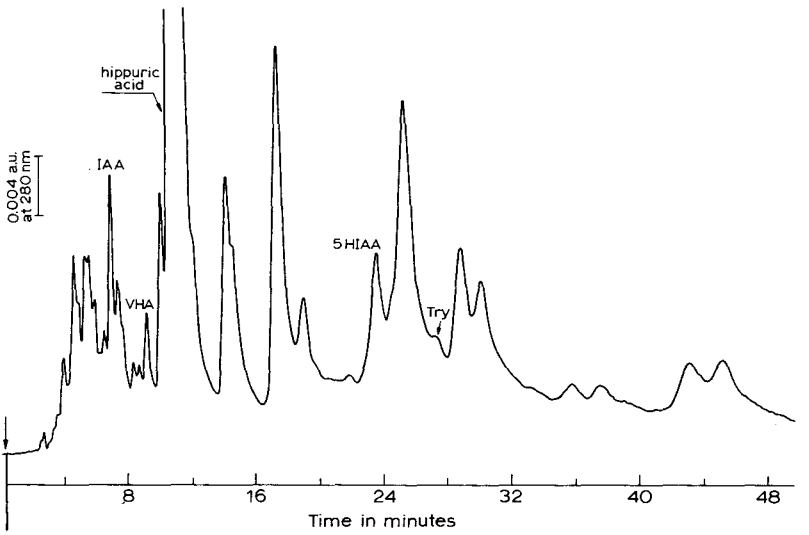

Fig. 7. Chromatogram of the extract (fraction A) of a urine sample from a healthy adult subject. Volume injected: 2.5 μl. Chromatographic conditions as in Fig. 2. For abbreviations see Table I.

It was necessary to employ extraction procedures for the substances of interest in the urine prior to chromatography since the number of UV-absorbing urinary constituents was very high. However, these procedures do offer the advantage that such a multicomponent mixture is fractionated into subgroups of given chemical affinities, for example neutral compounds, acids and bases, so as to obtain simpler chromatographic responses.

For this purpose we have devised a method for the extraction of organic compounds from urine using C_{18}-bonded silica microcolumns, which appears to us less laborious and more flexible than ethyl acetate extraction of acidified and NaCl-saturated urine [17, 22, 23].

Though both extraction methods are based on the same principle, i.e. liquid—liquid partition of the substances between two immiscible phases, the use of the C_{18} cartridges offers a more versatile system in as much as more than one fraction may be collected from a single specimen by multiple successive elutions with solvents of different strengths. Thus we obtained three fractions, substantially different from each other, from the urine of a case of neuroblastoma (Figs. 3—5). HVA and VMA were present in the first of these fractions (A), while the remaining two fractions yielded chromatograms with some high peaks, which have not yet been identified.

The fraction A obtained from the urine of a phenylketonuric patient was found to contain indole-lactic acid and 5-hydroxytryptophan, as well as some unknown substances (Fig. 5). Similarly, HVA was always present in the first fraction from urine samples of healthy subjects (Fig. 7).

As far as the selectivity of the present extraction method is concerned, we have obtained a clear-cut separation of some acids and neutral compounds (Table II), in fractions A and B, respectively, while the basic and very polar substances were not retained by the bonded phase.

The drawback of the chromatographic method described is that the strongly retained compounds, which yield relatively broad peaks, give poor detection responses if sensitivity is correlated only with peak height. Generally, the sensitivity limits, expressed in terms of peak height, were lower than in reversed-phase HPLC, probably because of bathochromic or ipsochromic effects of the organic mobile phase on the UV-absorbance maxima of the metabolites studied.

In conclusion, we believe that: (A) the HPLC technique described here, although still open to further improvement, constitutes a valuable system for the rapid and simple analysis of the metabolites considered; and (B) the use of the C_{18}-bonded silica cartridges further simplifies the analytical procedure and offers the possibility of obtaining more highly purified and fractionated samples for chromatographic analysis.

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IMPROVED GAS CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF CLONAZEPAM LEVELS IN PLASMA USING A NITROGEN-SENSITIVE DETECTOR

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SUMMARY

A gas—liquid chromatographic procedure (GLC) is described for the determination of clonazepam in plasma. The drug is extracted from buffered plasma at pH 9.0 with diethyl ether and then back-extracted into 6 *N* hydrochloric acid—6 *N* sulfuric acid (95:5) and hydrolyzed at 100°C to convert the drug into its benzophenone derivative. The benzophenone derivative of flurazepam is added to plasma as an internal reference standard. Drug derivatives are finally extracted from the neutralized aqueous phase and assayed by GLC. The present procedure makes use of a nitrogen-sensitive detector which is more stable and selective than the commonly employed electron-capture procedure. The sensitivity of the detector for clonazepam is 1 ng/ml.

INTRODUCTION

Clonazepam (Clonopin®, Roche), one of the relatively newer members of the benzodiazepine class of compounds, has been increasingly used in the treatment of seizure disorders. Therapeutic drug level monitoring of various anticonvulsant drugs has been a valuable tool in the management of epilepsy. However, pharmacokinetics of clonazepam, as well as its blood level data during chronic and single-dose administration, are relatively few [1—4]. Clonazepam is usually prescribed along with other common anticonvulsant drugs such as diphenylhydantoin, phenobarbital and succinimides. Data about the possible interactions of these anticonvulsants with clonazepam and its metabolism are very limited.

Gas—liquid chromatography (GLC) has been a valuable tool in therapeutic drug level monitoring because of its versatility in qualitative and quantitative

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analysis of a wide variety of drugs. In routine therapeutic drug level monitoring, a flame ionization detector (FID) is often used for drugs present in μg/ml concentrations in plasma. The sensitivity of FID is unsuitable for those anticonvulsant drugs which are present in plasma only in ng/ml quantities. Electron-capture (EC) detectors have been used for monitoring various drugs present in low concentrations in plasma, including clonazepam [5—10] but their use in routine laboratory work is difficult because of inherent instability and because of extremely high response to halogen-containing compounds which may be present as impurities. Since many drugs contain nitrogen, recently introduced nitrogen—phosphorus-sensitive detectors have been increasingly used in therapeutic drug level monitoring because of their selectivity and stability as compared to EC detectors.

Several investigators have published GLC procedures using EC detectors for clonazepam plasma level determinations [5—10]. These methods can be separated into two groups: (a) those that measure the unchanged drugs [7, 10, 11—13] and (b) those in which clonazepam is modified either by derivatization, e.g., methylation [6, 9, 14] and trimethylsilylation [15] or by degradation by acid hydrolysis [5, 6, 16]. Derivatization of clonazepam by methylation or by trimethylsilylation etc., is often difficult to reproduce and often yields varying products and sometimes decreased product yields. Conversion of benzodiazepinones to their respective benzophenones can be carried out with good yield and therefore is still the method of choice for the benzodiazepinone assay [17].

In our present procedure, we have modified the acid hydrolysis method of De Silva et al. [5] and have used a nitrogen-sensitive detector for routine GLC determination of clonazepam levels in plasma with acceptable accuracy and reproducibility.

EXPERIMENTAL

Reagent

All reagents were of highest grade purity available. Diethyl ether (Fisher Scientific, Pitsburgh, PA, U.S.A.), Spectranalysed was made peroxide free by passing through a column of basic alumina (Alumina Basic, Brockman Activity 1, Fisher Scientific). Ethyl acetate used was glass distilled grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Hydrochloric and sulfuric acid were of "Ultrex" grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). Borate—KCl—Na_2CO_3 buffer (1 *M*) pH 9.0 was prepared from analytical grade reagents [17]. Bromothymol blue reagent was a 0.1% solution in 50% ethanol. Sodium hydroxide (6 *M*) was prepared from analytical grade pellets (J.T. Baker). Clonazepam [7-nitro-5-(2-chloro-phenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one], flurazepam [7-chloro-1-(2-diethylaminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one] and other reference benzodiazepines were obtained as generous gifts from Hoffman-La Roche (Nutley, NJ, U.S.A.). Clonazepam (10 mg) was dissolved in 100 ml of methanol. One ml of this stock solution was diluted in deionized water to give a solution of 500 ng/ml of working solution. The aqueous solution was placed in an ultrasonic bath to ensure complete dissolution of clonazepam. The working solution was further

diluted in drug-free pooled plasma to concentrations of 10, 25, 50 and 100 ng/ml. Aliquots of prepared plasma standards and patient plasma samples containing various concentrations of clonazepam were kept frozen at −20°C and were used for day-to-day analysis over a period of six months. Freshly prepared spiked plasma standard solutions were used to compare the stability of clonazepam in storage. This comparison served as the basis for day-to-day quality control and for within-run and between-run accuracy determinations of the procedure.

All glassware was scrupulously cleaned with detergent in an ultrasonic bath, rinsed with regular glass-distilled water and finally with distilled deionized water and dried in the oven. The water used was distilled in glass, deionized and pretested for any spurious peaks with the nitrogen-sensitive detector.

Preparation of internal standard from flurazepam

The benzophenone of flurazepam (Dalmane®, Roche): 2-amino(diethylaminoethyl)-5-chloro-2′-fluorobenzophenone (ADACB) is prepared by hydrolysing 25 mg of flurazepam in 50 ml of 6 *N* hydrochloric acid—6 *N* sulfuric acid (95:5) in a boiling water bath for 60 min in a stoppered glass tube. The hydrolyzed solution is then cooled on ice, a few drops of bromothymol blue indicator added and neutralized with 6 *N* sodium hydroxide solution until just blue. The neutralized solution is extracted with cold peroxide-free diethyl ether (25 ml) three times. The ether extract is dried over anhydrous sodium sulfate and finally evaporated to dryness under a slow stream of nitrogen. The yellow hydrolysate is a mixture of the benzophenone of flurazepam and unhydrolyzed flurazepam. The material is then passed through a column of alumina (8 cm × 1.5 cm) and eluted with 10% ethyl acetate in benzene. Eight fractions (20 ml each) are collected. The benzophenone of flurazepam is eluted in the middle fractions (Nos. 3, 4 and 5) as indicated by its bright yellow color. The solvent is removed in a rotary evaporator and the residue weighed. The benzophenone of flurazepam (10 mg approx.) is then dissolved in 100 ml of methanol and dilutions are made in distilled deionized water to give a working solution for the internal standard of 50 ng/ml. One ml of this solution is added to the plasma samples as an internal reference standard. The purity of the benzophenone is established by GLC on different columns (3% SE-30 and 3% OV-17) and also by mass spectrometry. Mass spectrometry (electron impact technique) yielded an M^+ peak with an *m/e* value of 348.

Apparatus and GLC conditions

Analyses were performed using a Hewlett-Packard gas chromatograph, Model 5830A, modified and equipped with interchangeable flame ionization and nitrogen—phosphorus-sensitive detectors. Coiled glass columns (Supelco, Bellefonte, PA, U.S.A.), 1.8 m × 2 mm I.D., were packed with 3% SE-30 on 80—100 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) and baked overnight at 260°C, with a helium carrier gas flow-rate of 30 ml/min. The chromatographic conditions for separation of the benzophenones of clonazepam and flurazepam are as follows: carrier gas helium with a flow-rate of 30 ml/min; column oven temperature, 215°C; injection port temperature (on column) 270°C; air flow-rate to collector, 50 ml/min;

hydrogen flow-rate to collector, 3 ml/min; and the collector voltage varied from 12 to 16 V.

General extraction procedure

In a 15-ml PTFE-lined screw-capped centrifuge tube are placed 50 ng of the internal standard, 1.0 ml of plasma (clonazepam standard solutions or patient plasma) and 2 ml of borate buffer. Diethyl ether (10 ml) is added and the tubes are shaken in a reciprocating shaker for 10 min. The tubes are then cooled on ice, and centrifuged for 7 min. The ether phase is then carefully transferred into another tube containing 2 ml of 6 *N* hydrochloric acid—6 *N* sulfuric acid (95:5) and the tubes are shaken for 10 min. After cooling on ice the tubes are centrifuged and the ether layer is siphoned off. The acid layer is washed with 5 ml of diethyl ether which is aspirated and discarded and the tubes are placed in a boiling water bath loosely capped to allow the dissolved ether to escape. After 5 min the caps are tightly closed and the tubes are left in the bath for another 45 min. After hydrolysis the tubes are cooled on ice, a drop of the indicator added and the contents neutralized with 6 *N* sodium hydroxide until just blue. Diethyl ether (4 ml) is then added to the tubes, the tubes are shaken for 10 min, cooled on ice and centrifuged for 10 min. The ether phase is carefully transferred into small (3-ml) conical glass-stoppered tubes. The ether is evaporated under a slow stream of nitrogen at 50°C. The residue is then redissolved in 25 μl of ethyl acetate and 5—7 μl are injected into the gas chromatograph.

A standard curve is obtained by analyzing plasma standards of clonazepam in the concentration range of 10, 25, 50 and 100 ng/ml. Following chromatography, the peak height ratios and the integrator printouts for the area are compared in order to determine the accuracy of the procedure. Peak height ratios are used to calculate the concentrations of unknowns and the relative standard deviations of calibrators for within-run and day-to-day run accuracy.

RESULTS AND DISCUSSION

Fig. 1 shows the benzophenone derivatives of the commonly used benzodiazepinones and medazepam (benzodiazepine); the latter is not converted to its benzophenone derivative on treatment with strong acid. The relative retention times (*RRT*) of the benzophenones with respect to medazepam on a 3% SE-30 column are also shown (medazepam retention time is approximately 4 min). Fig. 2 shows the gas—liquid chromatograms of drug-free plasma to which were added 50 ng of clonazepam and 50 ng of the internal standard (ADACB) and then carried through the extraction procedure, and of plasma samples from three patients who are on a combination of anticonvulsant drugs including clonazepam. The early peaks in the chromatograms are possibly due to the acid hydrolysis breakdown products of other drugs (anticonvulsants) and endogenous nitrogen-sensitive substances of the plasma. The findings exemplified in Figs. 1 and 2 and the data in Table I show that commonly prescribed anticonvulsant drugs do not produce any interfering peaks in the assay for clonazepam. Thus, we have used this procedure to analyze a large number of patient plasma samples for clonazepam and found no instance in which the clonazepam peak in

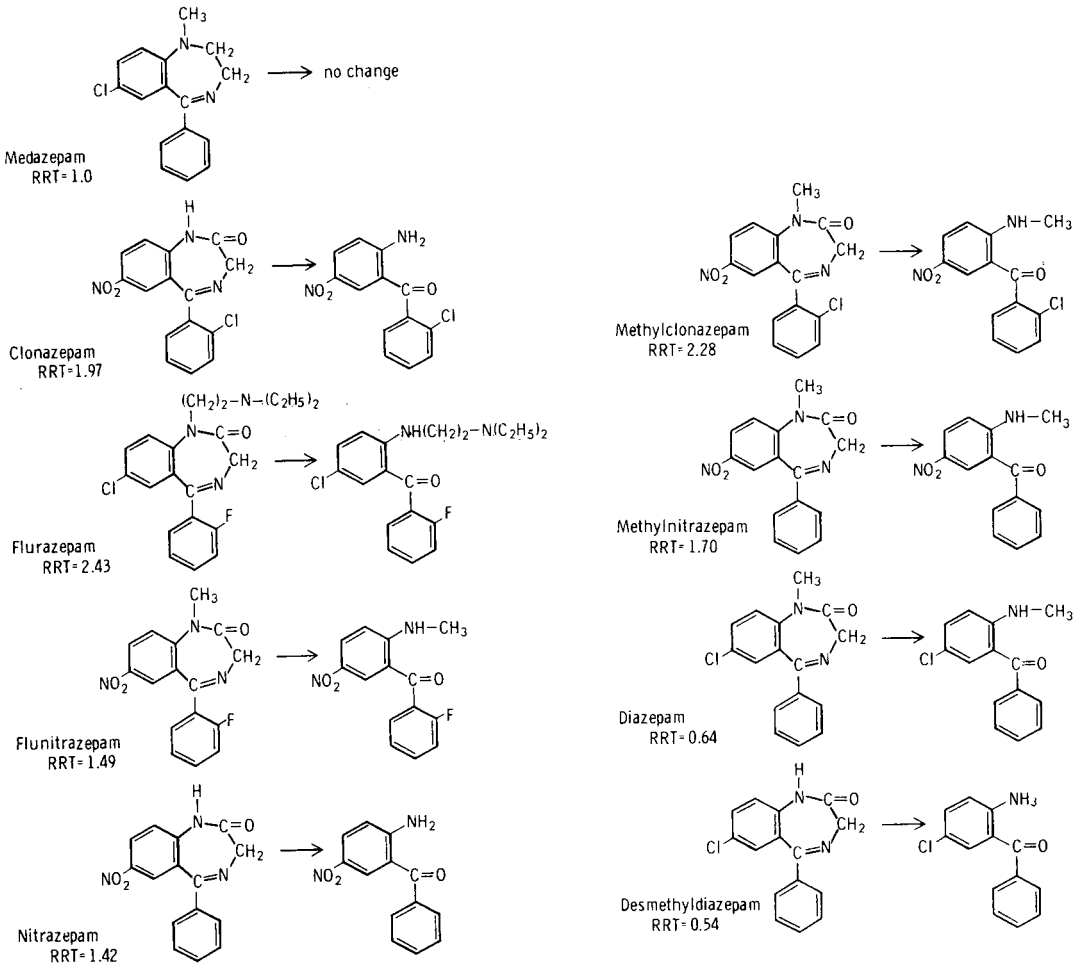

Fig. 1. Benzophenone derivatives of commonly used benzodiazepinone drugs and their *RRT* with respect to medazepam on a 3% SE-30 column.

the chromatogram is obscured by the presence of another drug or by any other endogenous nitrogen-containing compound.

It is evident from Figs. 1 and 2 and Table I that several benzodiazepinones other than clonazepam could easily be detected and estimated in plasma by the present procedure but this was not investigated further. The results of clonazepam analyses are shown in Table I which also includes the quantitative data of the three chromatograms shown in Fig. 2b, c and d. The recoveries of the benzophenone of clonazepam and the internal standard were satisfactory (86 ± 4%, n = 6 ± S.E.M.). The retention time for the clonazepam peak in the chromatogram is about 8 min and for the internal standard is about 10 min under standard assay conditions. Other benzodiazepinones (methylclonazepam) could also, in theory, be used as internal reference standards but we have found that the benzophenone of flurazepam consistently produced the most sym-

TABLE I

CLONAZEPAM CONCENTRATION IN PLASMA OF PATIENTS WHO ARE ON MULTIPLE ANTICONVULSANT DRUG THERAPY

Patient	Clonazepam* (ng/ml)	Phenobarbital (μg/ml)	Dilantin (μg/ml)	Tegretol (μg/ml)	Mysoline (μg/ml)	Valproic acid (μg/ml)	Tridione** (μg/ml)	Zarontin (μg/ml)
A	22.5	—	11.9	—	—	—	—	—
B	12.7	23.5	11.3	—	—	—	—	—
C	27.3	—	—	5.2	—	—	—	—
D	24.6	21.4	—	—	—	1.0	800.0	—
E	16.5	42.8	14.2	14.2	8.0	69.7	—	—
F	46.0	—	19.0	—	—	—	—	—
G	—	—	—	—	—	—	—	—
H	2.0	—	—	—	—	79.7	—	65.7
I	54.0	19.2	—	—	—	—	980.0	—
J	32.8	41.2	—	—	—	—	46.8	—
K	14.0	15.8	5.6	—	—	—	—	22.9
L	26.0	20.2	7.8	—	8.1	113.6	—	—
M	15.5	16.3	9.3	—	8.2	—	—	—
N	20.1	31.0	30.3	4.0	12.0	—	—	—
O	12.1	30.3	12.1	8.1	—	81.4	—	—
P	17.1	—	10.3	—	—	102.0	—	82.2
Q	80.0	—	—	—	—	—	—	—
b	17.3	—	10.1	—	—	94.0	—	94.0
c	11.2	18.3	13.8	—	4.1	99.3	—	—
d	2.05	—	3.7	—	—	4.2	—	—

*All drugs except clonazepam were assayed by GLC with FID using a modified Kupferberg procedure [18].
**Values for dimethadione.

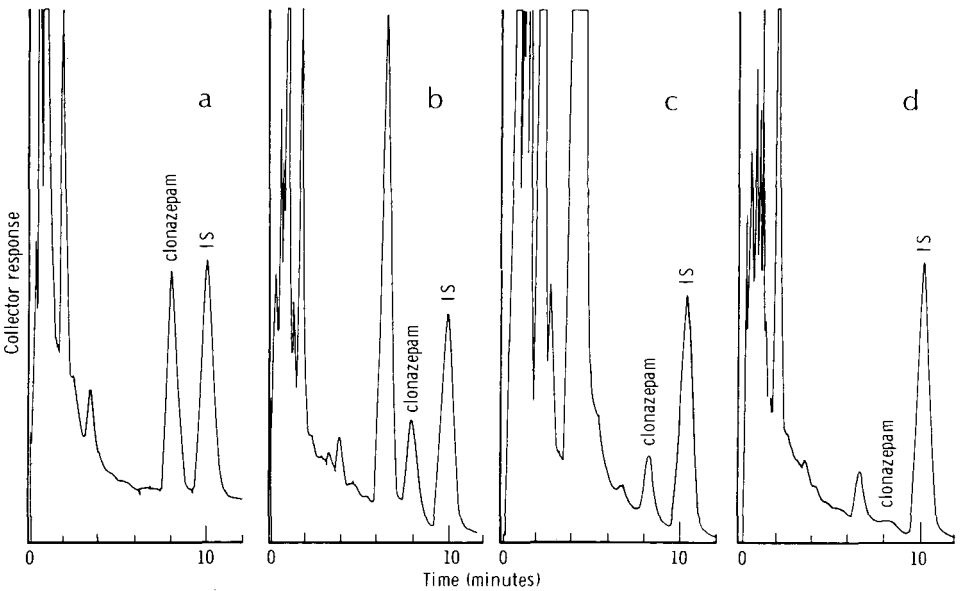

Fig. 2. Chromatograms of (a) blank plasma with added clonazepam (50 ng) and (b, c, d) three patient plasmas all assayed in the same manner after adding 50 ng internal standard, IS (see Table I).

metrical peak. The linearity of the entire procedure was demonstrated by replicate analyses of the standard plasma solutions of clonazepam containing 10, 25, 50 and 100 ng/ml. The ratio of peak heights of clonazepam benzophenone (ANCB)/internal standard was plotted against concentrations. The range of linearity was excellent from 10 to 100 ng/ml. The precision data for the assay were obtained using spiked plasma samples as well as patient plasma samples containing clonazepam. The within-day reproducibility of the method was obtained with plasma standards containing 25, 50, and 100 ng/ml of clonazepam. The day-to-day reproducibility for the procedure was obtained using similar concentrations in control plasma as well as in patient plasma samples. These control and patient plasma samples contained 16.5, 28.2 and 69.5 ng/ml of clonazepam. The results are summarized in Table II.

At concentrations less than 5 ng/ml the accuracy is poor and not clinically useful. Plasma samples containing concentrations over 100 ng/ml can be determined accurately by repeating the assay using 0.5 ml of the plasma or less. Although the relationship between plasma concentration of clonazepam and therapeutic response is not fully established, 20—70 ng/ml in plasma is usually accepted as the optimal range for clinical effectiveness.

Benzodiazepinones are usually converted to their respective benzophenone derivatives upon mineral acid hydrolysis [5, 6]. We found that benzodiazepinones with a long side-chain attached to nitrogen I of the ring (as in the case of flurazepam) undergo only incomplete hydrolysis even under the most rigorous conditions. This finding has not been reported by previous investigators. Therefore, it is important to use the benzophenone of flurazepam as the internal standard instead of the drug itself.

TABLE II

PRECISION DATA FOR CLONAZEPAM ASSAY

Plasma standards are 25, 50 and 100 ng/ml; patient plasma values 16.5, 28.2 and 69.5 ng/ml.

	n	Level 1			Level 2			Level 3		
		Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)
Within-day										
Fresh plasma	20	24.6	0.14	0.56	49.5	0.19	0.38	97.8	0.83	0.85
Frozen plasma	10	24.3	0.16	0.65	49.2	0.23	0.46	98.0	0.80	0.81
Between-day										
Fresh plasma	12	24.7	0.13	0.52	49.0	0.26	0.53	98.1	0.80	0.81
Frozen plasma	6	24.1	0.18	0.74	48.8	0.31	0.63	97.5	0.93	0.95
Patient plasma frozen	6	15.8	0.05	0.36	27.6	0.10	0.36	68.5	0.13	0.20

The specificity of the present procedure has been well tested in the presence of other anticonvulsant drugs, and virtually no interference has been found. Clonazepam levels found in patient plasma varied from several ng/ml to 80 ng/ml; the latter value was from a patient (child) who had been prescribed clonazepam for two years without any other co-medication and whose seizures are under complete control.

The benzophenone of flurazepam, prepared for use as internal reference standard, was found to be pure and devoid of any contamination from the parent drug. Once prepared (about 10 mg), the internal standard is sufficient for analyzing 2000 samples.

Flurazepam, if co-administered with clonazepam will interfere with the present assay procedure for clonazepam. However, it is very unlikely that the two drugs will be prescribed for concurrent use. Of the 500 or more patient plasma samples analyzed so far in our laboratory none was found to contain flurazepam as a co-medication.

In a separate series of experiments the benzophenone of clonazepam was prepared; the response of the nitrogen-sensitive detector was found to be linear with increasing concentrations.

The procedure is specific for clonazepam and no other commonly prescribed benzodiazepinones will interfere. The metabolites of clonazepam (Fig. 3), i.e., the 7-amino derivative and 3-hydroxyclonazepam will produce the same benzophenone derivative as that produced by clonazepam, upon hydrolysis. However, under the conditions of the assay these metabolites are not extracted; thus after addition of 150 ng/ml of 7-amino clonazepam and 7-acetamidoclonazepam to drug-free plasma no peak corresponding to the benzophenone breakdown product of clonazepam was noted.

Our findings substantiate the observations of previous authors [5, 6]. The 3-hydroxy metabolite of clonazepam is not found in significant amounts in plasma [6, 19] and washing of the acid phase by diethyl ether eliminates most of these metabolites [5] and reduces the changes of substantial interference.

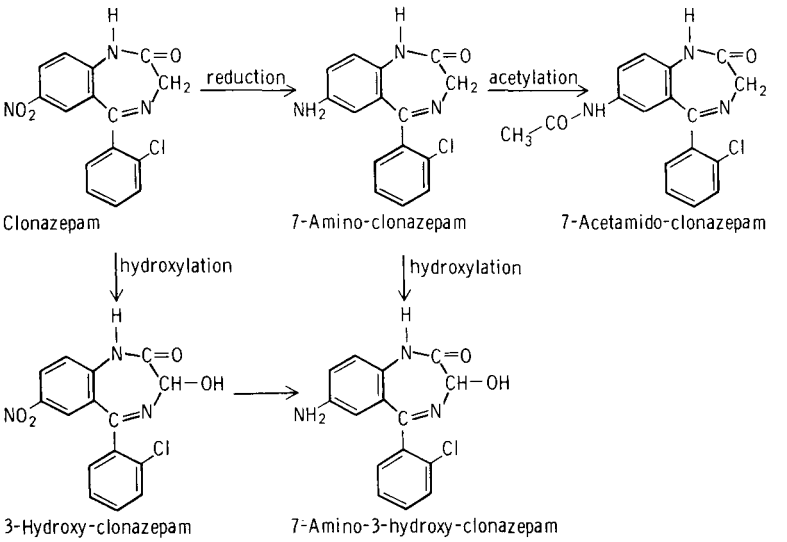

Fig. 3. Possible metabolic pathway of clonazepam in man (Eschenhof [19]).

Our present method based on the technique of mineral acid hydrolysis developed by De Silva et al. [5], is relatively simple and uses the nitrogen-sensitive detector for GLC analysis. The method may be used for routine analysis since it eliminates the problems associated with the more commonly employed EC technique.

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THE ANALYSIS OF ARILDONE IN PLASMA, URINE AND FECES BY GAS—LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

The analysis of arildone in plasma, urine and feces by gas—liquid chromatography with electron-capture detection is described. O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine is the derivatizing agent for the plasma and urine analysis; 3-nitrophenylhydrazine is utilized for fecal analysis. The mean (± S.E.) minimum quantifiable level of arildone was 1.4 (± 0.2) ng/ml in urine, 6.4 (± 0.1) ng/ml in plasma, and 12.6 (± 1.0) ng/g in feces. The chromatographic response was linear in the range of 0 and 10—120 ng/ml for plasma, 0 and 2.5—20 ng/ml for urine and 0 and 25—250 ng/g for feces. The estimated overall precision of the assay was 5.5%, 6.4% and 8.9% in urine, plasma and feces, respectively.

INTRODUCTION

Arildone, 4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione, is a member of a new class of antiviral agents which has shown activity against both DNA and RNA viruses; it was particularly effective against herpes simplex virus types 1 and 2 [1, 2]. Arildone has been reported to inhibit the uncoating of polio-virus in infected HeLa cells and, therefore, preventing the viral-induced inhibition of host cell protein synthesis; arildone does not inhibit either the absorption or penetration of polio-virus into the cells in tissue culture [3]. The drug is currently the subject of clinical trials to evaluate its safety and efficacy in humans.

This report describes methods for the quantitative analysis of arildone in human plasma, urine, and feces. The plasma and urine methods involve derivatization with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine [4] and analysis

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by gas—liquid chromatography (GLC) with electron-capture (EC) detection. The fecal analysis employs 3-nitrophenylhydrazine as the derivatizing reagent prior to GLC analysis with EC detection.

EXPERIMENTAL

Materials

Arildone (Fig. 1, I) and the internal standard (Fig. 1, II) were synthesized at Sterling-Winthrop Research Institute. Hexane (ChromAR; Mallinckrodt, St. Louis, MO, U.S.A.) was distilled at atmospheric pressure before use. The O-(2, 3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was synthesized at Sterling-Winthrop Research Institute or, more recently, purchased from Sigma (St. Louis, MO, U.S.A.). 3-Nitrophenylhydrazine hydrochloride (3-NPH), 98% (Aldrich, Milwaukee, WI, U.S.A.) was used as received. All other chemicals were reagent grade, with the exception of cyclohexane which was practical grade, and used without further purification.

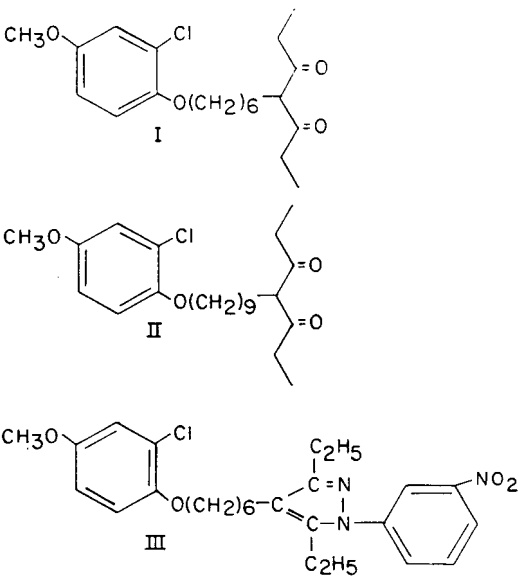

Fig. 1. Structural formulae of (I) arildone, 4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione; (II) internal standard, 4-[9-(2-chloro-4-methoxyphenoxy)nonyl]-3,5-heptanedione; (III) $C_{26}H_{32}N_3O_4Cl$ (M.W. 485).

Preparation of samples and standards

Spiked samples, to be analyzed under single-blind conditions, were prepared in human control plasma, urine and fecal homogenates [feces—triple distilled water (1:4, w/v)], coded and randomized. One set of samples in each biological medium was analyzed upon preparation; the other set was frozen for a minimum of four days at −4°C. Fresh standards, in the sample medium to be analyzed, were prepared on the day of analysis of each set of samples.

Plasma. Duplicate standards were prepared by adding appropriate volumes of an arildone stock solution (2 ng/μl in methanol) and 1.0 ml of human control

plasma (oxalate anticoagulant) to clean, unsilanized tubes to give final concentrations of 0 and 10—120 ng/ml of plasma. Two sets of quadruplicate samples were prepared, in the same manner as the standards, to give final arildone concentrations of 0, 15, 30, 52 and 84 ng/ml.

A third set of standards and samples was prepared, coded, extracted and derivatized by one analyst. The derivatized standards and samples were analyzed by GLC—EC under single-blind conditions by a second analyst. The arildone concentrations of the samples were 0, 13, 26, 32, 46 and 74 ng/ml of plasma.

Urine. Duplicate standards were prepared by adding appropriate volumes of an arildone working solution (1 ng/μl in methanol) and 2.0 ml of human control urine to clean, unsilanized tubes to give final arildone concentrations of 0 and 2.5—20 ng/ml of urine. The tubes were capped and thoroughly mixed. Two sets of triplicate samples were prepared, in the same manner as the standards, to give final arildone concentrations of 0, 3.75, 6.5, 11.0, 13.5 and 19.0 ng/ml of urine.

Feces. Duplicate standards were prepared by adding 5 ml of human control fecal homogenate (1 g feces, 4 ml triple distilled water) and appropriate volumes of an arildone stock solution (2 ng/μl in acetonitrile) to give final arildone concentrations of 0 and 25—250 ng/g of feces. The tubes were capped and thoroughly mixed. Two sets of quadruplicate samples were prepared in the same manner as the standards to give final arildone concentrations of 0, 54, 82, 106 and 178 ng/g of feces.

Assay procedure

Plasma and urine. To 1.0 ml of plasma (or 2.0 ml of urine) were added 145 ng (or 109 ng for urine) of an internal standard stock solution (145 ng per 15 μl or 109 ng per 20 μl in methanol) and 10.0 ml of hexane. The tube was shaken, centrifuged and placed in a dry ice—acetone bath to freeze the aqueous layer. The hexane was decanted into a clean tube and evaporated to dryness in a heating block with the aid of a stream of dry air. The residue was treated with 200 μl of the derivatizing reagent solution [2 mg PFBHA per 200 μl in glacial acetic acid—ethanol (5:95)]. The tube was capped, and the mixture was allowed to react for 90 min at 90°C. The reaction mixture was then evaporated to dryness in a heating block with the aid of a stream of dry air. The residue was partitioned between 200 μl of cyclohexane and 200 μl of 10% acetic acid. A 2-μl aliquot of the cyclohexane phase was analyzed on a gas—liquid chromatograph equipped with an electron-capture detector (Hewlett-Packard Model 5710A).

Feces. Sixty μl of an internal standard stock solution (435 ng per 60 μl in methanol) were added to each tube containing fecal homogenate. The contents were thoroughly mixed, and 100 μl of 5 *F* sodium hydroxide were added. The contents were thoroughly mixed, 10 ml of hexane were added, and the tube was placed on a rotary mixer for 30 min. After centrifugation, the aqueous phase was frozen in a dry ice—acetone bath, and the hexane phase was decanted into a clean tube. The hexane was evaporated to dryness at about 60°C with the aid of a stream of dry air. The residue was dissolved in 4 ml of acetonitrile and extracted three times with 2 ml of hexane. The hexane was aspirated and discarded.

To the acetonitrile phase were added 0.5 ml of 0.5 *F* ammonium hydroxide in methanol and 0.2 ml of the derivatizing solution (400 μg 3-NPH per 0.2 ml methanol). The tube was capped and heated for 15 min at 90°C. The solvent was evaporated to dryness at 60°C with the aid of a stream of dry air. The residue was partitioned between 200 μl hexane and 300 μl water. A 2-μl aliquot of the hexane phase was analyzed on a gas—liquid chromatograph, as above.

Chromatographic conditions

The column was a 2-ft. silanized glass column packed with 3% OV-1 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). The column temperature was 275°C (285°C for fecal analysis), the injector and detector temperatures were 300°C, and the carrier gas was 7% methane in argon flowing at 60 ml/min. These conditions gave retention times of approximately 50 sec for derivatized I and 100 sec for derivatized II.

Extraction efficiency

The recoveries of I and II from plasma, urine and feces were determined at two or three concentrations of I by comparing the peak heights of extracted standards with those of reference standards. Reference standards were prepared by adding appropriate amounts of I and II to hexane extracts of the biological media. Extracted standards were prepared by adding appropriate amounts of I and II to control human plasma, urine and fecal homogenate and extracting them according to the procedures outlined above. The reference standards (in extracts) and extracted standards were then carried concurrently through the appropriate derivatization procedures and analyzed by GLC—EC. The per cent recovery of I was determined by comparing the peak height of the arildone derivative peak in each extracted standard with the linear regression obtained from the peak heights of the arildone derivative in the reference standards. The per cent recovery of II was calculated according to Goldstein [5] by comparing the peak heights of the internal standard derivative in the extracted samples with the peak heights of the internal standard derivative in the reference standards.

Statistical analysis

Several statistical tests were applied to the analytical data. A regression analysis of the peak height ratios (I:II) obtained for the standards was performed to determine the linearity of the response with respect to concentrations. The resulting linear regression was used to estimate the concentrations of arildone in the prepared samples. The minimum quantifiable level (*MQL*) of the assay was determined from the regression line as that concentration whose lower 80% confidence limit just encompassed zero as determined by the inverse prediction [6].

The assayed levels from the determination of the prepared samples were expressed as per cent differences from the nominal values and analyzed by a two-way analysis of variance with replication to test for a concentration effect, a time effect and a concentration—time interaction. The resulting *F*-ratios were examined for significant sources of variation. The precision of the assay was determined from this analysis.

RESULTS

Plasma and urine assay

Representative chromatograms of extracted and derivatized plasma and urine samples are shown in Fig. 2B and C. Regression analysis on the standards indicated a linear relationship between peak height ratio (derivatized I:derivatized II) over the range of 0 and 10—120 ng/ml for plasma and 0 and 2.5—20 ng/ml for urine. A summary of the results of the regression analysis is presented in Table I.

The concentrations of the prepared plasma and urine samples were estimated by inverse prediction from the appropriate regression equation and are summarized in Tables II and III, respectively. The two-way analysis of variance of the nominal values for the urine samples indicated no significant sources (concentration, time or concentration × time) of variation at $P \leqslant 0.01$. An overall estimate of the urine assay precision, based on the variance of the repeat determinations within each concentration level, was 5.5%. The accuracy of the assay, defined by the ranges of the mean per cent differences from the nominal concentration levels, varied from −13.2% to +2.1%. The mean (± S.E.) *MQL* was 1.4 (± 0.2) ng/ml, $N = 3$.

The analysis of variance on the results for the plasma samples indicated no

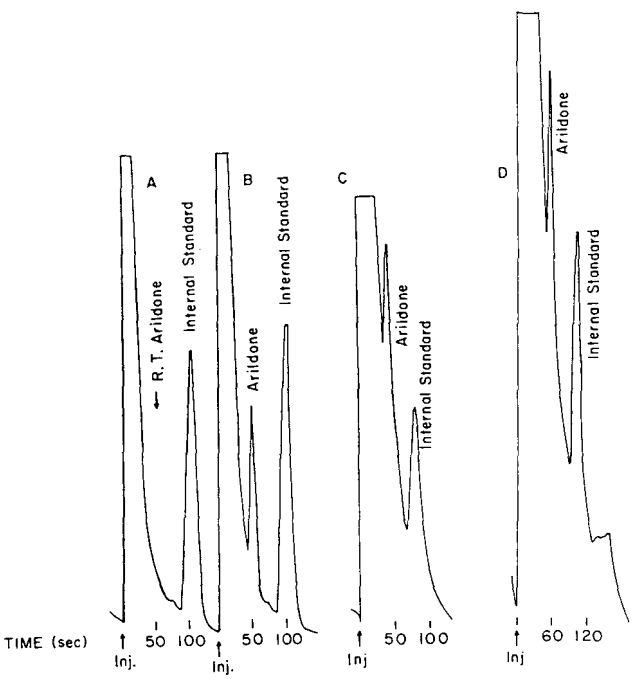

Fig. 2. Representative chromatograms (see text for chromatographic conditions). (A) Derivatized control urine extract, attenuation 128; (B) derivatized urine extract containing arildone, 11 ng/ml, attenuation 128; (C) derivatized plasma extract containing arildone, 26 ng/ml, attenuation 64; (D) derivatized fecal extract containing arildone, 100 ng/g, attenuation 128.

TABLE I

SUMMARY OF LINEAR STANDARD CURVE DETERMINATIONS

	Range (ng/ml)	No. of points	Slope* ± S.E.	Y_0** ± S.E.	*MQL* (ng/ml)
Urine					
Day 1	0—40	12	0.0318 ± 0.0010	−0.036 ± 0.023	1.2
Day 5	0—60	14	0.0284 ± 0.0009	−0.011 ± 0.028	1.7
Plasma					
Day 1	0—120	16	0.0371 ± 0.0008	−0.008 ± 0.058	5.4
Day 6	0—120	15	0.0406 ± 0.0013	0.038 ± 0.079	6.6
Day 7	0—120	16	0.0303 ± 0.0008	0.014 ± 0.051	5.9
Feces					
Day 1	0—250***	16	0.0086 ± 0.0002	−0.007 ± 0.033	13.6
Day 6	0—250	16	0.0100 ± 0.0002	−0.042 ± 0.033	11.6

*Change in peak height ratio per unit change in concentration.
**Y-axis intercept of the least-squares regression line; units are peak height ratio.
***ng/g of feces.

time effect or concentration × time interaction at $P \leqslant 0.05$; however, a significant concentration effect and a lack of agreement between the assayed and nominal values was observed. Since these observations had not been made during the development of the procedure, the third set of samples was prepared to determine if the preparation of the original samples was a significant factor. The data for this set of samples (Table II) showed no concentration effect at $P \leqslant 0.05$ and showed excellent agreement between the assayed and nominal values. The overall estimated precision of the plasma assay was 6.4% and the accuracy, based on the mean per cent differences of the assayed values from the nominal values for the last set of plasma samples, ranged from −5% to +8.5%. The mean (± S.E.) *MQL* of the plasma analysis was 6.0 (± 0.1) μg/ml, $N = 3$.

The extraction efficiency of arildone and of the internal standard was independent of the arildone concentration. From plasma, the mean extraction efficiency (± 95% confidence limits), determined at 100 ng/ml I and 290 ng/ml II, was 68 (± 24)%, $N = 4$ and 33 (± 12)%, $N = 4$ for I and II, respectively. From urine, the extraction efficiency, evaluated over the range of 5—30 ng/ml was 94.8 (± 2.0)%, $N = 14$ for I. The mean extraction efficiency for II, at a concentration of 54 ng/ml, was 86.7 (± 7.2)%, $N = 14$.

Fecal assay

A representative chromatogram of an extracted and derivatized fecal sample is shown in Fig. 2D. The regression analysis on the chromatographic peak height data for the standards indicated a linear response (Table I) in the range of 0 and 25—250 ng/g feces. The concentrations of the prepared samples, estimated from the regression analysis, are summarized in Table IV. No significant sources of variation were observed at $P \leqslant 0.05$. The estimated overall assay precision was 8.9% and the accuracy of the assay ranged from −9.2% to +1.6%. The mean (± S.E.) *MQL* of the fecal analysis was 12.6 (± 1.0) ng/g.

TABLE II

SUMMARY OF DATA FROM ANALYSIS OF ARILDONE IN PREPARED PLASMA SAMPLES (ng/ml)

Concentration level	Assayed level*	Assayed level**	Concentration level §§§	Assayed level §§§
0	<*MQL****	<*MQL*§	0	<*MQL*†
	<*MQL*	<*MQL*		<*MQL*
	<*MQL*	<*MQL*		<*MQL*
	<*MQL*	<*MQL*		
15	13.7	9.6	13	14.0
	11.5	11.6		12.7
	11.8	12.1		14.0
	11.5	13.8		
Mean	12.1	11.8	Mean	13.6
S.E.M. (%)	4.4	7.3	S.E.M. (%)	3.2
Mean difference (%) from freshly prepared samples		−2.5	Mean difference (%) from nominal	+4.4
30	25.5	29.3	26	26.9
	26.6	—§§		29.2
	27.7	27.8		28.5
	26.6	28.8		
Mean	26.6	28.6	Mean	28.2
S.E.M. (%)	1.7	1.3	S.E.M. (%)	2.4
Mean difference (%) from freshly prepared samples		+7.5	Mean difference (%) from nominal	+8.5
52	44.4	49.0	32	37.1
	49.2	46.5		29.9
	48.7	42.4		30.5
	45.5	48.8		
Mean	47.0	46.7	Mean	32.5
S.E.M. (%)	2.5	3.3	S.E.M. (%)	7.1
Mean difference (%) from freshly prepared samples		−0.6	Mean difference (%) from nominal	+1.6

(Continued on p. 220)

TABLE II (continued)

Concentration level	Assayed level*	Assayed level**	Concentration level §§§	Assayed level §§§
84	92.9	77.8	46	42.7
	88.3	86.6		—§§
	96.9	92.6		44.7
	92.1	85.4		
Mean	92.6	85.6	Mean	43.7
S.E.M. (%)	1.9	3.6	S.E.M. (%)	2.3
Mean difference (%) from freshly prepared samples		−7.6	Mean difference (%) from nominal	−5.0
			74	76.0
				77.6
				79.3
				77.3
			Mean	77.6
			S.E.M. (%)	0.8
			Mean difference (%) from nominal	+4.8

*Assayed immediately after preparation.
**Frozen for 5 days before analysis.
***MQL = 5.4 ng/ml.
§MQL = 6.6 ng/ml.
§§ Sample lost.
§§§Samples and standards prepared by same analyst.
†MQL = 5.9 ng/ml.

TABLE III

SUMMARY OF DATA FROM ANALYSIS OF ARILDONE IN PREPARED URINE SAMPLES (ng/ml)

Concentration level	Assayed level*	Assayed level**
0	<*MQL****	<*MQL*§
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
3.75	3.5	3.2
	3.7	3.4
	3.4	3.2
Mean	3.5	3.3
S.E.M. (%)	2.5	2.0
Mean difference (%)	−5.6	−13.2
6.5	5.4	5.8
	6.5	5.7
	5.1	6.0
Mean	5.7	5.8
S.E.M. (%)	7.5	1.5
Mean difference (%)	−12.6	−10.2
11.0	10.8	9.4
	10.0	9.7
	10.6	10.2
Mean	10.5	9.8
S.E.M. (%)	2.3	2.4
Mean difference (%)	−5.0	−11.1
13.5	12.6	15.0
	13.3	13.4
	13.1	12.9
Mean	13.0	13.8
S.E.M. (%)	1.6	4.6
Mean difference (%)	−3.6	+2.1
19.0	19.1	18.7
	17.5	18.2
	17.8	19.8
Mean	18.1	18.9
S.E.M. (%)	2.7	2.5
Mean difference (%)	−4.5	−0.5

*Assayed immediately after preparation.
**Frozen for 4 days before analysis.
***MQL = 1.2 ng/ml.
§MQL = 1.7 ng/ml.

TABLE IV

SUMMARY OF DATA FROM ANALYSIS OF ARILDONE IN PREPARED HUMAN FECAL SAMPLES (ng/g OF FECES)

Concentration level (ng/g)	Assayed level fresh*	Assayed level frozen**
0	<*MQL****	<*MQL*§
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
	<*MQL*	<*MQL*
54	54	50
	58	37
	47	53
	55	56
Mean	54	49
S.E.M. (%)	4.4	8.5
Mean difference (%)	−0.9	−9.2
82	84	82
	82	82
	72	76
	89	77
Mean	82	79
S.E.M. (%)	4.4	2.0
Mean difference (%)	−0.3	−3.4
106	98	96
	96	106
	115	114
	110	103
Mean	105	105
S.E.M. (%)	4.4	3.6
Mean difference (%)	−1.2	−1.2
178	172	180
	178	175
	168	166
	205	176
Mean	181	174
S.E.M. (%)	4.6	1.7
Mean difference (%)	+1.5	−2.1

*Analyzed upon preparation.
**Frozen for 5 days before analysis.
***MQL = 13.6 ng/g.
§MQL = 11.6 ng/g.

The mean (± S.E.) extraction efficiency for I over the range of 25—250 ng/g was 100 (± 2)%, N = 15. The mean (± 95% confidence limit) recovery for II (290 ng/g) was 77.8 (± 10.4)%.

DISCUSSION

Arildone presented several challenges for analytical method development.

The anticipated levels in biological media are in the ng/ml (or ng/g) range or lower, necessitating a highly sensitive method. This was complicated by the neutral character of the molecule which precluded the use of a back-extraction for elimination of interferences. In addition, derivatization of the β-diketone groups generally led to the formation of a mixture of *cis*- and *trans*-isomers. In the plasma and urine procedures, the PFBHA derivatization gave rise to two chromatographic peaks for both I and II. The use of the short GLC column merged the peaks due to isomers so that single peaks were observed for each I and II.

Binding to glassware and the GLC column was observed for both arildone and the internal standard. Use of silanized glassware aggravated the problem, but the presence of plasma or a plasma extract minimized binding to glassware. The GLC column required conditioning by injecting several samples containing high concentrations of the derivatives prior to injection of the standards and samples.

The assay has been useful for the analysis of arildone in human, rabbit, monkey or rat plasma; human or rat urine; and human feces. The analysis of dog plasma, however, revealed an interfering peak with the retention time of the derivative of the internal standard, II. For the analysis of dog plasma, 4-[6-(2-chloro-4-methoxyphenylamino)hexyl]-3,5-heptanedione, may be used as the internal standard.

In the analysis of fecal samples, PFBHA gave numerous derivatives of endogenous fecal components which could not be removed from the sample and which interfered with the GLC analysis. Derivatization of I with 3-NPH gave a single derivative identified by mass spectrometry as having a molecular weight corresponding to Fig. 1, III. This derivatization, coupled with the hexane—acetonitrile step in the clean-up allowed the analysis of fecal samples at levels as low as 15 ng/g of feces.

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CLINICAL ANALYSIS FOR THE ANTI-NEOPLASTIC AGENT 1,4-DIHYDROXY-5,8-BIS{{2-[(2-HYDROXYETHYL)AMINO]ETHYL}-AMINO} 9,10-ANTHRACENEDIONE DIHYDROCHLORIDE (NSC 301739) IN PLASMA

APPLICATION OF TEMPERATURE CONTROL TO PROVIDE SELECTIVITY IN PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An analytical method is described which permits monitoring of plasma levels of the anti-tumor agent 1,4-dihydroxy-5,8-bis{{2-[(2-hydroxyethyl)amino]ethyl}amino}9,10-anthracenedione dihydrochloride (DHAD) following its intravenous administration to cancer patients. The drug cannot be efficiently extracted from plasma into water-immiscible solvents, but is effectively separated from the biological matrix by retention on hydrophobic XAD-2 beads packed in a disposable glass cartridge. DHAD is subsequently selectively eluted from this column and then analyzed by reversed-phase partition chromatography with spectrophotometric detection of the analyte. Resolution of overlapping bands during high-performance liquid chromatographic separation was achieved by systematic optimization of mobile phase, ion-pairing agent and temperature. A possible explanation for the observed selectivity provided by temperature adjustment is offered. Plasma levels in the range of 75—3000 ng of DHAD per ml (7.5—300 ng applied to the column) can be analyzed with a precision of $< \pm 10\%$. Total recovery of drug from plasma is ca. 95%.

INTRODUCTION

The anthracenedione, 1,4-dihydroxy-5,8-bis{{2-[(2-hydroxyethyl)amino]-ethyl}amino}9,10-anthracenedione dihydrochloride (NSC 301739; DHAD) is an analog of adriamycin that has shown significant antitumor activity in several animal tumor systems [1—3]. Preliminary studies indicate that DHAD is less

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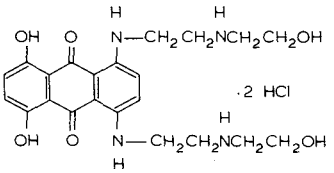


cardiotoxic than adriamycin in the rat cardiotoxicity model [4], thus, this drug represents a candidate agent with potential effectiveness against solid tumors and leukemia without the dose limiting cardiotoxicity associated with adriamycin. Initial clinical studies (Phase I) with DHAD indicate leukopenia and thrombocytopenia are dose limiting and suggest more rigorous clinical studies (Phase II) be carried out at an initial dose of 12 mg/m^2 repeated at 21—28-day intervals [5] to evaluate the therapeutic value of the drug. As part of these studies, the distribution and elimination of drug must be defined, necessitating drug level monitoring in plasma.

The purpose of the present study was to develop a clinically-useful assay for DHAD. Since the initiation of this work two papers concerned at least in part with the analysis of DHAD have appeared [6,7]. In the first, the authors describe a gradient elution HPLC procedure for separating nine aminoanthraquinone analogs. Temperature changes were also used to enhance resolution. While these authors proposed that the method was suitable for analysis in biological fluids, they did not address the problem of interference by biological compounds. Additionally, the utilization of a system which is neither isocratic nor isothermal would not be particularly suitable for routine clinical use.

In the report of Ostroy and Gams [7], which appeared subsequent to the completion of our own work, a method is described which utilized solvent extraction of DHAD and isocratic separation using high-performance liquid chromatography (HPLC). The major criticisms of the method are (a) the authors' failure to consider the instability of DHAD in plasma in designing the final analytical method and (b) the relatively inefficient recovery of DHAD and imprecision observed as a result of the tedious procedure for extraction of the drug from biological fluids.

Since our own studies have unequivocally demonstrated that DHAD is rapidly degraded in plasma, the validity of the method of Ostroy and Gams [7] is in question.

The present paper describes a method which appears to be suitable for clinical analysis of DHAD in plasma. It involves stabilization of drug in the biological matrix, separation of drug from biological fluid by retention on XAD-2 beads, elution from the resin and subsequent HPLC analysis with spectrophotometric detection.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, a Model 710A Waters Intelligent Sample Processor (WISP) and a Model 440 Absorbance Detector. Peak areas were obtained mechanically with a Model 1810L10 Polar Planimeter (Dietzgen, Des

Plaines, IL, U.S.A.) and electronically with a Varian Assoc. (Palo Alto, CA, U.S.A.) Model 111-C Chromatography Data System (CDS 111-C) interfaced with the 10-V output of the Model 440 Absorbance Detector. The WISP initiated the CDS 111-C integration cycle upon automatic injection, and was programmed to make triplicate 100-μl injections (at slow syringe speed) with a 20-min run time per injection. The CDS 111-C was programmed for an initial peak width of 20 sec and a stop time of 18 min. For the developmental work, a Waters Assoc. Model U6K injector was used in place of the WISP and a Waters Assoc. Model 660 Solvent Programmer was utilized for mobile phase optimization. Separation was obtained with a Waters Assoc. μBondapak C_{18} column (30 cm × 3.9 mm I.D.; 10 μm particle size) and column eluent was monitored at 254 nm at a sensitivity of 1.0 a.u.f.s.

Materials

DHAD was supplied as the dihydrochloride salt by the National Cancer Institute and was used as obtained. The sodium salts of butane-, pentane-, hexane- and octanesulfonic acid (Eastman Kodak, Rochester, NY, U.S.A.) and ammonium dihydrogen phosphate AR (Matheson Coleman and Bell, Norwood, OH, U.S.A.) were used as obtained. Methanol (Fisher, Springfield, NJ, U.S.A.) and 2-propanol (J.T. Baker, Phillipsburg, NJ, U.S.A.) were HPLC grade. Amberlite XAD-2 resin (20—50 mesh) (Rohm and Haas, Philadelphia, PA, U.S.A.) was reduced to 100—200 mesh by dry milling the resin in a Micro Mill (Lab Apparatus, Cleveland, OH, U.S.A.). The pulverized resin was wet sieved with acetone through 100- and 200-mesh sieves (Fisher). The 100—200-mesh fraction was collected and thoroughly washed with acetone to exclude particles smaller than 200 mesh.

All water was distilled in glass following mixed bed deionization. Disposable glass transfer pipets (9 in.) (Rochester Scientific, Rochester, NY, U.S.A.) and glass wool (Corning Glass Works, Corning, NY, U.S.A.) were used as received. All other chemicals were reagent grade and used without further purification. The plasma used (Community Blood Center, Kansas City, MO, U.S.A.) was "recovered human plasma" containing citrate—phosphate—dextrose anticoagulant and was stored at 4°C.

Separation of DHAD from plasma

Disposable columns were prepared by slurry packing 9-in. disposable pipets (containing a small plug of glass wool in the tip) with 150 mg of 100—200-mesh XAD-2 beads suspended in 3—4 ml of methanol. After the bed settled, a second glass wool plug was introduced at the top of the bed to maintain its integrity upon application of aqueous samples. Columns were then sequentially washed with 4—5 column volumes of methanol, distilled water, and 0.05 *M* phosphate buffer (pH 7.4), and stored in this buffer until used.

Freshly prepared plasma samples containing DHAD were stabilized [adjusted to pH 5.3 and made 0.5% (w/v) in ascorbic acid] by the immediate addition of 500 μl of a 5% ascorbic acid solution [prepared in 0.1 *M* citrate buffer (pH 3.0)] to 5-ml plasma samples immediately after the samples were withdrawn. Duplicate 2-ml portions of these samples were then applied directly to the XAD-2 columns and washed with 5-ml portions each of 0.05 *M* ammonium dihydrogen

phosphate (pH 2.7) solution. DHAD was subsequently eluted from the column with 1.75 ml of 2-propanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (30:70) into a 2-ml volumetric flask and adjusted to volume with the elution solvent.

Chromatography

The eluate from the XAD-2 column containing DHAD was chromatographed as octanesulfonate ion pairs on an octadecylsilane bonded phase column. The chromatographic system was optimized by systematically varying temperature, hetaeron and methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) composition. The optimum isocratic system for the resolution and quantitation of DHAD in plasma samples was found to consist of methanol—buffer (45:55) containing 6 m*M* sodium octanesulfonate. The flow-rate was maintained at 2.0 ml/min and the column was thermostatted by immersion in a water bath maintained at 49 ± 1°C. All analyses were performed with 100-μl injection volumes.

Quantitative analysis

The concentration of DHAD in a sample was quantitated by comparison of the mean peak area of triplicate injections (100 μl) of the column eluent with a calibration curve prepared in the same manner using samples containing known concentrations of DHAD in plasma. The calibration curve was prepared by the analysis of duplicate samples of DHAD in plasma at each of eight concentrations ranging from 75 to 3000 ng/ml. Samples were prepared by the addition of 0.375—15 μg of DHAD dissolved in 500 μl of 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) to sufficient plasma to give a final volume of 5 ml. Ascorbic acid solution (500 μl) was then added and 2-ml aliquots of the final solution were carried through the analysis sequence described above. A calibration curve of DHAD in mobile phase was prepared simultaneously by spiking mobile phase with DHAD (as described for the plasma standard curve) and assaying directly by HPLC. Peak areas were calculated directly with the CDS-111C and measured with a polar planimeter to correlate electronic integration units with peak areas in mm^2.

Stability studies

The stability of DHAD in plasma and plasma adjusted to pH 5.3 [containing 0.5% (w/v) ascorbic acid] at a concentration of 1000 ng/ml was investigated at ambient (ca. 25°C), refrigerator (4°C) and freezer (−17°C) temperatures. Samples stored at these temperatures were assayed for DHAD content at regular time intervals.

RESULTS AND DISCUSSION

The plasma analysis of DHAD consists of four distinct, yet interrelated phases: (a) stabilization of the drug in the sample; (b) separation of drug from the biological matrix; (c) high-efficiency chromatographic separation of drug from potential contaminants; and (d) detection of the drug in the column

effluent with sufficient sensitivity to monitor patient plasma levels after therapeutic dosing at 12 mg/m^2 (ca. 330 μg/kg body weight).

Separation of DHAD from plasma or aqueous buffer

Initial attempts to remove DHAD from aqueous buffer solutions (pH 7.4) by direct extraction met with limited success. Extraction efficiency was better using proton-donating solvents (e.g., chloroform, dichloromethane) than proton-accepting solvents (e.g., diethyl ether, ethyl acetate) and could be further improved by the addition of a second proton-donating species (1-pentanol) to the extractant. However, such extractions were very nonspecific and incomplete, and furthermore, DHAD was shown to have limited stability in plasma (at pH 7.4). DHAD could be quantitatively extracted from pH 4.0 buffer into 1-pentanol as a heptafluorobutyrate ion aggregate. (The stoichiometry of this ion aggregate has not yet been elucidated.) Extending this procedure to plasma, however, necessitated the introduction of a back-extraction of the ion aggregate into 0.05 *M* sulphuric acid to minimize interferences due to coextraction. As a result, in plasma, the overall recovery dropped to 72% and the method was found to be relatively inaccurate (± 11%) and imprecise (coefficient of variance ± 8%). These values are in agreement with the results of Ostroy and Gams [7] for chloroform extraction of DHAD from basic plasma after protein denaturation. Recoveries of 60—80% were reported, and the assay was determined to be reproducible within 10% [7]. In the present study, ion-pair extraction was judged impractical for routine clinical analysis because (1) of the incomplete recovery of DHAD from plasma, (2) the high polarity requirement of the extraction solvent results in non-selective removal of drug with co-extraction of plasma-derived substances, (3) the extent of sample handling leads to long analysis times and increased probability for errors and (4) the noxious odor of 1-pentanol necessitates extractions be carried out in a fume hood (an inconvenience in clinical settings). Therefore, other methods for the removal of DHAD from plasma were sought.

An alternative approach to separation of DHAD from plasma which proved more acceptable involved adsorption of the drug onto beads of the non-ionic resin, Amberlite XAD-2, packed in a short disposable column. The commercially-distributed material is only available in a 20—50-mesh size bead and 500 mg of the dry resin packed in a short cartridge failed to retain a 2-μg load of DHAD. However, reduction of particle size to 100—200 mesh provided an effective column packing for retention of DHAD present in plasma. A disposable column packed with 150 mg of small-particle XAD-2 resin completely retained up to 6 μg of DHAD present in a 2-ml plasma sample.

Plasma samples, containing DHAD, were adjusted to pH 5.3 ± 0.2 and made 0.5% (w/v) in ascorbate (to improve drug stability) and 2 ml of sample was gravity-fed through the XAD-2 column. DHAD was retained by the resin under these conditions and subsequently was eluted with a mixed organic solvent. To minimize co-elution of substances that may pose potential interferences to subsequent steps in the analysis sequence, the column was exhaustively washed with buffer prior to elution of drug. A solvent with minimum elution strength to displace 2 μg of DHAD from 150 mg of XAD-2 resin was systematically sought to further minimize contamination. An acidic eluent [0.05 *M* am-

monium dihydrogen phosphate (pH 2.7)] was chosen to present the amine functions in an ionized state, which would result both in minimizing the attraction of the drug for the hydrophobic surface of the resin and in stabilizing the drug against pH-dependent oxidative degradation. Elution strength was increased by the addition of 2-propanol to the buffer. Complete elution and recovery of the drug was achieved with minimum contamination of the sample using 1.75 ml of eluent at a composition of 30% 2-propanol in 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7). Higher concentrations of 2-propanol were less suitable since they caused co-elution of contaminants from the resin and adversely affected the shape and resolution of the HPLC bands due to the resulting increase in the strength of the injection solvent.

Chromatography

Separation of DHAD from plasma contaminants, chemical impurities and degradation products, which co-eluted from the XAD-2 columns, was achieved by isocratic reversed-phase paired-ion chromatography on an RP-18 column.

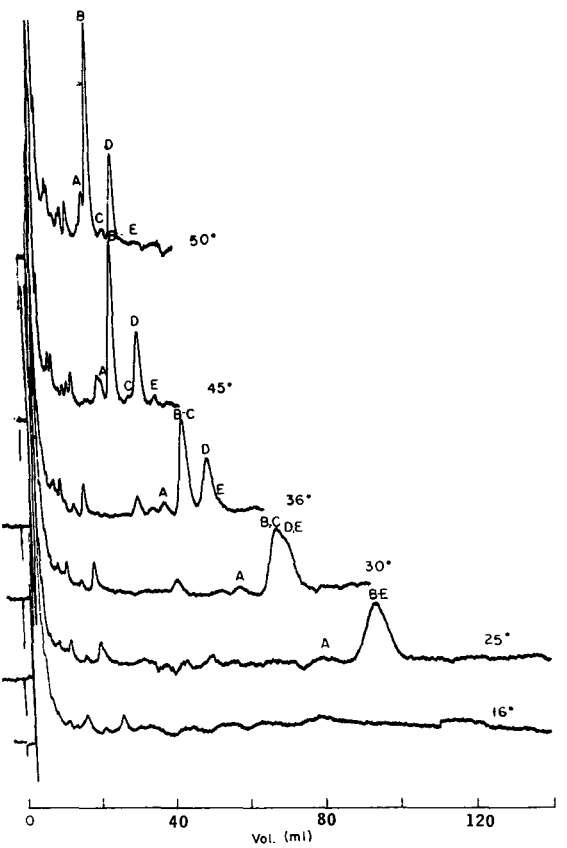


Fig. 1. Chromatograms of DHAD species obtained from a plasma sample carried through the analysis sequence using a mobile phase of 6 m*M* sodium octanesulfonate in methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45 : 55). A and C are impurities, B is DHAD and D and E are chemical degradation products of the parent drug.

Temperature effects

When chromatography was carried out at ambient temperature (25 ± 1°C), resolution was unsatisfactory using a variety of stationary phases (μ-CN, RP-18, RP-8), mobile phases (containing methanol, tetrahydrofuran, 2-propanol or acetonitrile as organic modifier), buffers of differing pH and ion-pairing agents (C_4—C_8 sulfonates). Day-to-day variations in room temperature appeared to have an effect on the quality of separation, prompting an investigation of temperature effects on chromatographic selectivity of the paired-ion system. An RP-18 column and a mobile phase of methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45:55) made 6 m*M* in sodium octanesulfonate was used to isolate DHAD from a plasma sample that had been previously carried through XAD-2 column clean-up. An apparent single peak (B—E), preceded by a shoulder (A) (Fig. 1) was observed at ambient temperature (25°C). As shown in Figs. 1 and 2, an increase in temperature resulted in an improvement in resolution as evidenced by the emergence of peaks C, D and E from under the DHAD peak (B).

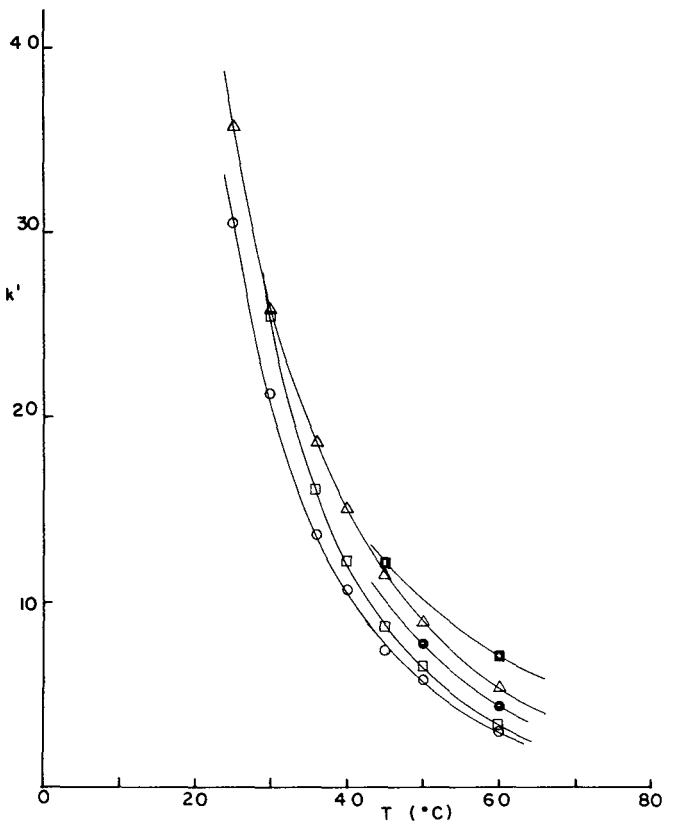


Fig. 2. Capacity factor (*k'*) for DHAD species vs. temperature for separation of DHAD (□), impurities A (○) and C (●), and degradation products D (△) and E (■) on an RP-18 column using methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45:55) containing 6 m*M* sodium octanesulfonate as mobile phase.

The dependency of capacity factor (k') on temperature is given by

$$k'_A = \Phi \exp(-\Delta H^0_A/RT) \exp(\Delta S^0_A/R) \quad (1)$$

where Φ is the phase ratio of the column and ΔH^0_A and ΔS^0_A are the enthalpy and entropy of transfer of solute A between chromatographic phases, respectively. In the present case, as for the partitioning of other ion aggregates [8], the distribution process appears to be enthalpically-controlled. The observed decrease in k' with increasing temperature (Figs. 1 and 2) indicates that the partitioning process for all five analytes (A—E) is exothermic. The magnitude of the enthalpy of transfer for the individual components is different as determined from Van 't Hoff plots (ln k' vs. the reciprocal of the absolute temperature) prepared from data for compounds A—E (Fig. 3). Thus temperature changes would be expected to affect k' for each analyte differently, providing a means for modifying selectivity, as exhibited in Figs. 1 and 2. Although similar temperature effects have been observed in reversed-phase chromatography [6,9—11] such behavior has not been described for reversed-phase paired-ion HPLC.

Temperature elevation also decreases the viscosity of the mobile phase and minimizes mass transfer effects in both stationary and mobile phases [12]. These effects are reflected in an increased plate count (N; Fig. 4) being observed with increased temperature. The dramatic increase is, in part, artifactual since it also represents the resolution of two or more superimposed bands. The decrease in chromatographic efficiency observed above 50°C can probably be attributed to (a) a loss in the effective number of theoretical plates (N_{eff}) caused by the decrease in k' with increasing temperature as described by eqn. 2 [13] and (b) extracolumn effects which become pronounced at $k' \leqslant 2$ [13]

$$N_{eff} = N\left(\frac{k'}{1+k'}\right)^2 \quad (2)$$

Temperature increases resulted in an increase in peak skewness, defined by the asymmetry factors of 1.2 at 25°C, 1.7 at 50°C, and 2.1 at 60°C. Thus, the change in peak shape that occurs with changes in temperature does not account for the loss in apparent plate count at higher temperatures. Optimum resolution and chromatographic efficiency was achieved at a temperature of 49 ± 1°C which was used for subsequent development of chromatographic separations.

Choice of ion-pairing agent

The k' values for the three DHAD-derived species and the two impurities increased with increasing length of the carbon chain of the alkylsulfonate hetaeron (Fig. 5 a—d) and with hetaeron concentration (Fig. 6). Increasing the chain length of the counter ion increased k' by increasing the activity coefficient of the hetaeron in the mobile phase, thus favoring the partitioning of the ion pair into the stationary phase. Neither butane-, pentane- nor hexanesulfonate (Fig. 5 a—c) offered resolution of the five species (A—E) within an acceptable range of k' values ($1 < k' < 10$) due to the large difference in partitioning behavior for peaks A—C and D—E. As the carbon chain length of the hetaeron was increased, k' for all five components could be made more similar

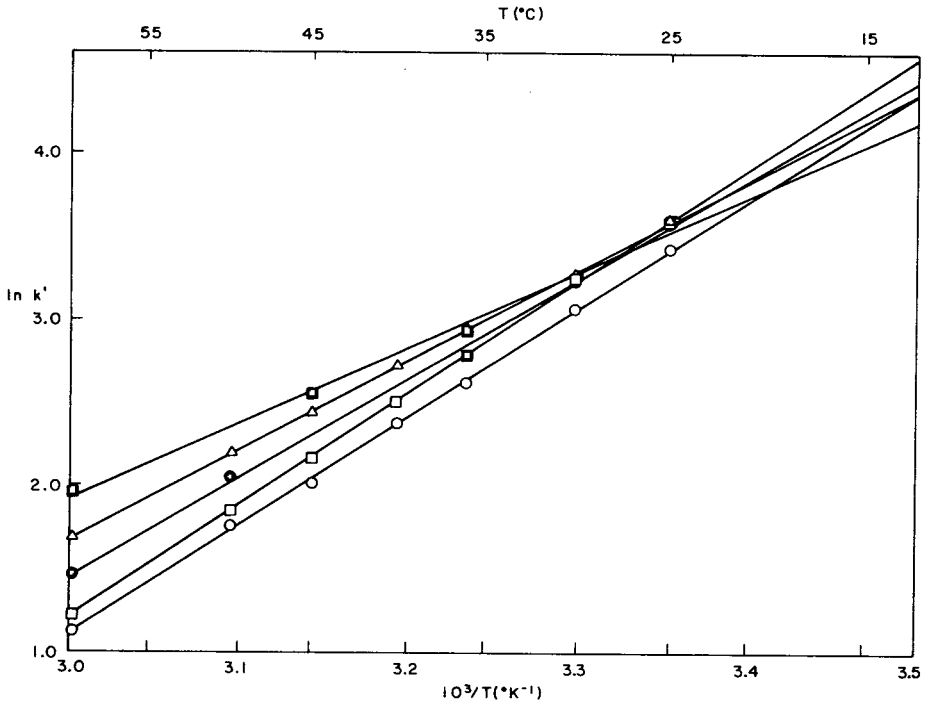


Fig. 3. Van 't Hoff plot for DHAD (□); impurities A (○) and C (●); and degradation products D (△) and E (■). Mobile phase: methanol—0.05 *M* $NH_4H_2PO_4$ (pH 2.70) (45:55) + 6 m*M* sodium octanesulfonate; flow-rate: 2.0 ml/min.

(while still maintaining peak resolution) by carrying out the separation at a higher concentration of organic modifier. Octanesulfonic acid provided acceptable resolution of all desired components (Fig. 5d).

The capacity factor of the ion pair is related to its partition coefficient, K_p, according to eqn. 3 [13]

$$k' = \frac{V_s}{V_m} K_p [B^-]^x_{mp} \quad (3)$$

where V_s and V_m are the volumes of the stationary and mobile phases, respectively. Thus, k' should be proportional to the concentration of the hetaeron, $[B^-]$, in the mobile phase. Plots of log k' vs. log $[B^-]_{mp}$ were linear with slopes close to 1, indicating the stoichiometry of DHAD to hetaeron in the ion aggregate was 1:1. As the octanesulfonate concentration was increased from 3 to 9 m*M* (Fig. 6), the k' value for DHAD increased at equivalent methanol concentrations. However, at higher hetaeron concentrations, resolution of neighboring bands decreased, apparently due to increasing similarity in the extent of ion-pairing of the different species. As the concentration of hetaeron increased, k' for DHAD increased faster than k' for the major degradation peak (D), thus providing an additional source of separation selectivity.

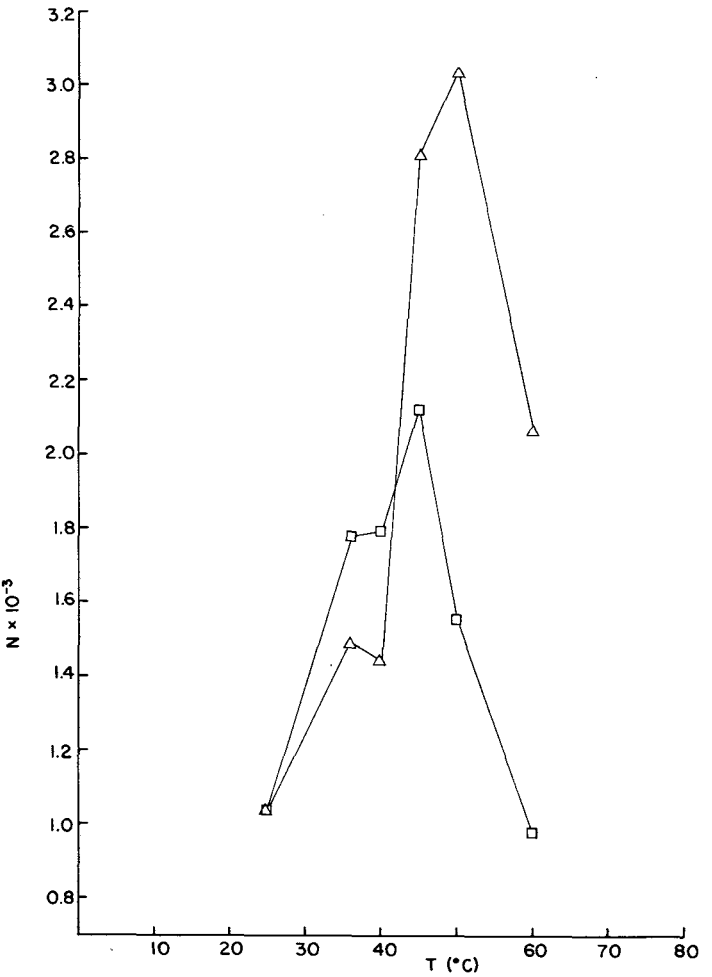


Fig. 4. Effect of temperature on plate number (N) calculated for DHAD (□) and its major degradation product, D (△).

Chromatographic results

Maximum resolution of components coupled with minimum analysis time was achieved using a mobile phase of methanol—0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) (45:55) made 6 m*M* in sodium octanesulfonate. A chromatogram of a plasma sample containing 500 ng of DHAD per ml that had been carried through the analysis sequence is shown in Fig. 7 and contrasted with a plasma blank, carried through the analysis scheme but containing no drug. No contamination is seen in the region in which DHAD elutes. Under the chromatographic conditions stated, DHAD elutes with a capacity factor (k') of 6.4 (V_R = 16.8 ml). The analytical column was calculated to possess ca. 2125 plates (N) (reduced plate height, h = 14.1). However, the peak asymmetry factor for the DHAD peak is ca. 1.7 suggesting that a significant positive error in plate count may have been produced in calculation due to use of the simplified expression, $N = 16\ (t_r/t_w)^2$ [13]. Peaks A (k' = 5.8) and C (k' = 7.7) are apparent

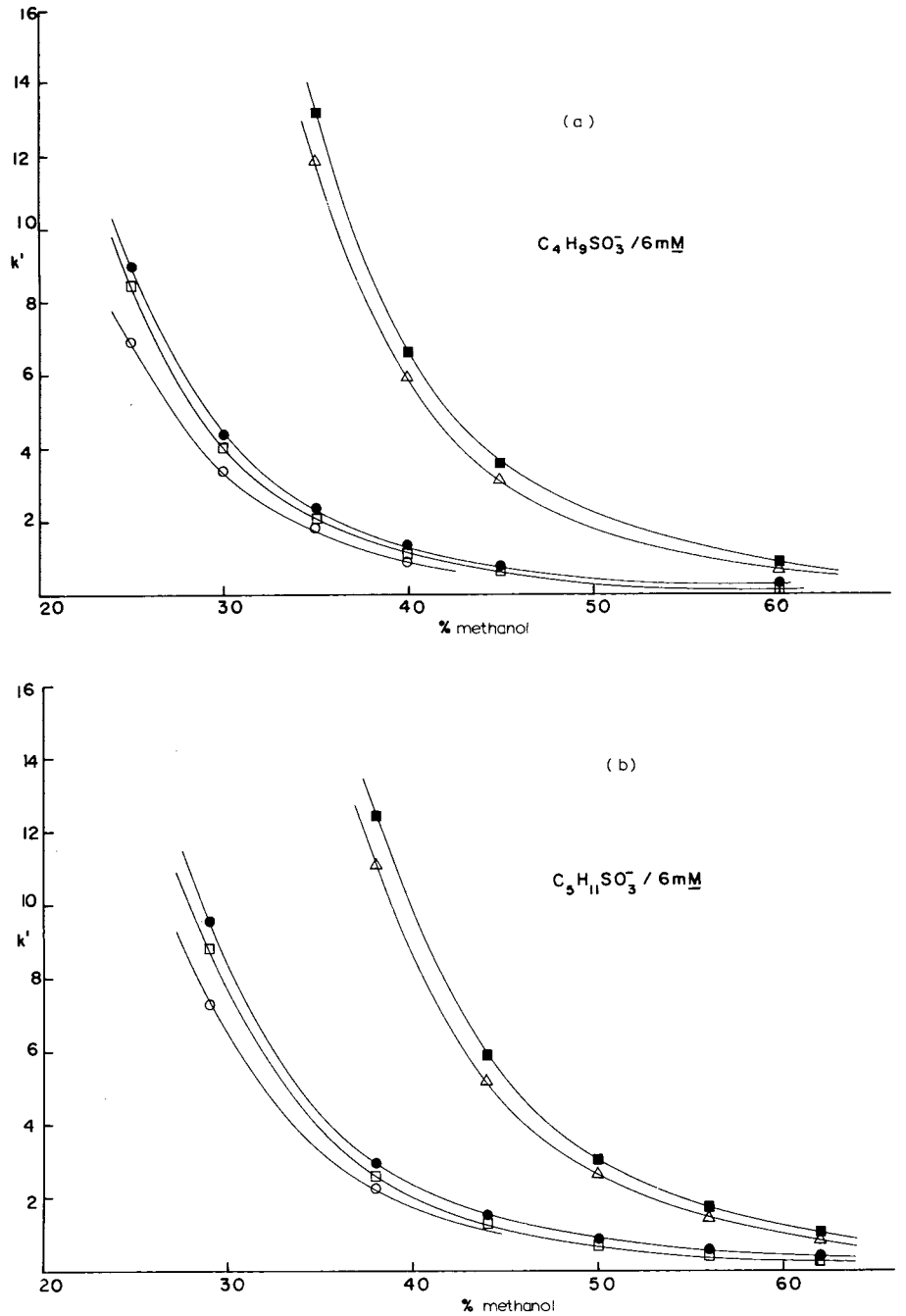
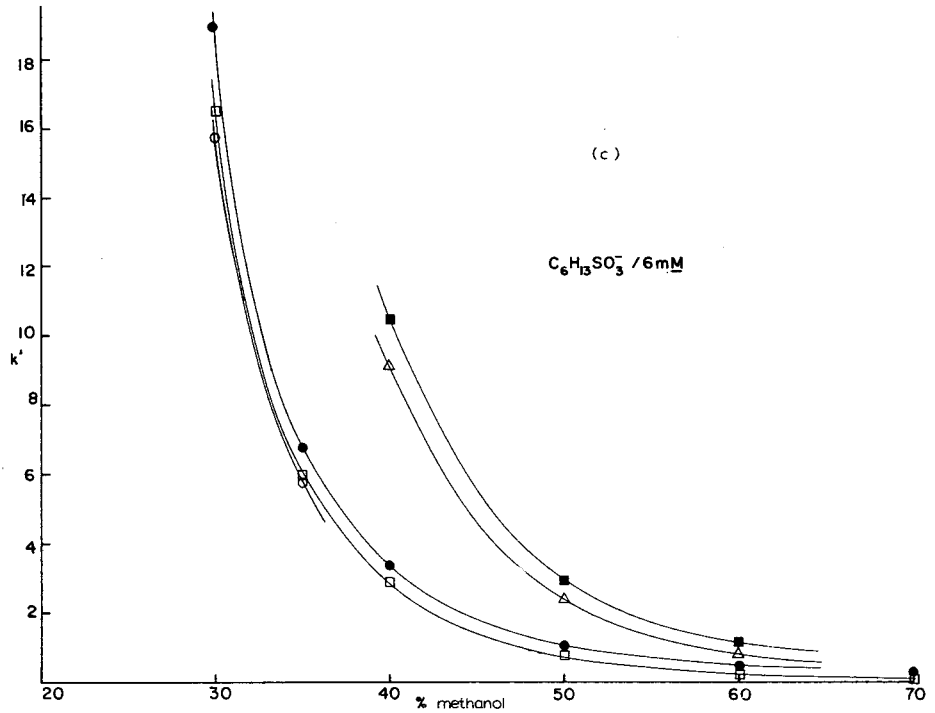


Fig. 5. Capacity factor (k') for impurities A (○) and C (●), DHAD (□), and its degradation products D (△) and E (■) vs. methanol concentration in mobile phase of 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) and 6 m*M* sodium alkanesulfonate. (a) Sodium butanesulfonate; (b) sodium pentanesulfonate; (c) sodium hexanesulfonate; (d) sodium octanesulfonate. Column thermostatted at 49 ± 1°C.



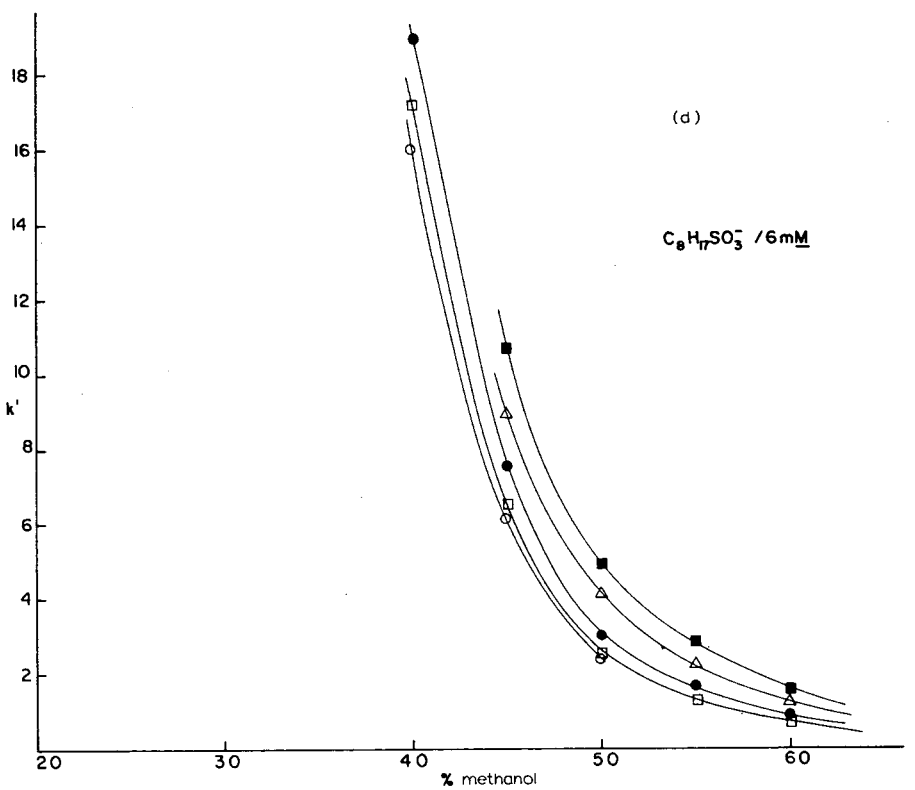


Fig. 5 (continued).

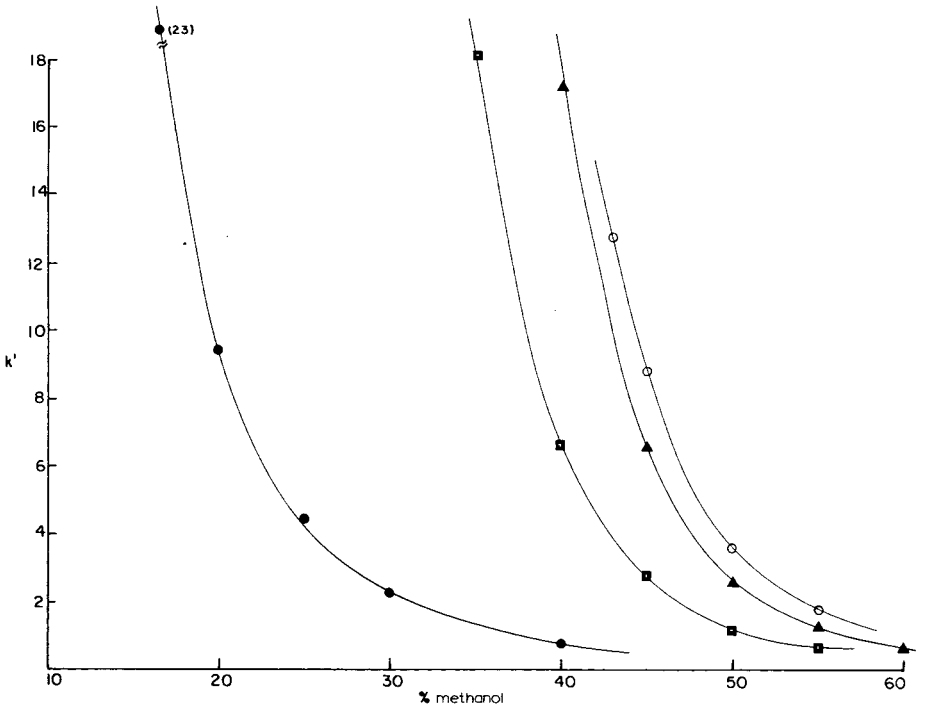


Fig. 6. Capacity factor (k') for DHAD vs. methanol concentration in mobile phase of 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) containing sodium octanesulfonate at concentrations of 0 (•), 3 (▪), 6 (▲) and 9 (○) m*M*. System thermostatted at 49 ± 1°C.

impurities in the drug sample. They are present immediately after the drug is dissolved in buffer or plasma and do not appear to change in intensity as a function of time. Components D (k' = 8.9) and E (k' = 10.2) were not initially present and their concentrations increase with time, concomitant with a decrease in the amount of DHAD present. Consequently, it appears that they are products of chemical degradation of DHAD.

Quantitative analysis

DHAD was quantitated in plasma by comparison of computer-calculated peak areas for the analyte to a standard curve constructed from the analysis of plasma samples containing known amounts of drug. The area of the DHAD peak was linearly related to DHAD concentration for 8 concentrations of drug in the range 75—3000 ng of DHAD per ml of plasma. Linearity of response was determined by least squares analysis of data points, and is described by the line $y = 4347\ x - 103890$ (correlation coefficient > 0.999), with the line crossing the abscissa at 24 ng/ml. A similar curve was prepared for DHAD in the HPLC mobile phase, yielding a line described by $y = 4583\ x - 11198$ (correlation coefficient > 0.999), with the line crossing the abscissa at 2.4 ng/ml. Absolute recovery of DHAD from plasma was evaluated by comparing the slopes of these two lines, and indicated an overall recovery of ca. 95%. Peak areas in mm^2 were measured via polar planimetry at a chart speed of 0.2 in./min and yielded lines

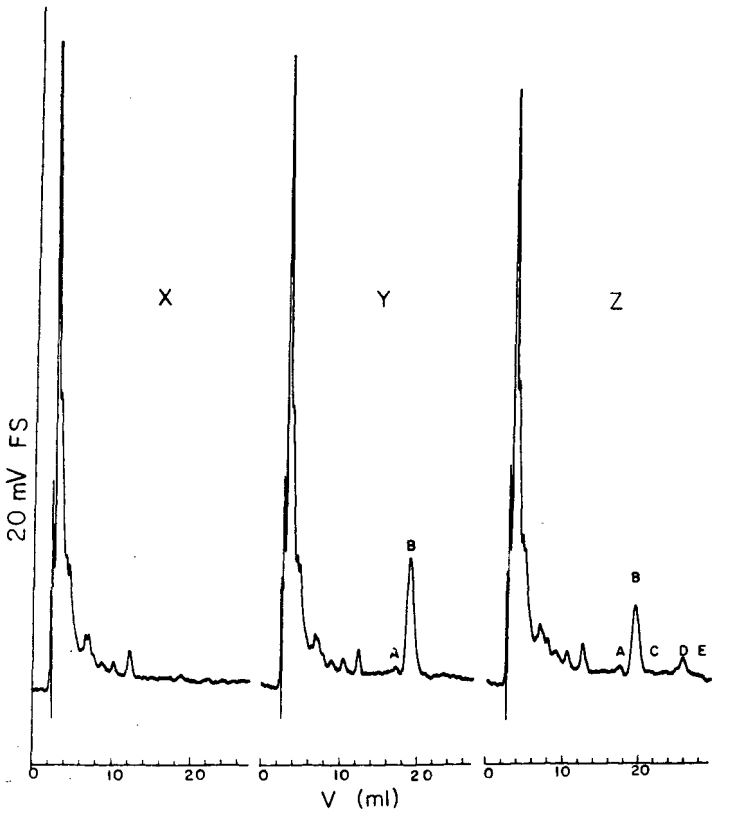


Fig. 7. Chromatogram of DHAD from plasma. Tracing X represents a blank, i.e. biological fluid not containing drug but carried through the analysis sequence; tracing Y is the result obtained from work-up of a fresh plasma sample containing 500 ng of DHAD per ml (50 ng drug placed on column). Tracing Z differs from Y only in that the sample had aged for 3 h at 37°C before work-up. Peaks A and C are impurities in the drug; B is DHAD and D and E are chemical degradation products of the parent drug. Separation was carried out on an RP-18 bonded phase column with 6 m*M* sodium octanesulfonate in methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45 : 55) as mobile phase. Flow-rate: 2 ml/min. System thermostatted at 49 ± 1°C.

of $y = 0.301\,x - 7.186$ and $y = 0.317\,x - 0.751$ for the curves from plasma and mobile phase, respectively. Detection limits were approximately 75 ng/ml plasma (ca. 7.5 ng drug applied to the column in 100-μl injection volume) at the 3σ level, as determined by the analysis of plasma samples supplemented with drug at these levels. Plasma samples at the 75 ng/ml level could be analyzed with a precision of ± 10%, whereas samples at 3000 ng/ml could be analyzed with ± 2% precision.

Stability studies

At room temperature (25°C), DHAD was found to be unstable in aqueous solution, degrading in an apparent first-order manner with $t_{\frac{1}{2}} \approx 130$ h in 0.05 *M* phosphate buffer (pH 7.4) and $t_{\frac{1}{2}} \approx 24$ h under similar conditions in

plasma. The loss of drug could be reduced by storing samples at lower temperatures. When refrigerated (4°C), the $t_{\frac{1}{2}}$ of DHAD was extended to 6 days (60% loss in one week) and at freezer temperature (—17°C), 13% of drug was lost in one week ($t_{\frac{1}{2}} \approx 36$ days). This instability makes proper storage conditions imparative to prevent drug loss from the sample prior to analysis. Degradation probably involves oxidation of the phenylenediamine moiety to the corresponding quinoneimine which is subject to hydrolysis to yield the quinone [14]. Since such reactions are normally pH-dependent [15], it was felt that stability could be enhanced by decreasing pH. In plasma adjusted to pH 5.3, the $t_{\frac{1}{2}}$ for loss of DHAD at room temperature increased by a factor of 2.5 (to ca. 60 h) relative to that in plasma at pH 7.4. Further reduction of pH was not practical since at pH < 5.3, precipitation of the biological material occurred, seriously complicating homogenous sampling of the plasma.

Since degradation was presumed to involve oxidation, the use of antioxidants to further stabilize DHAD in plasma samples was studied. The first candidate, sodium bisulfite (2.6%, w/v) rapidly reacted ($t_{\frac{1}{2}} \approx 20$ min) with DHAD in phosphate buffer (pH 7.4) and was, therefore, unsuitable. Ascorbic acid proved to be a much more useful reagent, but due to its instability at neutral pH [16], acidification of plasma samples concurrent with the addition of ascorbate was necessary. In plasma adjusted to pH 5.3, the addition of 0.5% (w/v) ascorbate stabilized the system and resulted in less than 1% loss of DHAD in 48 h at room temperature. At refrigerator temperatures (ca. 4°C), less than 4% loss of DHAD was observed in one week in these same plasma samples. To determine whether or not ascorbate could reduce the oxidized product of DHAD back to parent drug, thereby introducing potential positive deviations in analysis, ascorbate was added to a plasma sample (pH 5.3) containing DHAD which had been allowed to degrade to 50% of its initial concentration. Over a 24-h period at room temperature, ascorbate failed to regenerate significant quantities of DHAD, but did prevent its further degradation thus demonstrating its effectiveness as a stabilizer for DHAD in plasma. In view of these results, it is clear that in all clinical studies, freshly obtained plasma samples should be immediately stabilized through pH adjustment and addition of ascorbic acid.

In summary, a rapid clinical method is described for monitoring the anticancer agent DHAD in plasma. The drug was separated from plasma constituents by its retention on a disposable glass column packed with XAD-2 beads. Subsequent elution followed by isothermal and isocratic HPLC analysis of the eluent from the XAD-2 column (with spectrophotometric detection) provided a sensitive and specific means for measuring DHAD at levels $\geqslant 75$ ng/ml in plasma with a precision of ± 10%. Although DHAD is unstable in biological media, a procedure for stabilization involving addition of ascorbic acid together with acidification provides adequate sample stability. In a previous report [7] of a clinical method for DHAD, the drug stability in plasma was not addressed and results obtained may be in question. Overall, the method presented in this paper appears to offer significant advantages over the works presented thus far in the literature [6,7] and may, therefore, be more applicable for the routine clinical analysis of DHAD.

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CHROMBIO. 731

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF NITROXOLINE IN PLASMA AND URINE

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of nitroxoline in 50-μl plasma and urine samples.

A structural analogue of nitroxoline, 8-hydroxyquinoline, was added to the eluent in order to suppress peak asymmetry. Several parameters of the eluent were studied for the optimisation of the chromatographic system.

Plasma concentration—time curves were constructed for three volunteers after they had received an oral dose of 100 mg of nitroxoline. Plasma half-life was about 1 h. Within 12 h, about 1% of the dose was excreted in the urine as free nitroxoline and about 30% as conjugated metabolite of the parent compound.

INTRODUCTION

Nitroxoline (8-hydroxy-5-nitroquinoline) is used in the treatment of urinary tract infections of Gram-negative and Gram-positive microorganisms.

In the literature only ultraviolet—visible spectrophotometric determinations of nitroxoline in plasma [1] and in urine [1, 2] have been reported. Pharmacokinetic data of nitroxoline in man are scarce. Recently some data have been published concerning nitroxoline levels in urine, of both the conjugated and the unconjugated drug [2].

The aim of this study was to develop a high-performance liquid chromatographic (HPLC) method for the analysis of nitroxoline in plasma and urine, of sufficient sensitivity to allow the analysis of small plasma samples collected from finger-pricks. The usefulness of the method was tested by analyzing plasma and urine samples of three volunteers who had received an oral dose of nitroxoline.

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EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system, Model U6K injector and Model 440 absorbance detector operated at 436 nm.

A μBondapak C_{18} column (Waters Assoc.), 30 cm × 3.9 mm I.D., particle size 10 μm, was used.

Chemicals

Nitroxoline was a kind gift from Roussel Laboratories B.V. (Hoevelaken, The Netherlands).

Chloroform, methanol, 8-hydroxyquinoline, disodium phosphate and picric acid (all of "pro analysis" grade) were purchased from Merck (Darmstadt, G.F.R.).

Extraction of plasma and urine

To 50 μl of plasma (or urine) in a 7-ml centrifuge tube were added 15 μl (or 4 μl) of 4 *M* hydrochloric acid. The resulting solution was extracted with 2 ml of a solution of the internal standard in chloroform (picric acid, 5 μg ml^{-1}). After mixing on a Vortex mixer for 30 sec and subsequent centrifugation at 2500 *g* for 2 min, the chloroform phase was transferred to another centrifuge tube. The chloroform layer was evaporated to dryness under a stream of nitrogen at 30° C. The residue was reconstituted in 50 μl of eluent by mixing on a Vortex mixer for 15 sec; 20 μl of this solution were injected into the HPLC system.

Chromatography

The eluent consisted of 8-hydroxyquinoline (0.1%, w/w), aqueous phosphate buffer (0.2 *M*, pH 7.4); 8%, w/w), methanol (35%, w/w) and water. The flow-rate was 1.5 ml min^{-1}. Chromatography was performed at ambient temperature.

In vivo studies

Three volunteers received an oral dose of 100 mg of nitroxoline each. Blood samples were collected by finger-prick into 1-ml plastic vials containing heparin sodium after 1, 2, 3, 4, 6, 8, 12, and 24 h. The samples were spun down at 2500 *g*; the plasma was collected and stored at −20° C prior to analysis. Conjugated nitroxoline in urine was determined after refluxing the samples for 1 h at pH 0.5. The samples were then extracted and analyzed as described above.

RESULTS AND DISCUSSIONS

With some frequently used chromatographic systems — reversed-phase, ion-exchange and dynamic ion-exchange — strongly tailing peaks of nitroxoline were observed. Typical chromatograms are presented in Fig. 1a—c. This tailing is possibly caused by a strong interaction of nitroxoline with the free silanol

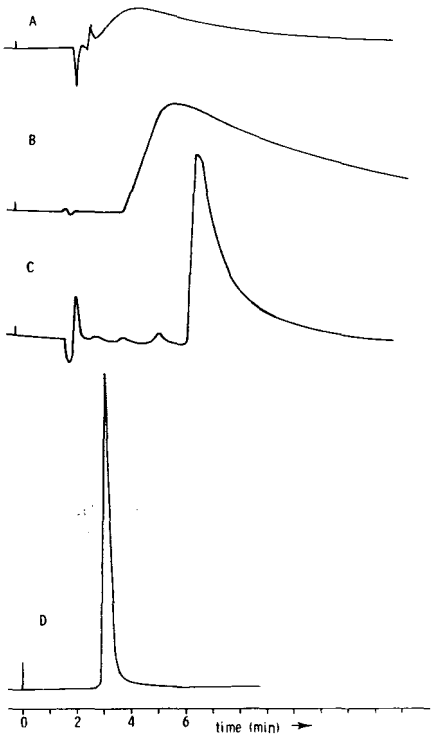

Fig. 1. Chromatograms of nitroxoline in different chromatographic systems. (A) Strong anion exchange. Column Partisil SAX, 25 cm × 4.6 mm I.D.; particle size 10 μm (Whatman, Clifton, NJ, U.S.A.);.eluent, aqueous phosphate buffer (pH 7.2, 0.05 *M*) 75% (w/w), methanol 25% (w/w). (B) Dynamic ion exchange. Column, μBondapak C_{18}; eluent, cetrimide 0.125% (w/w), aqueous phosphate buffer (pH 7.7, 0.2 *M*) 4% (w/w), methanol 62.5% (w/w) and water. (C) Dynamic ion exchange. Column, μBondapak C_{18}; eluent, cetrimide 0.1% (w/w), 1,2-diaminocyclohexane-N,N,N¹,N¹-tetraacetic acid (Titriplex IV, Merck) 0.1% (w/w), aqueous phosphate buffer (pH 7.4, 0.2 *M*) 7.3% (w/w), methanol 55% (w/w) and water. (D) Column, μBondapak C_{18}; eluent, 8-hydroxyquinoline 0.1% (w/w), aqueous phosphate buffer (pH 7.4, 0.2 *M*) 8% (w/w), methanol 50% (w/w) and water.

groups of the column packing material. This tailing was suppressed by the addition of 8-hydroxyquinoline, a structural analogue of nitroxoline (Fig. 1d). Another cause for the strongly tailing peaks could be the presence of trace elements, which are well-known impurities in silica gel [3]. When Titriplex IV, a complexing agent for metal ions, was added to the eluent in the dynamic ion-exchange system, a significant decrease of tailing was observed (Fig. 1b and c). It is therefore possible that the complex-forming properties of 8-hydroxyquinoline are at least partly responsible for the improved symmetry of the nitroxoline peaks.

The composition of the eluent — pH, ionic strength and 8-hydroxyquinoline concentration — was optimized by considering peak symmetry. For the determination of nitroxoline a phosphate concentration of 16 mmol was chosen, because at this concentration the curve of phosphate concentration versus peak symmetry is almost flat (Fig. 2). At higher concentrations the risk of precipitation is evident. Fig. 3 shows the effect of the pH of the phosphate

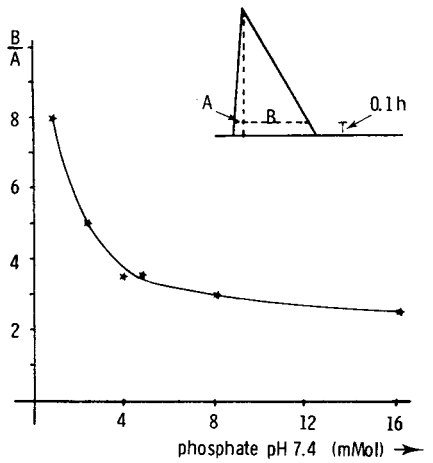

Fig. 2. Influence of the phosphate concentration in the eluent on the peak symmetry of nitroxoline. Eluent: 8-hydroxyquinoline 0.1% (w/w), methanol 35% (w/w) and water.

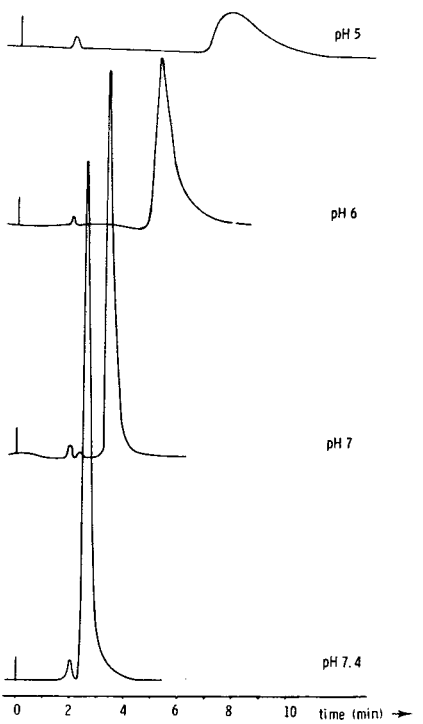

Fig. 3. Influence of the pH of the aqueous phosphate buffer on the chromatographic behaviour of nitroxoline. Eluent: 8-hydroxyquinoline 0.1% (w/w), aqueous phosphate buffer (0.2 *M*) 8% (w/w), methanol 35% (w/w) and water.

buffer on the chromatographic behaviour of nitroxoline. For the determination pH 7.4 was chosen, because at this pH the peak symmetry is acceptable and the detector response at 436 nm is close to the maximum response of the anionic form of the drug. At higher pH values the column life is the limiting factor.

A concentration of 0.1% 8-hydroxyquinoline in the eluent was sufficient to reduce the tailing to a minimum (results not shown). The concentration of methanol in the eluent did not affect the peak symmetry, so this parameter was used to optimize the analysis time. A concentration of 35% (w/w) methanol was chosen for the determination. The chromatographic analysis was performed within 5 min at this methanol concentration. The best day-to-day reproducible chromatograms were obtained when, at the end of each day, the column was washed with methanol containing 1% glacial acetic acid.

A very simple method for the determination of nitroxoline in plasma would be the direct injection of the plasma supernatant after precipitation of the proteins with acetonitrile. However, chromatograms obtained in this way showed a peak at the dead time and a negative peak after about 12 min (k' = 6.3). Apparently acetonitrile stripped some of the 8-hydroxyquinoline off the column; the negative peak is then caused by re-loading of the column resulting in a temporary decrease in the concentration of 8-hydroxyquinoline in the eluent. This effect could not be suppressed by adding 8-hydroxyquinoline to the acetontrile. Although these peaks did not interfere with the nitroxoline and internal standard peaks in the chromatogram, the analysis time was much prolonged. Moreover, the nitroxoline peaks showed more tailing in the presence of the acetonitrile. Obviously, the chromatographic system is disturbed by the addition of acetonitrile. It was therefore decided to include an extraction step in the procedure, which created the possibility of reconstituting the nitroxoline in the eluent after evaporation of the organic layer.

Picric acid was chosen as the internal standard. The stability of this compound under the preceding conditions was studied; no decomposition or loss of picric acid was observed, not even when after the evaporation of the chloroform layer the residue was heated for 2 h at 40°C. Fig. 4a shows the absolute recovery of nitroxoline and picric acid from serum after the addition of different volumes of 4 *M* hydrochloric acid. In Fig. 4b the pH values of serum are shown as a function of the amount of added 4 *M* hydrochloric acid. The absolute recovery was found to be 95% for nitroxoline and 85% for picric acid, when 15 μl of 4 *M* hydrochloric acid were added to 50 μl of serum. With different batches of serum and plasma no significant differences in pH were found upon addition of hydrochloric acid.

Fig. 5 shows chromatograms obtained from the in vivo experiment. Following the procedure (50-μl samples, 20-μl injection volumes) the detection limit (signal-to-noise ratio is 3) was 80 ng ml^{-1} plasma. For the in vivo experiment two calibration curves (concentration range 0.0792—39.6 μg ml^{-1}) were constructed by analyzing serum or urine samples with varying amounts of nitroxoline. The results were, for serum, $y = 0.0412x + 0.0020$ ($r^2 = 0.9996$, $n = 14$) where y = peak height ratio of nitroxoline/internal standard and x = concentration of nitroxoline in μg ml^{-1}, and for urine $y = 0.0406x - 0.0093$ ($r^2 = 0.9998$, $n = 4$). The reproducibility of the method was examined at two

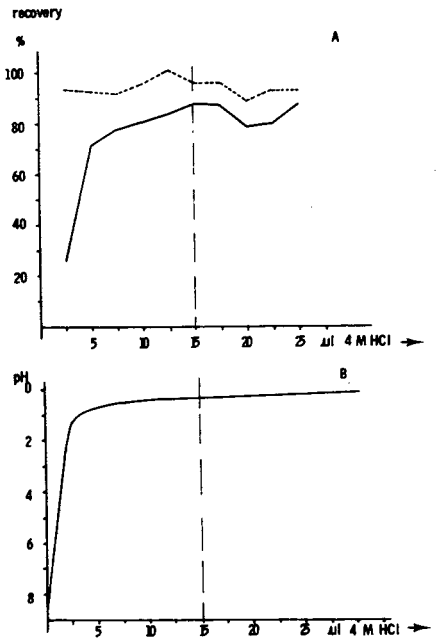

Fig. 4. (A) Recovery of nitroxoline (— — —) and picric acid (——) from 50 µl of serum, and (B) pH, both as a function of the added volume of 4 *M* hydrochloric acid.

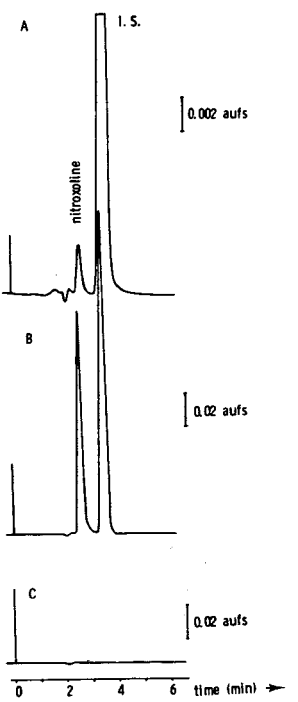

Fig. 5. Chromatograms obtained from (A) a volunteer plasma sample (0.4 µg ml^{-1} nitroxoline), (B) a spiked serum sample (15.8 µg ml^{-1} nitroxoline) and (C) a volunteer plasma blank. I.S. = internal standard.

concentrations, 0.2 μg ml^{-1} and 20 μg ml^{-1}, by analyzing eight serum samples at each concentration. The coefficients of variation were 7.6% at 0.2 μg ml^{-1} and 3.5% at 20 μg ml^{-1}.

Fig. 6 shows the plasma concentration—time curves of nitroxoline for three volunteers. The plasma half-life of nitroxoline was about 1 h. After 8—12 h the plasma concentration of nitroxoline was decreased to the detection limit; for a more exact kinetic study of the elimination phase the sensitivity of the determination must be increased. This can be achieved, for example, by using 200-μl plasma samples (also obtainable by finger-prick) and injection of a larger portion of the final solution into the chromatographic system.

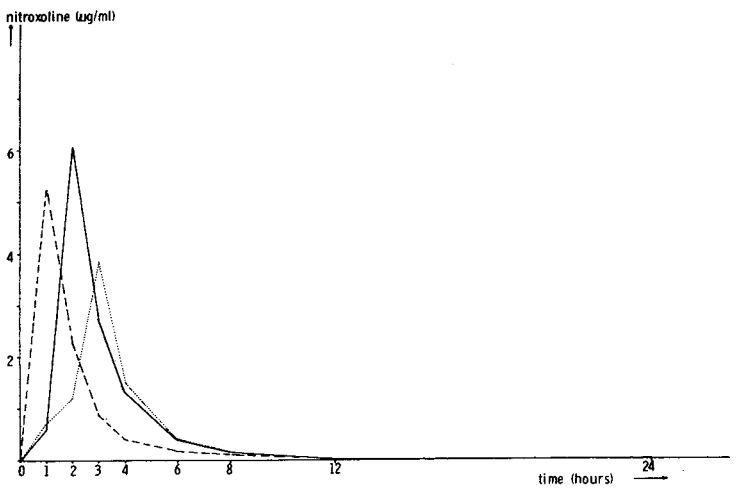

Fig. 6. Plasma concentration—time curves for three volunteers after an oral dose of 100 mg of nitroxoline.

Table I shows the nitroxoline recovery from the 12-h urine samples collected from the three volunteers, and the values for free and conjugated nitroxoline reported in the literature [2].

TABLE I

RECOVERY OF NITROXOLINE IN URINE (12 h) AFTER ORAL ADMINISTRATION OF 100 mg

Volunteer	Nitroxoline (free) (%)	Nitroxoline (conjugated) (%)
A	1.2	23.3
B	0.3	41.1
C	0.3	24.1
Literature [3]	1.5*	48*

*Oral dose, 200 mg.

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CHROMBIO. 736

DETERMINATION OF D-PENICILLAMINE IN SERUM BY FLUORESCENCE DERIVATIZATION AND LIQUID COLUMN CHROMATOGRAPHY

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SUMMARY

A simple and fast method for the determination of D-penicillamine in serum is described. The analysis is based on a fluorescence derivatization of the sulfhydryl group combined with a reversed-phase liquid chromatographic separation and fluorescence detection. Before derivatization the serum proteins are precipitated with ethanol and removed by centrifugation. As derivatizing agent 5-dimethylaminonaphthaline-1-sulfonylaziridine is used which reacts selectively with thiols under defined reaction conditions. The detection limit is in the pmol range; 50—300 ng of D-penicillamine can be determined with a relative standard deviation of 7—8%. Thus the method permits a simple determination of D-penicillamine in serum at therapeutic levels.

INTRODUCTION

D-Penicillamine (D-PA) is used for the treatment of polyarthritis [1], cystinuria [2], poisoning caused by heavy metals [3] and Wilson's disease [4]. Additionally, synergistic effects in the metabolism of organic mercury compounds [5] and in the treatment of other pathogenic conditions have been

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described [6—8]. Despite its frequent use the pharmacological effects of D-PA have not been sufficiently investigated. This is partly due to the lack of selective and sensitive methods for its analysis.

For determination of D-PA the properties of its functional groups are used. Colorimetric analysis can be carried out after complexation with $FeCl_3$ [9] or with 5,5'-dithiobis-2-(nitrobenzoic acid) [10]. Polarographic determination [11] and separations using automatic amino acid analyzers [12—14] have been described. For detection, reaction with H_2PtCl_2—KI [12] or ninhydrin [13, 14] is used. Faster analysis is possible by high-performance liquid chromatographic (HPLC) separations on cation resins combined with electrochemical detection [15].

HPLC has proved to be very useful for the determination of many substances in biological fluids due to the speed and selectivity of separation. Sometimes detection is a problem; the required detection limit can in some cases only be achieved by chemical derivatization. For D-PA selective derivatization of the thiol group seemed to be most promising. Recently, the use of 5-dimethylaminonaphthaline-1-sulfonylaziridine (dansylaziridine) for pre-column derivatization of thiols was described [16]. The derivatives are stable and can be separated by reversed-phase liquid chromatography within a short time. The fluorescence is linear over a wide range and permits trace determination of D-PA.

EXPERIMENTAL

Materials

All solvents used were of analytical grade quality (Merck, Darmstadt, G.F.R.). 5-Dimethylaminonaphthaline-1-sulfonylaziridine puriss. biochim. was purchased from Fluka (Buchs, Switzerland). For ion-exchange separations Dowex 50W-X2, 200—400 mesh (Serva, Heidelberg, G.F.R.) packed in polystyrol columns (Sarstedt, Nümbrecht-Rommelsdorf, G.F.R.) was used. The stationary phase for HPLC was LiChrosorb RP-18, 7 μm particle size (Merck), packed in 150 × 3.2 mm I.D. steel columns.

Apparatus

A Waters Assoc. 6000A pump in combination with a Perkin-Elmer LC 1000 fluorescence detector (excitation filter 338 nm) was used for HPLC determinations. The fluorescence emission was measured at 540 nm or by means of a 430 nm cut-off filter. For sample injection a Waters U6K injection system (injection volume 100 μl) was used. All separations were carried out isocratically at room temperature, thermostating being unnecessary. Peak area calculations were done with a Hewlett-Packard 3380A integrator.

Procedure

As a result of optimization studies the following procedure for sample clean-up and derivatization is recommended. One millilitre of ethanol is dispensed into a 10-ml vial that can be tightly closed; then 0.5 ml of serum stabilized with a 0.3 *M* aqueous solution of sodium EDTA (10:1) is added. The vial is closed and shaken well for 2 min. After centrifugation for 3 min at 2700 *g*, 1 ml of the clear supernatant is pipetted into another vial, and 2 ml of phosphate

buffer (pH 8.2, 0.067 *M*) and 0.2 ml of dansylaziridine solution (10 mg of dansylaziridine per ml of methanol) are added. The closed vial is placed in a thermostated water-bath at 60°C for 1 h. After this reaction time the solution can be directly injected into the liquid chromatograph. The slight opalescence observed in some solutions can be removed by centrifugation (5 min at 2700 *g*).

RESULTS AND DISCUSSION

Sample preparation

Since many compounds with sulfhydryl groups are present in biological fluids such as serum, direct determination without previous clean-up is not possible. Pre-separation of the proteins from the low molecular weight compounds is necessary. One possibility is the use of short ion-exchange columns such as those described for the determination of iodinated amino acids in serum [17]. Serum (0.2 ml) is pipetted onto the top of the cation-exchange column. The resin (Dowex 50W-X2, 200—400 mesh, H^+) is filled to a height of 15 mm in a polystyrol column, 6 mm I.D. The proteins are eluted with 10 ml of 0.02 *M* NaOH, the amino acids being concentrated at the bottom of the column by this procedure. The amino acids can then be eluted with a mixture of ethanol—5% ammonia (1:1). The elution is carried out by applying two 0.5-ml portions of this solvent. The first 0.5-ml portion is discarded; the second 0.5 ml contains the D-PA and can be directly used for derivatization. The recovery for a D-PA concentration of 12 μg/ml serum was 42.4 ± 8.3%. As the ion-exchange columns are used only once, cross-contamination of different samples is avoided. The method gives reproducible results but for routine analysis the amount of time and material is not negligible. Therefore we tried to work out a simpler and faster alternative.

This second method is a combination of precipitation and extraction. The high molecular weight proteins are precipitated by an organic solvent which serves at the same time as an extracting agent for the amino acids present in the serum. The procedure is described under Experimental. Two solvents were compared: ethanol and tetrahydrofuran (THF). The results are given in Table I. With THF the extraction yields are higher, but a serious disadvantage is the low precision found with this solvent. The reason for the poor reproducibility using THF could be difficulties in the derivatization reaction or the presence of traces of peroxides. Ethanol was therefore preferred in the subsequent investigations.

Thiols can be easily oxidized or complexed [18]. Therefore tests of the stability of free penicillamine in serum were made. Saetre and Rabenstein [15] described a stabilizing effect of EDTA. EDTA has the ability to complex traces of heavy metals which catalyze the oxidation of free thiol groups. The influence of this agent was checked in order to determine suitable storage conditions for samples that cannot be analyzed immediately. The results of the study are summarized in Fig. 1. Freshly centrifuged pooled serum was used as a matrix and 20 μg of D-PA per ml were added. For the stabilized solutions, 0.1 ml of aqueous sodium EDTA solution (0.3 *M*) was pipetted into 1 ml of serum. The stability of stabilized and non-stabilized solutions was compared at 4°C

TABLE I

RELATIVE PEAK HEIGHTS FOR PENICILLAMINE AFTER EXTRACTION WITH ETHANOL AND THF FROM SERUM

The standard deviations are calculated from eight trials each. For conditions of extraction and derivatization see Experimental. For chromatographic conditions see Fig. 3.

D-Penicillamine (μg/ml serum)	Relative peak height	
	Ethanol	THF
2	7 ± 0.4	8 ± 1.0
10	34 ± 1.7	37 ± 3.9
25	89 ± 4.6	97 ± 9.2
50	168 ± 8.7	177 ± 19.1

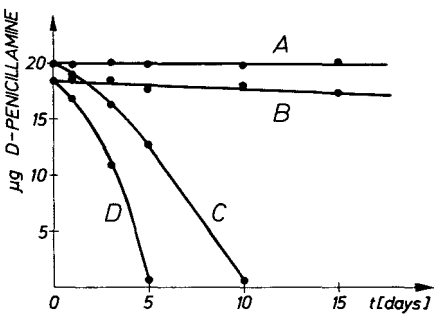

Fig. 1. Stability of D-penicillamine in serum (20 μg/ml) under various conditions. (A) Serum stabilized with sodium EDTA, stored at −18°C. (B) Serum without sodium EDTA, stored at −18°C. (C) Serum stabilized with sodium EDTA, stored at +4°C. (D) Serum without sodium EDTA, stored at +4°C.

and −18°C. Storage in the refrigerator (4°C) is not sufficient, whereas at −18°C the stabilized solution shows no loss of D-PA over a period of 15 days.

Derivatization

An account of the derivatization reaction and optimization of the conditions was published recently [16]. The maximum yield is reached at pH 8.2 with a minimum of 2.7-fold molar reagent excess using a reaction time of 1 h at 60°C. Under these conditions only free sulfhydryl groups are derivatized; weaker nucleophils such as amines or alcohols do not react. This finding is an important presupposition for the selective determination of D-PA in the complex matrix of serum. The derivatives are stable; no degradation could be observed in a week.

As all thiols present in serum are derivatized, the minimum amount of reagent required had to be found empirically. The results for a study in which pooled serum containing 29 μg/ml D-PA was used is summarized in Fig. 2. This figure shows the peak heights gained for the derivative of D-PA by HPLC determination. A minimum of 0.2 ml of reagent (corresponding to 2 mg of dansylaziridine) is necessary for complete derivatization. It is interesting to note that

the method for sample clean-up (ion exchange or precipitation) has no influence on the amount of reagent. Removal of the excess reagent is not necessary; the separation is done by the following HPLC determination.

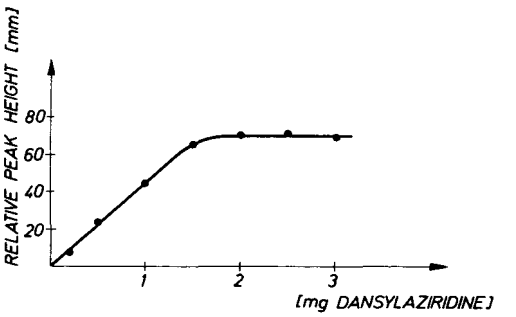

Fig. 2. Derivatization of D-penicillamine after extraction from pooled serum (29 μg D-PA per ml serum) with various amounts of dansylaziridine (10 mg of reagent per ml of methanol).

Chromatography of the derivatives

One of the most serious disadvantages of D-PA analysis by amino acid analyzers is the time required, which can be several hours [12, 13]. Faster separations are possible by reversed-phase liquid chromatography. The derivatives formed from amino acids containing thiol groups and dansylaziridine show an amphoteric character: both acidic and basic functional groups are present that can be used for ion-pairing. Good separations were achieved with a 1:2 mixture of acetonitrile and phosphate buffer (pH 8.2, 0.033 *M*) with the addition of 0.05% ethylenediamine as mobile phase and LiChrosorb RP-18 as stationary phase. Typical chromatograms are shown in Fig. 3. The two chromatograms show the results for a pure solution of D-PA and for serum from a polyarthritic patient treated with D-PA. The patient was given 250 mg of D-PA orally 2 h before the sample was taken. The derivatives of the amino acids are eluted before the reagent peak within a few minutes.

The derivatization has two advantages: first the selectivity of the reaction, second the selectivity and sensitivity of the fluorescence detection. UV and fluorescence spectra of dansylaziridine, which are nearly identical with the spectra of the derivatives [16], are given in Fig. 4. The UV absorption at 254 nm or 345 nm could also be used for detection, but in this region many other compounds present in serum show strong UV absorption and interfere in the chromatogram. In addition to higher selectivity, measurement of the fluorescence is more sensitive than UV detection. For excitation of fluorescence a 338-nm filter can be used (the optimum wavelength is 345 nm), the emission is measured at the maximum of 540 nm. The detection limit under these conditions is 85 pmol of penicillamine (signal-to-noise ratio of 3:1). Using a 430 nm cut-off filter for measuring emission, the detection limit can even be lowered to 12 pmol of penicillamine. The cut-off filter causes no loss of detection selectivity because no fluorogenic substances other than the dansylated derivatives are present.

Linear calibration curves were achieved by the addition of known amounts of D-PA to pooled serum. In Table II the results for the biologically relevant concentration range are summarized. The calibration curve passes through the origin of the coordinates and is linear up to 16 pmol penicillamine (correlation coefficient = 0.999). The relative standard deviation in the concentration range 50—300 ng per 100 μl (= injection volume) is 7—8%.

Besides the analysis of penicillamine, this method offers a possibility for the determination of other thiol compounds such as cysteine and glutathione. Investigations into the analysis of the reduced and oxidized forms of these thiols in biological fluids are in progress.

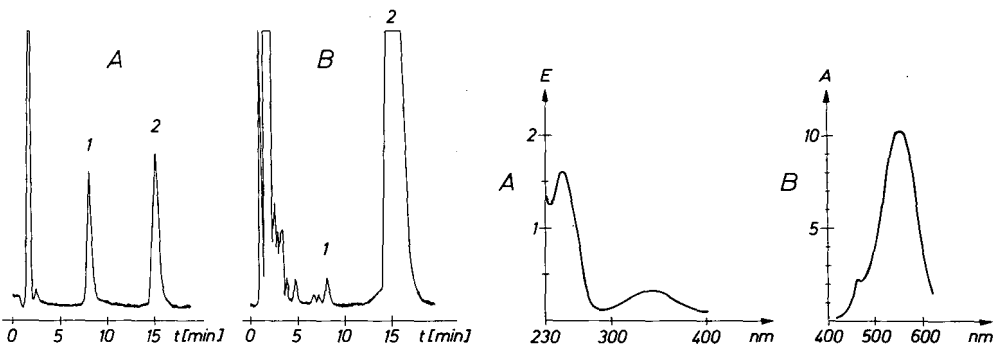

Fig. 3. Chromatographic determination of D-penicillamine after derivatization with dansylaziridine. (A) D-PA standard solution. (B) Serum from a patient treated with D-PA. Peaks: 1 = S-(2-dansylaminoethyl)-penicillamine; 2 = dansylaziridine. Solvent: acetonitrile—phosphate buffer (pH 8.2, 0.033 M) (1 : 2) + 0.05% ethylenediamine. Column: LiChrosorb RP-18. Flow-rate: 1 ml/min at 1700 p.s.i. Injection volume: 100 μl. Detection: λ_{exc} 338 nm; λ_{em} cut-off filter 430 nm.

Fig. 4. UV (A) and fluorescence emission (B) spectra of dansylaziridine (2.57×10^{-5} M) in methanol.

TABLE II

DETERMINATION OF D-PENICILLAMINE FROM SERUM AFTER DERIVATIZATION WITH DANSYLAZIRIDINE

Calculations of peak areas were made with a Hewlett-Packard 3380 A integrator.

D-Penicillamine (ng per 100 μl*)	Digits	S_{rel}** (%)
12.8	5,830	15.6
25.6	11,580	12.2
48.0	21,910	7.9
96.0	43,640	6.8
152.0	69,320	7.5
304.0	138,290	6.7

*100 μl = injection volume.
**S_{rel} = relative standard deviation (n = 7).

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CHROMBIO. 739

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRITHIOZINE AND ITS NEUTRAL METABOLITES IN HUMAN PLASMA AND URINE

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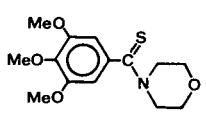
SUMMARY

A high-performance liquid chromatographic method for the quantitation of the new antisecretory and antiulcer drug trithiozine in human plasma and urine is reported. The procedure is simple and precise; it allows the simultaneous determination of therapeutic doses of the drug and its three main metabolites, namely, 4-(3,4,5-trimethoxythiobenzoyl)tetrahydro-1,4-oxazine S-oxide, 4-(3,4,5-trimethoxybenzoyl)tetrahydro-1,4-oxazine, and 2-hydroxy-4-(3,4,5-trimethoxybenzoyl)tetrahydro-1,4-oxazine.

INTRODUCTION

Trithiozine (I) was selected in a systematic research program on new alkoxythiobenzamides [1, 2]. The compound displays considerable antisecretory and antiulcer activity and is devoid of anticholinergic, antihistaminic, ganglioplegic and cardiovascular activity [3, 4]. Its efficacy has been documented in several clinical trials [5—7].



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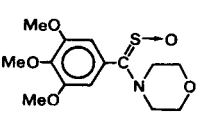

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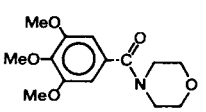

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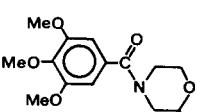

IV

A preliminary gas—liquid chromatographic (GLC) method for the determination of (I) has already been described for rat and dog plasma and urine [8]. Metabolic studies showed that the main neutral metabolites are the amide (III), the morpholinol (IV) and the sulphur-oxide (II). This last compound is converted by the GLC method [9] into the amide (III). In order to overcome this handicap, a high-performance liquid chromatographic (HPLC) method for the simultaneous quantitation of (I) and its main neutral metabolites was developed. The method was applied to the determination of plasma and urine levels of the four compounds in a volunteer after the oral administration of (I).

EXPERIMENTAL

Reagents and chemicals

The reference compounds [4-(3,4,5-trimethoxythiobenzoyl)tetrahydro-1,4-oxazine (I), 4-(3,4,5-trimethoxythiobenzoyl)tetrahydro-1,4-oxazine S-oxide (II), 4-(3,4,5-trimethoxybenzoyl)tetrahydro-1,4-oxazine (III), 4-(3,4,5-trimethoxybenzoyl)tetrahydro-2-hydroxy-1,4-oxazine (IV) and 4-(3,5-dimethoxythiobenzoyl)tetrahydro-1,4-oxazine (internal standard)] were synthesized in our laboratories as described elsewhere [1, 2, 8, 10, 11]. Their purity was tested by thin-layer chromatography: a single spot was detected by UV fluorescence quenching, except for (II) which cannot be completely purified from (I) and (III) even by several crystallizations or by liquid column chromatography [10—12].

Chloroform LiChrosolv and methanol LiChrosolv were obtained from Merck (Darmstadt, G.F.R.); double-distilled water and buffer solution (pH 10.0) were from Carlo Erba (Milan, Italy). Methanol and water were filtered before use through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The siliconizing agent was Dri-Film SC 87 (Pierce, Rockford, IL, U.S.A.). Separating phase filters (Whatman, Maidstone, Great Britain) were used after extraction.

All the compounds were dissolved in methanol for the external calibration curves and in pH 10.0 buffer for the internal calibration curves.

Glassware

The test-tubes were cleaned with sulphochromic mixture, rinsed with water, dried in an oven and then silanized in a 10% toluene solution of Dri-Film SC 87. After 2 h, the glassware was air dried and used for analysis.

Chromatographic conditions

A Hewlett-Packard analytical liquid chromatograph (Model 1080) was combined with an LC-55 variable-wavelength ultraviolet detector (Perkin-Elmer) operating at 254 nm. A stainless-steel column (25 × 4.6 mm I.D.) packed with LiChrosorb RP-8 (10 μm) (Merck) and connected with a precolumn dry-packed with Perisorb RP-8 (30—40 μm) (Merck) was used. The mobile phase consisted of mixtures of methanol and double-distilled water with gradient elution: 30% methanol for 3 min, gradient to 50% methanol in the following 3 min, then 50% methanol for 5 min. The flow-rate was 2.5 ml/min at 30°C.

Analytical procedure

External calibration curves. To aliquots of (I), (III) and (IV) ranging from 1 to

30 μg, 1 ml of internal standard (10 μg/ml) was added and the mixture was evaporated to dryness under a stream of nitrogen at 30°C. Methanol (100 μl) was added and 20 μl of this solution were injected. The external calibration curve for (II) was done separately because (I) and (III) are always present as impurities. The exact amount of (II) in the synthetic sample was calculated by subtracting the quantities of (I) and (III), calculated from their calibration curves, from the weighed amount.

Determination of (IV) as methyl ether. An aliquot of (IV) in methanolic solution (from 5 to 50 μg/ml) was evaporated to dryness under nitrogen in screw-capped tubes and then treated with 2 ml of anhydrous 3 *N* HCl—methanol in an oil bath at 60°C for 20 min. To the mixture was added 1 ml of internal standard; the solution was then evaporated, and the residue was dissolved in 100 μl of methanol; 20 μl were injected.

Internal calibration curves: urine. To aliquots of 4 ml of urine, 1 ml of internal standard (10 μg/ml) and 1 ml of a solution of (I) at concentrations ranging from 2 to 80 μg/ml were added. The mixture was brought to a volume of 7 ml with buffer (pH 10.0). Extraction was performed with 10 ml of chloroform by mechanical shaking for 20 min. After centrifugation at 2900 *g* for 5 min, the organic phase was filtered through a phase-separation filter and then evaporated to dryness at 30°C under nitrogen. The residue was redissolved with 100 μl of methanol and 20 μl were injected.

The same procedure was followed for (II), (III) and (IV); in the last case, the chloroform phase, after being reduced to dryness, was treated with 2 ml of anhydrous 3 *N* HCl—methanol as previously described.

Internal calibration curves: plasma. To 2-ml aliquots of plasma, 1 ml of internal standard (10 μg/ml) and 1 ml of a solution of (I) at concentrations ranging from 1 to 40 μg/ml were added. The volume of the mixture was brought to 4 ml with buffer and extracted with 9 ml of chloroform. Subsequent handling was as described for urine. Metabolites II, III and IV were treated in the same way.

Peak area ratios of compounds to internal standard were measured and plotted against their concentrations.

Application

A healthy male volunteer (R.R., 70 kg, 30 years' old) was administered a single oral dose (400-mg capsules of Tresanil^{R})★. Blood samples, collected at 0, 1, 2, 4, 6, 8, 10 and 24 h after administration, were mixed with heparin and centrifuged. The separated plasma was immediately analyzed in order to avoid decomposition of (II) [12].

Urine was collected every 8 h for 48 h. An aliquot of 4 ml was taken at each time for the quantitation of (I), (II) and (III), and a second 4-ml aliquot taken for quantitation of (IV).

RESULTS AND DISCUSSION

The separation of trithiozine from its three main metabolites was optimized by using gradient elution (Fig. 1). Under such conditions there are no inter-

★Tresanil^{R}, I.S.F. — Icar Leo.

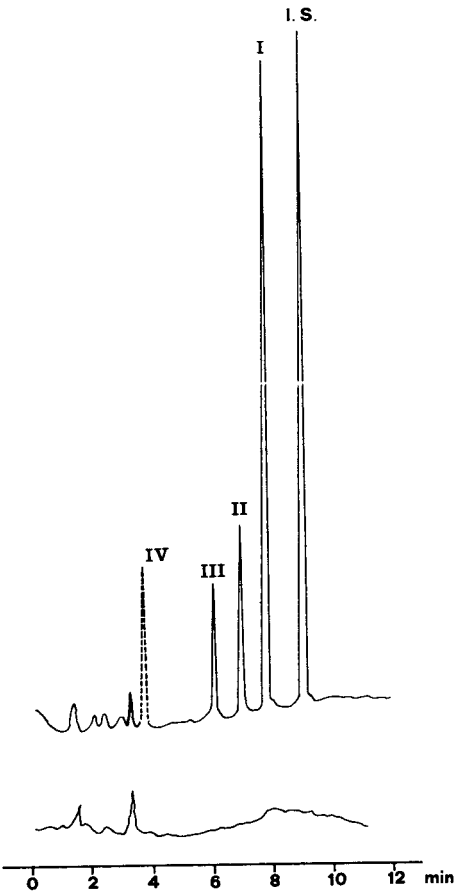

Fig. 1. HPLC profile of a neutral plasma extract (man, 400 mg of trithiozine, single oral dose). The dotted line has been used to show the separation of (IV) though it is only present in human plasma in amounts below the limit of sensitivity of the method. I.S. = internal standard.

ferences with plasma blank. The retention times are 3.90, 5.91, 6.72 and 7.70 min for (IV), (III), (II) and (I), respectively.

The internal calibration curves for the quantitation of the four compounds in plasma are reported in Fig. 2. Each point is the mean of three values. Good linearity was found in plasma with concentrations ranging from 0.5 to 20.0 μg/ml. The detection limits were empirically estimated to be 0.1 μg/ml for all the compounds. The recoveries, over the whole concentration range, and the reproducibility of the method calculated with the pooled standard deviation are reported in Table I.

Because of the presence of interfering substances in the urine, which vary between individuals and with time of collection, it was often impossible to separate compound (IV) as such from the urine blank. Therefore (IV) was derivatized to its methyl ether, as described in the Experimental section. Although this compound has a retention time too close to that of (II) (7.42 min), its

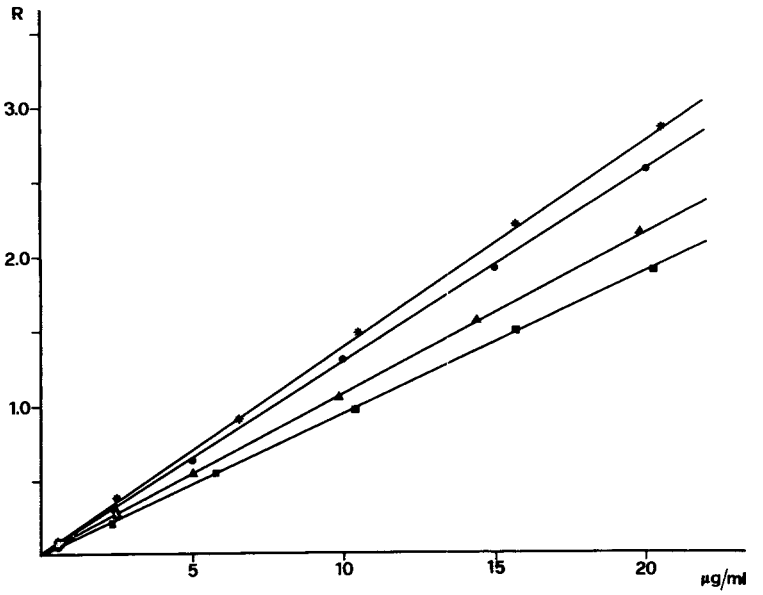

Fig. 2. Internal calibration curves of trithiozine (∗) and metabolites II (▲), III (●) and IV (■) in human plasma.

TABLE I

RECOVERIES AND REPRODUCIBILITY IN THE DETERMINATION OF TRITHIOZINE AND ITS NEUTRAL METABOLITES

Compound	Recovery (%)		Pooled standard deviation*	
	Plasma	Urine	Plasma	Urine
I	75.0	98.0	2.8	1.7
II	75.0	86.6	1.7	1.5
III	98.0	96.0	2.1	1.7
IV	73.0	78.5**	2.3	1.7

*Pooled standard deviation = $\frac{\Sigma(s_i/x_i)}{n} \times 100$, where s_i = standard deviation, x_i = mean value at point i, n = number of points on the calibration curve.

**Detected as methyl ether.

quantitation was possible given the complete degradation of (II) under the derivatization conditions [9]. A typical chromatographic profile of a urine extract before and after derivatization is shown in Fig. 3. The internal calibration curves for the quantitation in urine are reported in Fig. 4. Each point is the mean of three values. The detection limits were estimated to be 0.1 μg/ml for all the compounds. The recoveries, over the whole concentration range, and the reproducibility of the method are listed in Table I.

Plasma concentration—time curves of (I), (II) and (III) for a single subject following oral administration of 400 mg of trithiozine, and the cumulative

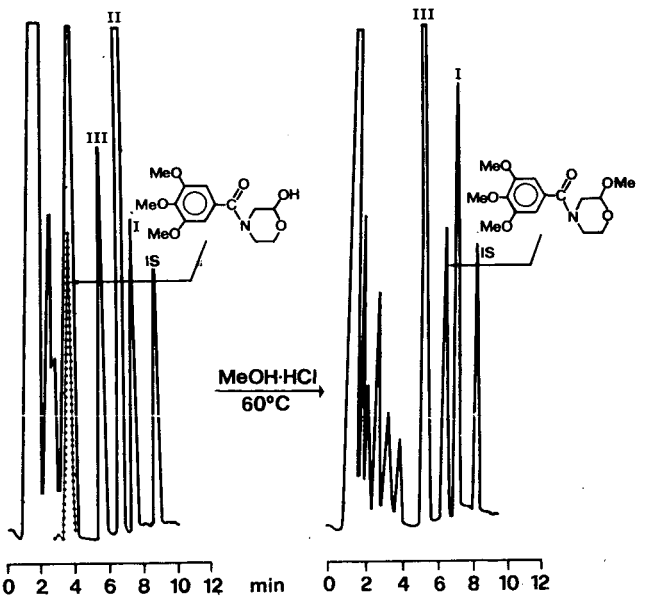

Fig. 3. HPLC profile of a urine extract before (left) and after (right) derivatization.

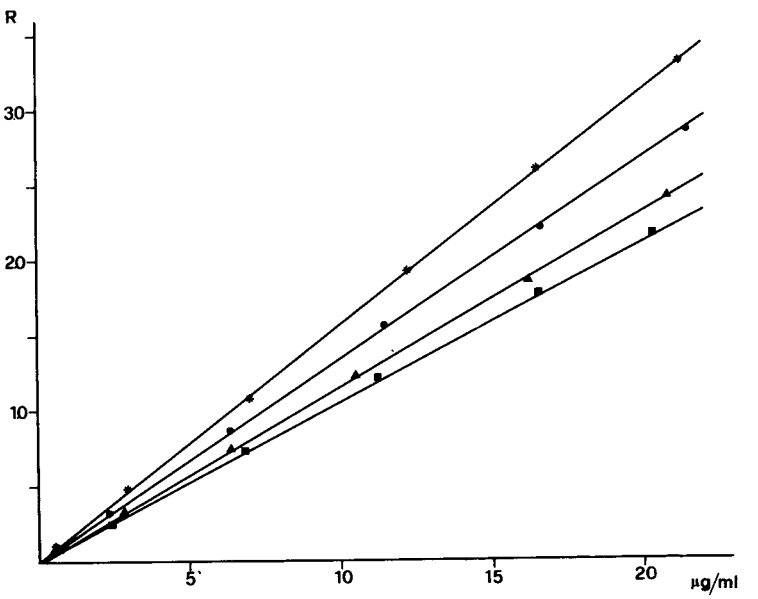

Fig. 4. Internal calibration curves of trithiozine (∗) and metabolites II (▲), III (●) and IV (■) in human urine.

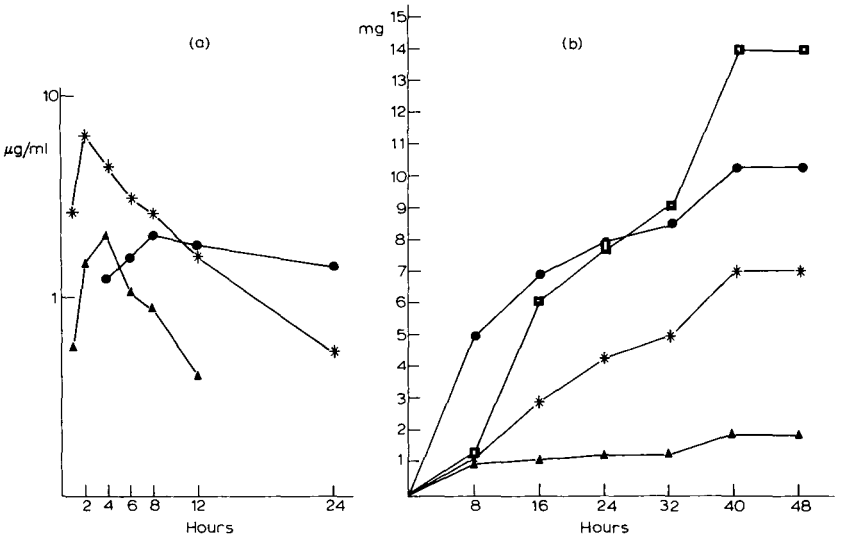

Fig. 5. Human plasma levels (a) and urinary cumulative excretion (b) of trithiozine (*) and its metabolites II (▲), III (●) and IV (■).

urinary excretion of (I), (II), (III) and (IV) were determined as shown in Fig. 5. In this case (IV) in plasma was found to be below the limit of sensitivity of the method. In contrast to the GLC procedure described elsewhere [8], the HPLC method permits the quantitation of the unchanged drug and its main neutral metabolites without formation of artifacts and is simple, accurate and precise enough for routine monitoring of clinical samples.

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CHROMBIO. 725

RAPID MICROANALYSIS OF ANTICONVULSANTS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A rapid microanalytical method is described for phenobarbital, phenytoin, primidone and carbamazepine utilizing high-performance thin-layer chromatography (HPTLC). This procedure incorporates a single extraction of a 50-μl plasma sample. One tenth of the extract is concentrated and applied to the HPTLC plate by a Contact Spotter, chromatographically separated and quantitated by in situ ultraviolet reflectance densitometry. The coefficient of variation is less than 4% (n = 8), the extraction efficiency is approximately 95% and the minimum detectable amount of pure drug standards applied to and developed on the HPTLC plate is 5 ng or less for all four anticonvulsants.

INTRODUCTION

A variety of methods have been reported for the determination of anticonvulsant drugs in blood plasma which, with the exception of immunoassay techniques [1], are for the most part based on gas [2] or liquid [3] chromatographic separations. Column chromatographic procedures are usually quite adequate where the number of samples to be examined is modest and the analysis time is not critical. However, if these factors are an important consideration, the ability to process many samples simultaneously becomes a definite advantage. It is for this reason that thin-layer chromatography (TLC) with in situ densitometric quantitation is an attractive alternative to column chromatography, particularly where many samples must be assayed in a short period of time.

Methods utilizing TLC in blood level determinations have been described for most of the important anticonvulsant drugs [4—8]. High-performance thin-layer chromatography (HPTLC), however, permits even greater speed of

analysis than realized with conventional TLC [9], and perhaps more importantly offers significant improvement in sensitivity of detection [10]. It is this latter feature which has led to the development of this microanalytical method for the determination of phenobarbital, phenytoin, primidone, and carbamazepine in blood plasma.

This procedure offers many important advantages over existing procedures [4—8]. It incorporates a single, rapid extraction of a 50-μl plasma sample, fifteen extracts may be concentrated and applied simultaneously to the HPTLC plate by means of contact spotting [11], and after development the drugs are measured by in situ ultraviolet (UV) reflectance densitometry. The method lends itself to the analysis of large numbers of samples as well as to emergency situations where a rapid and accurate assay is required.

MATERIALS AND METHODS

Apparatus

Samples were extracted in 1.5-ml Eppendorf polypropylene test tubes (Brinkmann Instruments, Westbury, NY, U.S.A.). HPTLC plates (silica gel 60 F254, E. Merck, Darmstadt, G.F.R.) were pre-cleaned by overnight development in absolute ethanol in the presence of ammonia vapor. A Shimadzu Model C-910 dual-wavelength TLC scanner (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used for densitometric determinations, and samples were applied to the HPTLC plates with a Contact Spotter (Clarke Analytical Systems, Sierra Madre, CA, U.S.A.). All glassware was silylated by a vapor phase method [12].

Reagents

Drugs used in preparation of standards were phenobarbital (Merck, Rahway, NJ, U.S.A.), phenytoin (Aldrich, Milwaukee, WI, U.S.A.), primidone (Ayerst Labs., New York, NY, U.S.A.), and carbamazepine (Ciba Pharmaceuticals, Summit, NJ, U.S.A.). The internal standard was *p*-tolylbarbital (Aldrich Chemical).

Chloroform, isopropanol, and ammonium hydroxide were ACS grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Absolute ethanol was from U.S. Industrial Chemicals (New York, NY, U.S.A.).

Preparation of standard solutions and plasma

Dissolve each drug in absolute alcohol to provide stock solutions of the following concentrations: phenobarbital, 1.0 g/l; phenytoin, 1.0 g/l; primidone, 4.0 g/l; carbamazepine, 0.20 g/l; and *p*-tolylbarbital (internal standard), 1.0 g/l. Introduce 0.5-, 1.5-, and 2.5-ml volumes of each stock solution (with the exception of the internal standard) into three 50-ml volumetric flasks. Evaporate the solvent with gentle heating under a flow of nitrogen and then fill to volume with drug-free plasma. Mix for 2 h, pipette 0.5-ml volumes into 1.5-ml polypropylene test-tubes and freeze for future use. These standards contain the following concentrations of phenobarbital, phenytoin, primidone, and

carbamazepine for the 0.5-, 1.5-, and 2.5-ml original volumes respectively: 10, 10, 4 and 2 mg/l; 30, 30, 12, 6 mg/l; and 50, 50, 20 and 10 mg/l.

Prepare a spotting standard by diluting 50 μl of each drug stock solution and 25 μl of the internal standard solution to 10 ml in a volumetric flask with ethyl acetate containing 0.01% octanol. The concentration of drugs in this solution is equivalent to the central points of the calibration curves produced by extracts of the plasma standards and thus serves as a external standard to monitor the extraction procedure.

Dilute 250 μl of the internal standard stock solution to 100 ml in ethyl acetate. This will be used as the plasma extraction solvent.

Procedure

Pipette 50 μl of the plasma standards and the patient plasma into 1.5-ml polypropylene test-tubes and add 300 μl of ethyl acetate containing the internal standard to each. Vortex the contents of the tubes for 1 min, allow to stand for an additional 10 min, and centrifuge for 5 min at 1000 *g*. Remove 30-μl aliquots of the upper solvent layers and deposit these in the concave sample indentations of the Contact Spotter alongside a 30-μl volume of the spotting standard. Evaporate under nitrogen flow at the low temperature setting. Transfer the residues to a 5 × 10 cm pre-washed HPTLC plate.

Develop the plate in a pre-equilibrated filter-paper lined chamber containing chloroform, together with a separate beaker of concentrated ammonium hydroxide. This solvent system moves the carbamazepine to an R_F of 0.4 and leaves the remaining anticonvulsants at the origin. Measure the carbamazepine by reflectance densitometry at 285 nm wavelength.

Re-develop the plate in chloroform—isopropanol—ammonium hydroxide (80:20:1), again in a pre-equilibrated tank with ammonium hydroxide present in a separate beaker. Two 7-min developments are usually required for complete separation. Scan the developed plate in the dual wavelength mode using 285 nm as the reference and 215 nm as the sample wavelengths.

RESULTS AND DISCUSSION

The in situ UV absorption spectra of the four anticonvulsants are shown in Fig. 1. At 285 nm no other drugs, metabolites or components from plasma extract of patients have been found which interfere with the detection and measurement of carbamazepine. Dual wavelength measurement for the remaining anticonvulsants is preferred over a single wavelength because of the greater signal gain required at 215 nm. Baseline noise due to surface irregularities in the HPTLC plate, light source variations, etc., are reduced significantly by compensation with a reference wavelength. As is the case with carbamazepine, no components have been found to interfere with the measurement of phenobarbital, phenytoin, primidone, and the internal standard using this system.

Fig. 2 shows the HPTLC separation of a plasma extract containing carbamazepine scanned at 285 nm and the remaining drugs scanned at 215 nm with the reference wavelength at 285 nm. The standard curves of the peak height ratios of the four drugs to the internal standard at concentrations

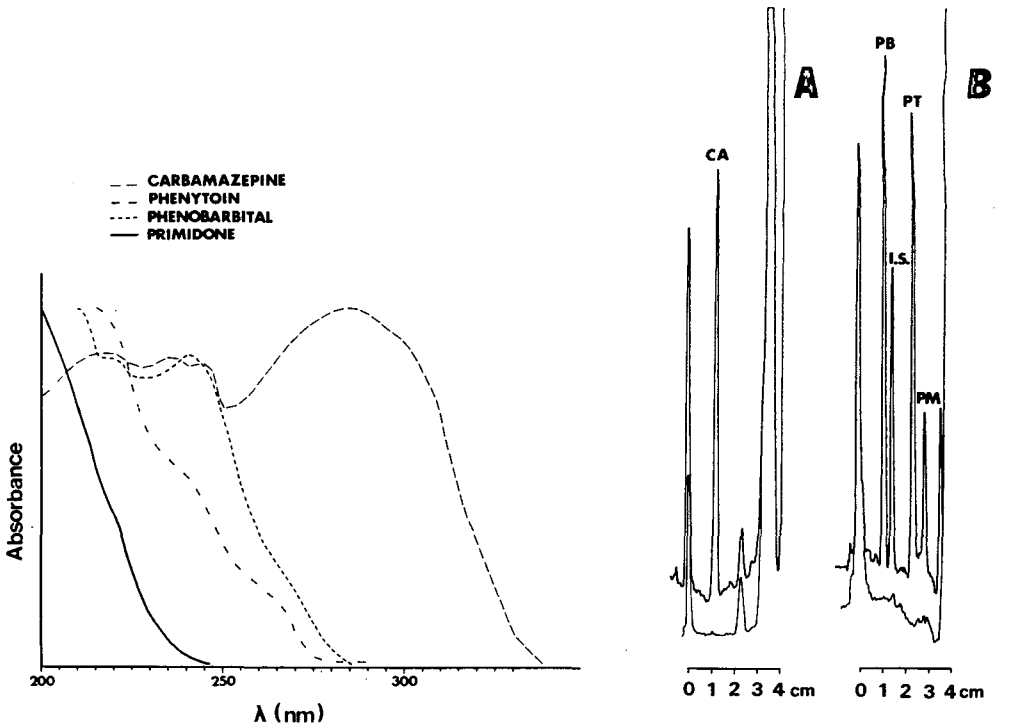

Fig. 1. UV absorption spectra obtained by scanning a HPTLC plate on which the four anticonvulsants had been spotted and chromatographically separated.

Fig. 2. (A) Chromatographic separation after first development system of a plasma extract containing 5 mg/l of carbamazepine (CA) and a blank plasma extract; HPTLC plate was scanned by reflectance densitometry at 285 nm. (B) Chromatographic separation after second solvent system of plasma extract containing 40 mg/l of phenobarbital (PB), *p*-tolylbarbital (internal standard, I.S.), 20 mg/l of phenytoin (PT), and 10 mg/l of primidone (PM) and a blank plasma extract; plate scanned by dual wavelength reflectance densitometry (285 nm reference and 215 nm sample wavelengths).

covering the therapeutic ranges are shown in Fig. 3. Each point represents the average of three determinations, and in each case the difference between values and the mean is less than 3%. Within-run precision obtained by processing eight aliquots of a single plasma sample through the complete procedure is shown in Table I. The extraction efficiency for all four anticonvulsants is greater than 95%, and minimum detectable amounts of pure drug standards applied to the HPTLC plates, developed, and scanned are approximately: phenobarbital, 5 ng; phenytoin, 3 ng; primidone, 3 ng; and carbamazepine 1 ng. With the extraction of 50 μl of plasma the minimum detectable amount is 2, 2, 2, and 0.5 μg/ml for phenobarbital, phenytoin, primidone and carbamazepine respectively. If greater sensitivity is required a larger amount of plasma may be used or a larger amount of the ethyl acetate extract may be placed on the HPTLC plate.

HPTLC plates containing fluorescent indicator are used in this procedure, because, aside from the obvious advantage of permitting observation of the development process, separation between phenobarbital and the internal

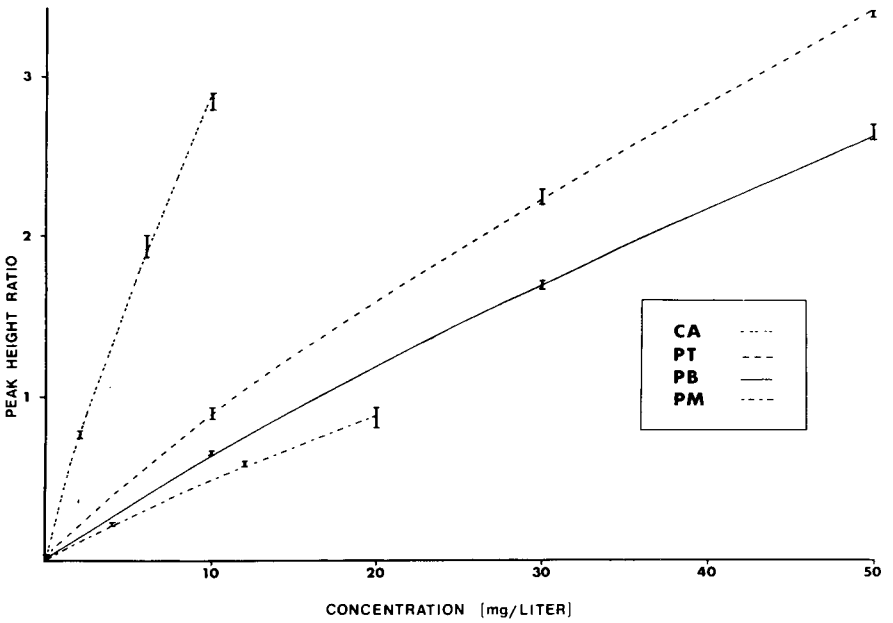

Fig. 3. Calibration curves of carbamazepine (CA), phenytoin (PT), phenobarbital (PB), and primidone (PM) expressed as the peak height ratio to the internal standard as a function of plasma concentration.

TABLE I

REPLICATE DETERMINATIONS OF ANTICONVULSANTS IN PLASMA

Replicate determination of a single plasma sample containing phenobarbital (PB), phenytoin (PT), primidone (PM), and carbamazepine (CA).

No.	PB	PT	PM	CA
1	31.5	32.0	13.4	6.3
2	30.1	31.3	13.0	5.8
3	29.7	31.3	13.2	5.8
4	29.7	31.2	13.4	6.0
5	29.0	31.0	13.4	6.2
6	29.2	30.3	13.7	6.1
7	28.8	30.3	13.2	6.1
8	29.4	29.2	12.0	5.8
$\bar{X}$	29.7	30.8	13.2	6.0
S.D.	0.85	0.68	0.51	0.20
CV (%)	2.9	2.8	3.9	3.3

standard is somewhat improved over that of non-fluorescent plates. Occasionally these compounds do not achieve baseline separation, but this problem can be remedied by adjusting the amount of ammonium hydroxide in the developing solvent or by developing one additional time in the final solvent system.

Clean HPTLC plates are of course quite important for successful determinations at the relatively short wavelengths at which phenobarbital, phenytoin,

and primidone are measured. It is therefore necessary to clean the plates of any components adsorbed from the atmosphere or from packaging materials prior to use. This is easily accomplished by developing the plates overnight in ethanol in an ammonia atmosphere. After this treatment the plates may be stored in a sealed glass container until needed. Development solvents were used as received without contributing any significant contamination.

A single plasma sample with a standard curve can be extracted, chromatographed and quantitated within 2 h. A much larger number of samples can be processed in approximately the same period of time since the extraction and chromatography are carried out concurrently. The densitometric scanning and quantitation is a sequential process which requires approximately one additional minute per sample.

It should be noted that even with a plasma volume of 50 μl, only one-tenth of the sample extract is actually used for a determination. Thus, with some refinement of the method, it should be entirely feasible to utilize blood samples collected from capillary sources, an important consideration with patients where venipuncture may be difficult.

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CHROMBIO. 720

Note

Excretion of *m*-hydroxymandelic acid in human urine

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Until recently, reports of octopamine in tissues and biological fluids have implied that either *p*-octopamine or a combination of octopamine isomers were being analyzed. In the last few years however techniques have been developed which permit the isomers of octopamine to be separated and quantitated [1—4]. The natural occurrence of *m*-octopamine in tissues suggests that its oxidatively deaminated metabolite, *m*-hydroxymandelic acid (*m*-HMA), might also occur and be excreted in urine. Very recently Midgley et al. [5] have shown that *m*-HMA is a normal constituent of human urine.

Independently we have also developed a procedure for the identification and quantitation of *m*-HMA in urine based on the heptafluorobutyryl-methyl ester derivative of *m*-HMA. We have utilized the gas chromatography—mass spectrometry—integrated ion current technique (GC—MS—IIC) to identify and quantitate *m*-HMA in human urine and so have confirmed the finding of Midgley et al. [5]. In this paper we report the use of this method to assess the longitudinal excretion of *m*-HMA in a human male and to determine its average excretion in a human population.

EXPERIMENTAL

Materials

HPLC-grade solvents were purchased from Caledon Labs. (Georgetown, Canada), heptafluorobutyric anhydride from Pierce (Rockford, IL, U.S.A.) and *m*-HMA from Sigma (St. Louis, MO, U.S.A.). *m*-HMA was ring deuterated by heating 200 mg at 80°C in 3 ml 9% deuterium chloride in deuterium oxide for 24 h. After rotary evaporation of the solvent and back-exchange of the phenolic and carboxylic deuterium atoms, the resulting pale brown crystalline

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product was shown by mass spectrometry to contain 79.8% *m*-HMA-D_3 (ring).

Urines from a young, healthy male were collected onto 10 ml conc. HCl in polyethylene bottles over twelve consecutive 24-h periods, each 24-h collection starting with the first voiding of the day. After recording the total volume, a 250-ml aliquot was removed and stored at —16°C until analyzed. One 24-h urine sample from each of six other persons was collected and stored in a similar manner.

Methods

At the time of analysis, each urine sample was completely thawed and mixed before 3-ml aliquots were removed. To each aliquot were added 520 ng (nominal) of *m*-HMA-D_3 and about 0.5 g of sodium chloride. The samples were then extracted with ethyl acetate (4 × 2 ml), concentrated to about 200 μl in a stream of nitrogen at 50—60°C, transferred to a 0.3-ml Reacti-vial (Pierce), and then evaporated to dryness. A solution of HCl in methanol (about 15%) (150 μl) was added and the mixture allowed to stand at room temperature for 30 min. The solvent was then evaporated in a stream of nitrogen. The samples were dried by adding benzene (100 μl), mixing thoroughly, and evaporating in a stream of nitrogen. To the dried samples were added hexane (150 μl) and heptafluorobutyric anhydride (40 μl). The samples were then heated in a heating block at 65°C for 1 h, cooled and washed once with 100 μl phosphate buffer (pH 6.0). The hexane layer was withdrawn, the remaining aqueous layer washed once with hexane (100 μl) which was then combined with the original hexane solution. This hexane solution was then concentrated to about 30 μl and submitted for GC—MS—IIC analysis.

A blank sample (3 ml 2 *N* HCl) was carried through the above procedure. Some urines were analyzed in triplicate in order to determine the reproducibility of the method.

A calibration curve (which was linear at least over the range 10 ng—10 μg) was constructed by preparing and analyzing solutions of mixtures containing various amounts of the derivative of *m*-HMA acid and a constant amount of the derivative of *m*-HMA-D_3.

Instrumentation

A Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector was coupled by means of an S.G.E. jet separator to an AEI-MS902S mass spectrometer. A support-coated open-tube (SCOT) column (S.G.E., GSC-SP2250, 57 m) was installed in the chromatograph and the helium flow-rate set at 24 cm/sec. The chromatography conditions are: oven temperature, 130°C (isothermal); injector temperature, 200°C; detector temperature, 200°C. Under these conditions, the retention time for the authentic di-(heptafluorobutyryl)methyl ester of *m*-HMA is 8.2 min. *p*-HMA has a retention time of 9.4 min and is completely separated from *m*-HMA. The mass spectrometer was operated at a resolution of 7000 for the IIC measurements and the temperature of the electron impact (70 eV) source was 200°C.

The exact masses of the molecular ions and $[M - COOCH_3]^+$ ions of the derivatives of *m*-HMA and its D_3 analogue are located in the mass spectrometer by means of the reference gas, heptacosafluorotri-*n*-butylamine, and the decade

box ratio is set so that the instrument alternately records the signals from the endogenous acid and the internal standard, as described previously for the integrated ion current analyses of trace amines [6]. For the IIC analyses the $[M - COOCH_3]^+$ ions were used; the exact masses for these are 514.9964 (endogenous acid) and 518.0152 (internal standard).

RESULTS AND DISCUSSION

m-HMA in its unconjugated form has been identified and quantitated in the twelve urine samples obtained from a single individual (Table I) and in the seven different urine samples from different individuals (Table II).

TABLE I

LONGITUDINAL URINARY EXCRETION OF UNCONJUGATED *m*-HYDROXYMANDELIC ACID IN A HUMAN

Samples 1—3 were analyzed in triplicate, while samples 4—12 represent single estimations.

Sample No.	*m*-HMA (μg/24 h)
1	58.6 ± 5.7
2	64.6 ± 1.1
3	46.9 ± 1.8
4	55.3
5	82.5
6	53.5
7	40.4
8	70.4
9	68.6
10	70.7
11	100.3
12	48.5

TABLE II

URINARY EXCRETION OF UNCONJUGATED *m*-HYDROXYMANDELIC ACID IN A HUMAN POPULATION

Sample No.	*m*-HMA (μg/24 h)
1	58.6
2	127.6
3	53.4
4	65.5
5	68.5
6	24.4
7	15.0

The unambiguity of the identification rests on three factors. First, the *meta*- and *para*-isomers of the acid are separated completely (by 1.2 min) on the SCOT capillary column, so that overlapping of signals does not occur; this is an important point because *p*-HMA is present in quantities 40—50 times larger than the *meta*-isomer. Second, the retention times of authentic *m*-HMA and

the endogenous acid are identical. Finally, at the correct retention time, the correct high-resolution masses of the M^+ and $[M - COOCH_3]^+$ ions of the derivative of the endogenous acid were recorded by the mass spectrometer and in the same intensity ratio as for authentic acid.

The ion chosen for the IIC analyses was the $[M - COOCH_3]^+$ ion since its relative intensity is seven times greater than that of the molecular ion (6.7% as opposed to 0.9%), and yet it is still unique to *m*-HMA.

The reproducibility of the method is indicated by the small standard deviations obtained (see Table I) for those samples analyzed in triplicate.

In a recent paper, Fell et al. [7] have claimed that *m*-HMA is not normally present in urine in detectable amounts, although they did not provide any data, or references, to substantiate their claim. They did however show that quite large amounts of labelled *m*-HMA were excreted in urine following ingestion of labelled *m*-tyrosine. Maruyama et al. [8] identified, but did not quantitate, *m*-HMA in rat, guinea pig and rabbit urine following injection of tritiated *m*-octopamine. Karnassiotis and Kramer [9] found 173 μg/100 ml of *m*-HMA in the serum of human patients suffering with terminal renal insufficiency, a level that reduced to 114 μg/ml following dialysis. Karoum and Sandler [10] achieved, using a SCOT column, a good separation of *m*-HMA from other phenolic acids using standard solutions of their methyl ester trimethylsilyl ether derivatives. A chromatogram from a derivatized urine extract as presented by Karoum and Sandler in their paper, however, did not include a peak at the retention time for *m*-HMA. This is perhaps not particularly surprising since we have also observed that if the attenuation for the chromatograph is set so as to permit the major peaks to be on scale, then substances present in smaller amounts do not show up.

Midgley et al. [5] have reported values for urinary *m*-HMA in the range of 11—71 ng/mg creatinine. Our values (see Table I), converted to these units, are similar (28—53 ng/mg creatinine) for the longitudinal study; we did not measure creatinine values in the other study.

This identification of *m*-HMA in the unconjugated form and its relatively consistent excretion both in a single individual and across individuals argues in favour of an endogenous origin for *m*-octopamine and *m*-HMA and of course confirms our earlier identification of *m*-octopamine [1].

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CHROMBIO. 730

Note

Determination of the absolute configuration of some biologically important urinary 2-hydroxydicarboxylic acids by capillary gas—liquid chromatography

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The analysis of urinary organic acids has become an integral part of the screening program for inborn errors of metabolism in the paediatric clinical laboratory. Especially, the widespread availability of gas chromatography—mass

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spectrometry (GC—MS) systems has led to the discovery of a variety of "new" metabolic disorders [1].

Many of the organic acids studied are chiral molecules. Because different enantiomers will be metabolized by separate pathways, the need for methods for the assignment of the absolute configurations of these chiral acids became evident. In our laboratories methods were developed for the separation of DL-hydroxymonocarboxylic acids, including capillary gas—liquid chromatography (GLC) of diastereomers obtained by esterification of the enantiomers with (—)-menthol [2]. Application of this technique has led to the discovery of a patient with D-glyceric acidemia [3] and a patient with permanent D-lactic aciduria [4]. Furthermore, it was demonstrated that urinary 2-hydroxybutyrate has the L-configuration [5]. The absolute configuration of urinary 3-hydroxybutyric acid in patients with ketosis and lactic acidosis has been verified to be D [5]; the excretion of L-glyceric acid in patients with hyperoxaluria type II was also checked (unpublished results). However, this menthylation method was not suited for all groups of chiral hydroxy acids and therefore other procedures had to be developed.

In this paper the separation of the diastereomers of the O-acetylated di-(—)-2-butyl esters of DL-2-hydroxysuccinic acid (DL-malic acid), DL-2-hydroxyglutaric acid and DL-2-hydroxyadipic acid on a non-chiral phase will be described, together with some applications.

EXPERIMENTAL

Chemicals

DL-2-Hydroxysuccinic acid and zinc DL-2-hydroxyglutarate were obtained from J.T. Baker Chemicals (Deventer, The Netherlands) and Sigma (St. Louis, MO, U.S.A.), respectively. Sodium L-2-hydroxyglutarate and L-2-hydroxysuccinic acid were purchased from Fluka (Buchs, Switzerland). DL-2-Hydroxyadipic acid was synthesized from adipic acid [6], whereas L-2-hydroxyadipic acid was prepared from L-2-aminoadipic acid (Calbiochem, San Diego, CA, U.S.A.) [7]. Salts were converted into the corresponding acids by treatment with Dowex 50 X-8 (H^+) in water.

(—)-2-Butanolic 1 *M* HCl was prepared by bubbling dry HCl gas through (—)-2-butanol (Fluka; (—)-antipode about 94% [8]) and stored at —18°C in a desiccator. The same procedure was followed for (±)-2-butanolic 1 *M* HCl.

Preparation of O-acetylated di-2-butyl esters of 2-hydroxydicarboxylic acids

To an ampoule containing 5 mg of a 2-hydroxydicarboxylic acid, 0.5 ml of 2-butanolic 1 *M* HCl was added. After heating for 2 h at 100° C the solvent was evaporated under reduced pressure. The residue was acetylated in 1 ml of pyridine—acetic anhydride (1:1, v/v) for 30 min at 100° C. After evaporation in the presence of toluene the residue was dissolved in chloroform and analysed by capillary GLC.

Analysis of urinary 2-hydroxydicarboxylic acids

Organic acids, including 2-hydroxydicarboxylic acids, were extracted from urine with ethyl acetate and analysed quantitatively by GC of the correspond-

ing pertrimethylsilyl derivatives as reported earlier [2]. For the determination of the absolute configuration of 2-hydroxydicarboxylic acids the extracts were derivatized as described above (O-acetylated di-(—)-2-butyl esters). Additional purification procedures were not carried out.

Capillary gas—liquid chromatography

The O-acetylated di-2-butyl esters of 2-hydroxydicarboxylic acids and the urinary extracts treated with (—)-2-butanolic 1 *M* HCl and acetic anhydride were analysed by capillary GLC on SP-1000 as non-chiral stationary phase at 140°C (2-hydroxysuccinic acid) or 160°C (2-hydroxyglutaric acid and 2-hydroxyadipic acid) (carrier gas nitrogen flow-rate 1 ml/min; make-up gas nitrogen flow-rate 30 ml/min [2]).

Gas chromatography—mass spectrometry

The 75-eV mass spectra of the derivatized dicarboxylic acids were recorded on a Jeol JGC-20 KP/JMS-D100/W-JMA combination using 3% OV-225 on Chromosorb W HP, 100—120 mesh, as stationary phase (ion-source temperature 150°C; accelerating voltage 3 kV; ionizing current 300 μA). The oven temperature was dependent on the sample under investigation.

RESULTS

The gas chromatograms of the O-acetylated di-(—)-2-butyl esters of DL-2-hydroxysuccinic acid, DL-2-hydroxyglutaric acid and DL-2-hydroxyadipic acid on SP-1000 as non-chiral stationary phase are presented in Fig. 1a—c, respectively. In each case the two peaks, related to the D- and L-enantiomers, were identified by co-chromatography with the O-acetylated di-(—)-2-butyl esters of the corresponding L-2-hydroxydicarboxylic acids (Fig. 1d—f). The diastereomeric derivatives of DL-2-hydroxysuccinic acid elute at about 17 min at 140°C (resolution factor R_D/R_L = 1.02), whereas those of DL-2-hydroxyglutaric acid (R_D/R_L = 1.02) and DL-2-hydroxyadipic acid (R_D/R_L = 1.02) show retention times of about 14 min and 18 min, respectively, at 160°C. In all cases the peak derived from the L-enantiomer has the shortest retention time. For 2-hydroxyglutaric acid, the applied esterification procedure gives rise to lactone formation. The identity of the various peaks was verified by GC—MS using packed columns of OV-225 as stationary phase. The mass spectra of the various derivatives are depicted in Fig. 2.

Reaction of the L-2-hydroxydicarboxylic acids with (±)-2-butanol gives rise to four diastereomers, namely (L; +,+), (L; —,—), (L; +,—) and (L; —,+)*. However, capillary GLC of the O-acetylated samples on SP-1000 showed only two peaks for each dicarboxylic acid. The pictures are exactly the same as those given in Fig. 1a—c. On non-chiral stationary phases like SP-1000, (L; +,+) and (D; —,—) forms are eluted together, as are (L; —,—) and (D; +,+), (L; +,—) and (D; —,+), and (L; —,+) and (D; +,—). Using the data mentioned above, it can be

*(L; —,+) means L-2-hydroxydicarboxylic acid with a (—)-2-butyl group at the carboxyl function connected directly to the chiral centre of the acid and a (+)-2-butyl group at the additional carboxyl function, etc.

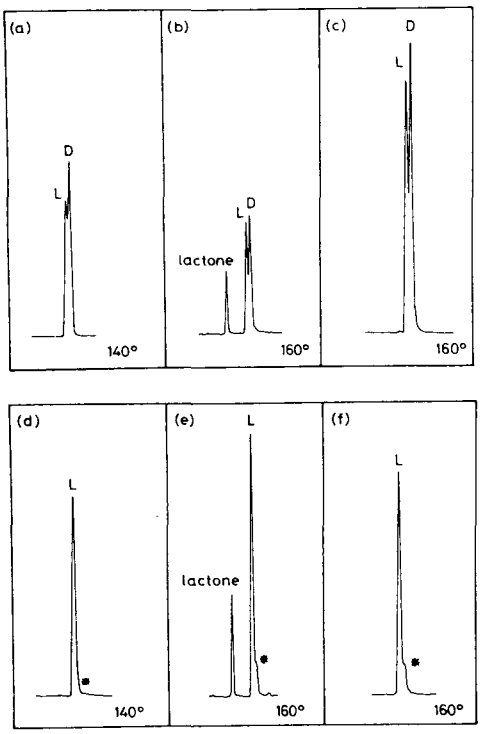

Fig. 1. Partial gas chromatograms on a SP-1000 WCOT capillary column (25 m × 0.3 mm I.D.) of the O-acetylated di-(—)-2-butyl ester derivatives of (a) DL-malic acid, (b) DL-2-hydroxyglutaric acid, (c) DL-2-hydroxyadipic acid, (d) L-malic acid, (e) L-2-hydroxyglutaric acid, and (f) L-2-hydroxyadipic acid (* indicates the shoulder obtained from the contaminating (+)-2-butanol in the (—)-isomer).

concluded that the first peaks correspond with the (L; —,—) diastereomers and the second ones with the (L; +,+) diastereomers. In view of the two peaks obtained instead of four, it is highly probable that the chiral ester function not directly attached at the chiral centre of the acid does not influence the chromatographic behaviour of the diastereomeric derivatives, so that the (L; —,+) form will coincide with the (L; —, —) form, and the (L; +, —) form with the (L; +, +) form.

The peaks of the O-acetylated di-(—)-2-butyl esters of the L-2-hydroxydicarboxylic acids (Fig. 1d—f) show a shoulder with a higher retention time, owing to the presence of a small amount of the (+)-enantiomer in the (—)-2-butanol sample [(—)/(+) = 94:6]. As expected (see above), the shoulders coincide with the O-acetylated di-(—)-2-butyl esters of the corresponding D-2-hydroxydicarboxylic acids.

BIOMEDICAL APPLICATIONS

The separation method developed has been applied to the determination of the absolute configuration of 2-hydroxydicarboxylic acids present in the urine of patients with various metabolic disorders.

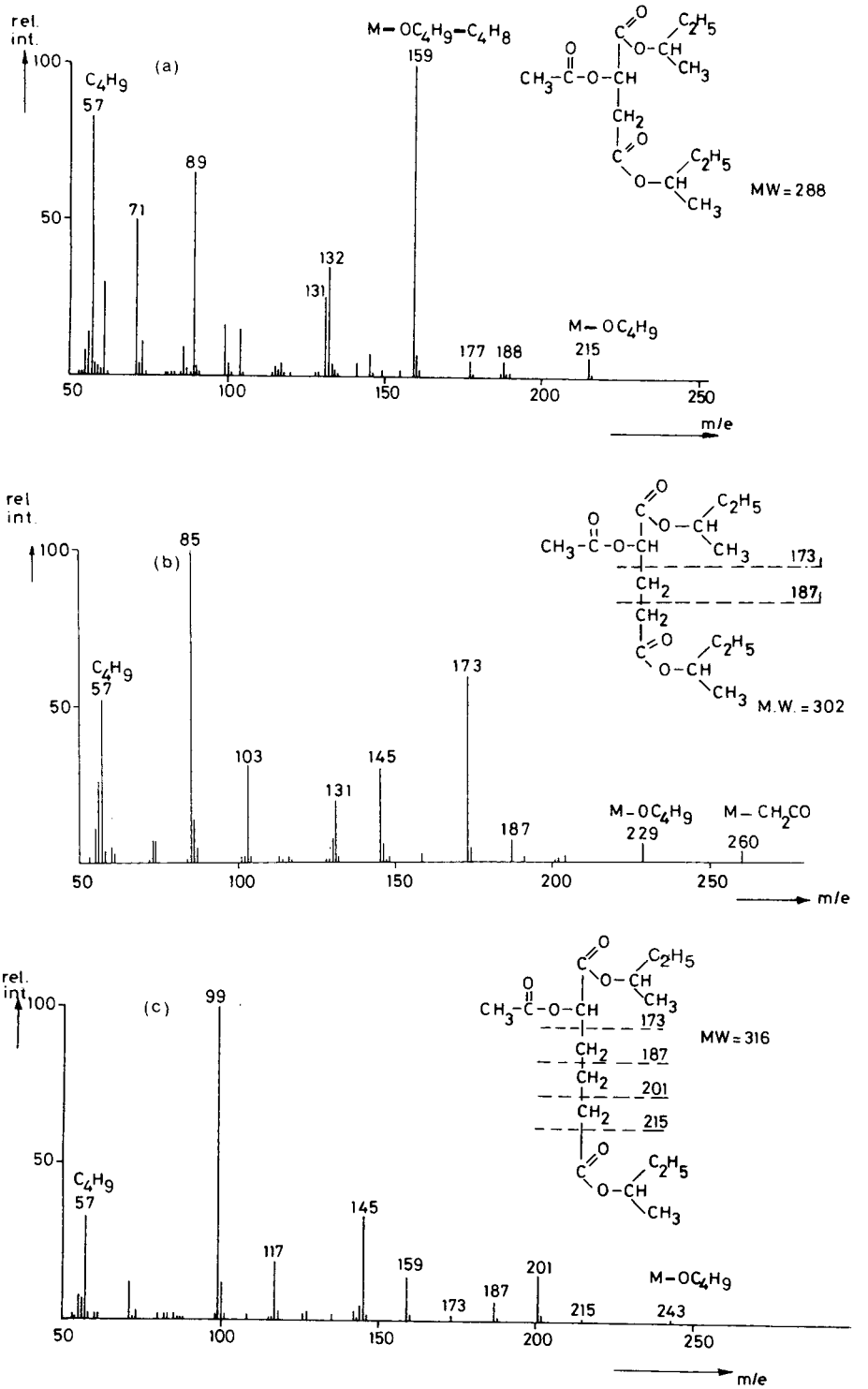

Fig. 2. 75-eV mass spectra of the O-acetylated di-(−)-2-butyl ester derivatives of (a) DL-malic acid, (b) DL-2-hydroxyglutaric acid, and (c) DL-2-hydroxyadipic acid.

2-Hydroxysuccinic acid (malic acid)

The profile of urinary organic acids was determined in two patients (aged 9 and 18 months) with lactic acidemia. In one of them a deficiency of pyruvate carboxylase was established in the liver. In addition to large amounts of lactic acid, substantial excretions of succinic, fumaric, 2-hydroxysuccinic, 2-oxoglutaric and 3-hydroxybutyric acids, and to a lesser degree of glutaric, adipic and 2-hydroxyglutaric acids were found. The urinary 2-hydroxysuccinate excretion was about 0.6 mmol/l in both patients. Capillary GC of the urinary extract treated with (—)-2-butanol and acetic anhydride showed 2-hydroxysuccinic acid to possess exclusively the L-configuration (Fig. 3a). The concentrations of 2-hydroxyglutaric acid in these samples were too low to allow determination of their absolute configuration directly.

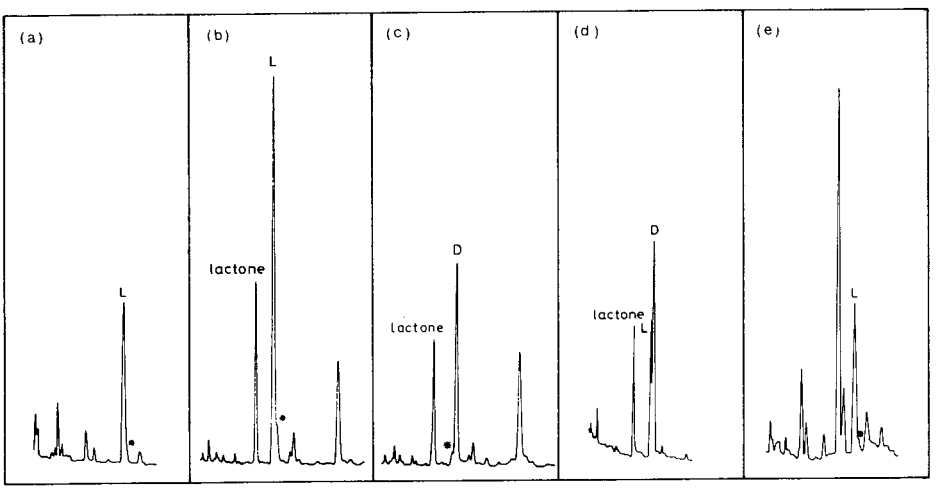

Fig. 3. Partial gas chromatograms of urinary extracts from different patients, derivatized with (—)-2-butanol and acetic anhydride. (a) L-Malic acid; (b) L-2-hydroxyglutaric acid; (c) D-2-hydroxyglutaric acid; (d) D- + L-2-hydroxyglutaric acid; (e) L-2-hydroxyadipic acid.

2-Hydroxyglutaric acid

Permanent excretion of increased amounts of 2-hydroxyglutaric acid (6 mmol/l) without the keto-analog was observed in a mentally retarded five-year-old boy. The capillary GC tracing of the derivatized urinary organic acids showed the presence of the O-acetylated di-(—)-2-butyl ester of L-2-hydroxyglutaric acid (Fig. 3b). There is no clue to the over-excretion of L-2-hydroxyglutarate in this patient [9].

By capillary GC the excessive excretion of D-2-hydroxyglutaric acid was demonstrated in two patients: a 13-month-old boy with hereditary fructose intolerance (fructose-1-phosphate aldolase deficiency) who excreted 0.6 mmol/l; and a 13-year-old boy suffering from egg allergy and protein-losing gastroenteropathy (5 mmol/l) (Fig. 3c). It has been proposed that the latter patient suffered from a defect of D-2-hydroxyglutarate dehydrogenase [10].

Analysis of the 2-hydroxyglutaric acid excreted by a five-month-old girl, admitted because of severe neurological abnormalities, convulsions, and failure to thrive, showed the simultaneous occurrence of both enantiomers. The

excretion of 2-hydroxyglutarate was variable, as was the ratio of the D- and L-enantiomers (Fig. 3d). Extended screening for metabolic disorders revealed that the girl excreted large amounts of uracil. In a brain biopsy specimen necrosis of both white and gray matter was demonstrated.

2-Hydroxyadipic acid

Two patients with 2-aminoadipic aciduria were studied: a girl of 4 [11] and a boy of 3. A reduced degradation of 2-oxo-adipate to glutaryl-CoA was demonstrated in one of the patient's fibroblasts. In addition to 2-aminoadipic acid, considerable amounts of 2-oxo-adipic acid and 2-hydroxyadipic acid were excreted. The latter compound proved to have the L-configuration in both cases (Fig. 3e). Because of the presence of interfering substances in one of the samples, the hydroxy acid was isolated from the urine by means of one-dimensional paper chromatography using 1-butanol—acetic acid—water (4:1:1, v/v) as solvent.

DISCUSSION

As has been demonstrated in this study, the optical isomers of the mono-hydroxylated dicarboxylic acids 2-hydroxysuccinic acid (malic acid), 2-hydroxyglutaric acid and 2-hydroxyadipic acid can be separated on the non-chiral stationary phase SP-1000 using the corresponding O-acetylated di-(—)-2-butyl ester derivatives. The resolution of the malic acid enantiomers has been reported earlier by Pollock and Jermany [12] after derivatization with 2-butanol, 3-methyl-2-butanol or 3,3-dimethyl-2-butanol and O-acetylation. Absolute configuration studies of malic and 2-hydroxyadipic acid using O-(—)-menthyloxycarbonyl methyl ester derivatives were reported in relation to the absolute configuration of unsaturated hydroperoxy fatty acids formed from certain unsaturated fatty acids by incubation with different lipoxygenases [13, 14]. For the determination of the absolute configuration of urinary chiral organic acids it is important that extensive purifications can be omitted. The possibility of using simple ethyl acetate extracts makes the approach more generally applicable in clinical chemistry. By GC both enantiomers of a compound can be analyzed simultaneously. The same holds for the analysis of series of chiral organic acids. The use of 2-butanol as an esterifying agent instead of the previously used menthol has the advantage of higher volatility of the derivatives, enabling the study of larger chiral organic acids. A minor drawback of the use of (—)-2-butanol is the presence of about 6% of (+)-2-butanol in all commercially available preparations of this reagent; on the other hand, the specific peak pattern so obtained can give direct information about the purity of the main GC peak.

The absolute configurations of the hydroxylated dicarboxylic acids in the patients' urine samples give rise to the following comments.

The absolute configuration of the urinary malic acid is the expected one. The reactions of the citric acid cycle are highly stereospecific and it has been known for a long time that the fumarase-catalyzed hydration of fumaric acid leads exclusively to L-malic acid. Hence it can be concluded that the excessive excretion of L-malic acid by patients with, for example, pyruvate carboxylase

deficiency is due to excessive production and/or loss of citric acid cycle intermediates.

The two forms of 2-hydroxyglutaric acid are undoubtedly produced via different metabolic pathways and it may even be questioned whether both compounds are of endogenous origin. It is tempting to relate the 2-hydroxyglutarate production to the availability of excess 2-oxo-glutarate, but only the last patient excreted substantial amounts of 2-oxo-glutarate and there appeared to be no correlation between the excretory levels of these two acids.

The urinary L-2-hydroxyadipic acid in patients with 2-aminoadipic aciduria is probably produced from 2-oxo-adipic acid by a non-specific enzyme. The finding that synthetic L-2-hydroxyadipate does not react with L-lactate dehydrogenase makes it improbable that this enzyme would mediate the in vivo formation of L-2-hydroxyadipate from 2-oxo-adipate.

Finally, it is evident from this study that a search for the enzymes responsible for the formation of 2-hydroxycarboxylic acids would be worthwhile for a better understanding of normal and especially of abnormal organic acid metabolism.

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Note

Separation of collagen types I and III by high-performance column liquid chromatography

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Two main principles have been applied in the past to this category of separations; namely, gel permeation chromatography and ion-exchange chromatography on cellulose-based ion exchangers [1]. In each case the so-called soft sorbents were used which do not offer the possibility of using high pressures and the separation therefore takes several days. The number of theoretical plates is low and the efficiency of the separation is also low. Recently, the so-called rigid sorbents have been introduced into the separation of proteins, allowing the application of small diameter particles and consequently elution under high pressure [2]. Sorbents in this category offer the possibility of applying the principles of gel permeation chromatography, ion-exchange chromatography and reversed-phase high-performance liquid chromatography (HPLC). The first successful separations were done by making use of controlled-pore glass: more recent are those derived from silica gel or various copolymers. In these sorbents the surface is covered with various forms of hydroxylated compounds. Of these so-called glycophases, those like LiChrosorb-Diol [3], TSK-SW gels [4] and Separon HEMA 1000 Glc [5] should be mentioned. These gels meet the demands of HPLC and offer separations of about two orders more rapid in comparison to the classical ones.

Recently a system of chromatographic procedures (multidimensional chromatography) was described for the separation of different types of collagen and constituent α-chains [6]. This system, though fairly efficient, exploits classical liquid column chromatographic procedures: two successive DEAE-cellulose chromatographic steps and two successive separations on Bio-Gel A 1.5 m. The success of this procedure is based on the preliminary removal

of proteoglycans during the first DEAE-cellulose run. The identification of collagen types I and III* in this system is based on the presence of the disulphide bonds in collagen type III; as these bonds are absent in collagen type I, the limited proteolysis of insoluble collagen type I results in two types of α-polypeptide chains [α_1(I) and α_2, molecular weight 100,000]. However, limited proteolysis of type III collagen, due to the disulphide bonds, results in the formation of $[\alpha_1(III)]_3$ trimer molecules (molecular weight 300,000). Instead of Bio-Gel separation, chromatography on Sepharose CL-6B of these two collagen types was suggested recently by ChandraRajan and Klein [8]. In addition, it is also possible to obtain fair separations of collagen types I and III by CM-cellulose chromatography (Epstein [9], Chung and Miller [10]), where $[\alpha_1(III)]_3$ molecules exhibit an intermediate mobility between α_1(I) and α_2 molecules.

The main disadvantages of all methods described up to now are: (1) the impossibility of distinguishing between $(\alpha_1)_2\alpha_2$ and $[\alpha_1(III)]_3$ molecules; (2) long separation time; and (3) difficulties in separating individual α-chains [α_1(I), α_1(III)] side by side.

In the present study we have used Separon HEMA 1000 Glc (a copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate covalently coated with glucose), because it has been found in preliminary experiments that this sorbent separates the compounds not only according to molecular size but under specified conditions it offers the possibility of separating compounds of equal molecular mass as well.

EXPERIMENTAL

Chromatographic separation

This was done using a Pye-Unicam liquid column chromatograph LC 20 equipped with the UV detector LC-3. For detection the wavelength was set at 230 nm. A stainless-steel column [11], 500 × 8 mm, packed with Separon HEMA 1000 Glc (12—17 μm; Laboratory Instrument Works, Prague, Czechoslovakia) was used [12]. The apparatus was operated at a flow-rate of 1.5 ml/min and 1.5 MPa over-pressure. The sensitivity of the detector was set at 0.04, the recorder speed was 0.25 cm/min. The whole separation lasted less than 30 min. Elution was carried out isocratically with 0.05 *M* Tris · HCl (pH 7.5) that was 2 *M* with respect to urea.

Pepsin solubilization of collagens

The method used follows almost completely the procedure described by ChandraRajan and Klein [8]. Skin (1 g) from two-month-old rat was finely cut with scissors, extracted overnight with chloroform—methanol (2:1, v/v) and then with methanol for 6 h at 4°C. The defatted tissue was suspended in 0.5 *M* acetic acid (10 mg/ml). Then crystalline pepsin (Worthington Biochemical Corp. Freehold, NJ, U.S.A.) was added at a concentration of 1 mg/ml [5] and the reaction mixture was stirred for 24 h at 8°C. The digest was then

*Since the nomenclature of collagen types and polypeptide changes that constitute the tropocollagen triple helix is rather complex, the reader not familiar with this is referred to ref. 7.

centrifuged at 30,000 *g* for 1 h at 4°C. The insoluble residue was redigested overnight with additional pepsin (the same enzyme:substrate ratio) and centrifuged as above. Supernatants were pooled and dialyzed overnight against 0.02 *M* Na_2HPO_4. The resulting collagen precipitate was collected by centrifugation at 30,000 *g* within 1 h. A flow scheme for this preparation is given in Fig. 1.

Denaturation of pepsin-solubilized collagen

The collagen precipitate was suspended (2—10 mg/ml) in, or dialyzed against, 2 *M* urea containing 0.05 *M* Tris · HCl (pH 7.5) and denatured by heating to 45°C for 30 min. When necessary the material loaded on the column

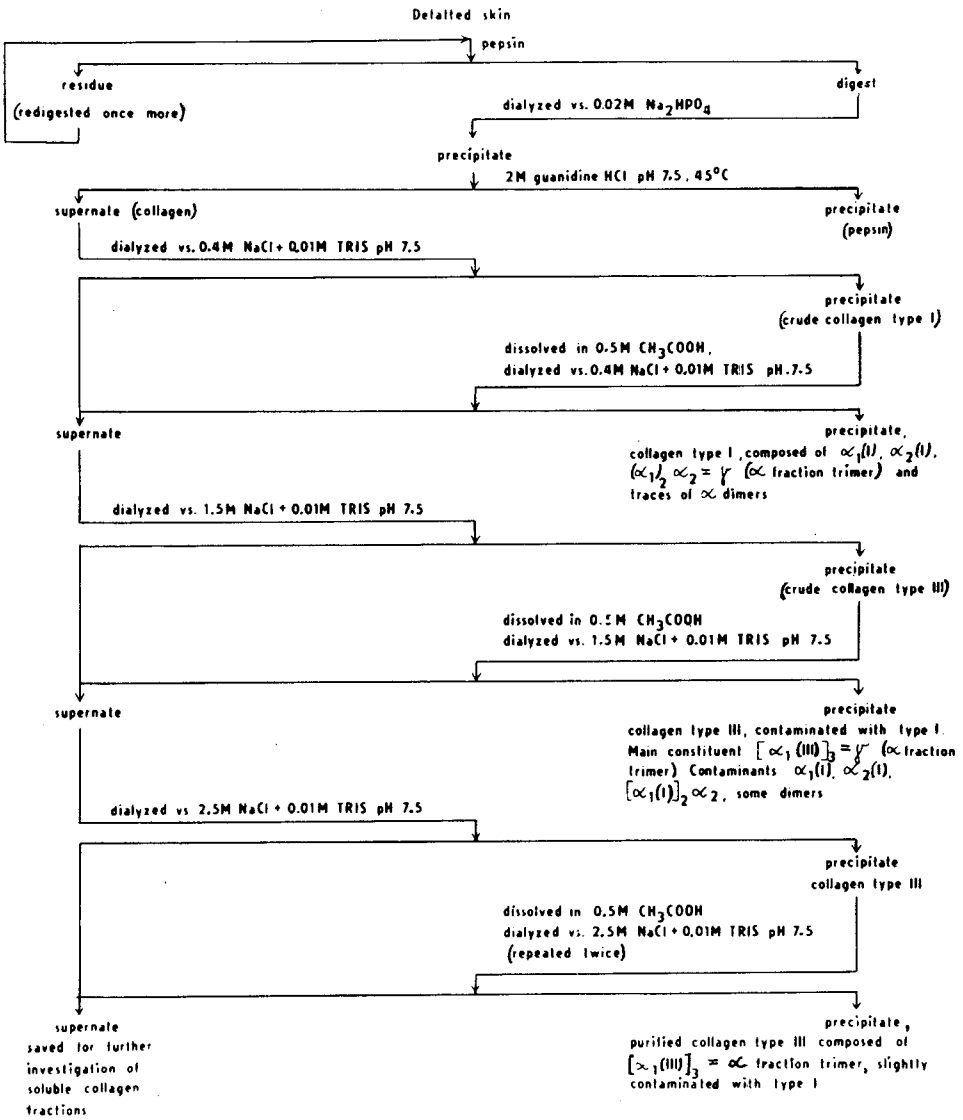

Fig. 1. Flow scheme for preparation of individual collagen types.

was concentrated by adding Aquacide IIA (flake polyethyleneglycol) B grade (Calbiochem, San Diego, CA, U.S.A.) and by removing the gel by brief centrifugation.

Cleavage of disulphide bonds

In order to avoid problems resulting from high UV absorbancy of mercaptoethanol in UV light, S—S bonds were cleaved by performic acid [12]. Denatured collagen was mixed (equal volumes) with concentrated formic acid, and hydrogen peroxide (30%) was added to a final concentration of 2%. The reaction mixture was left for 2 h at room temperature and then loaded onto the column.

Note: Individual collagen fractions isolated by column chromatography and fraction precipitation were checked by their amino acid composition, behaviour during SDS-slab gel electrophoresis [13] and CM-cellulose or Bio-Gel A 1.5 m chromatography [6]. Only those preparations that withstood the above criteria were used for high-resolution liquid column separations.

RESULTS AND DISCUSSION

The applicability of the separation procedure is illustrated in Figs. 2—4. As would be expected, preparations of type I collagen yielded α_1 and α_2 fractions with some β and γ material and slightly contaminated with some breakdown products (Fig. 2). Type III collagen preparations indicated the presence of $[\alpha_1(III)]_3$ which, in spite of the most cautious way of preparation, was contaminated with $\alpha_1(I)$ and $\alpha_2(I)$ material as well as with some other trimer. This material was indistinguishable both chromatographically and electro-

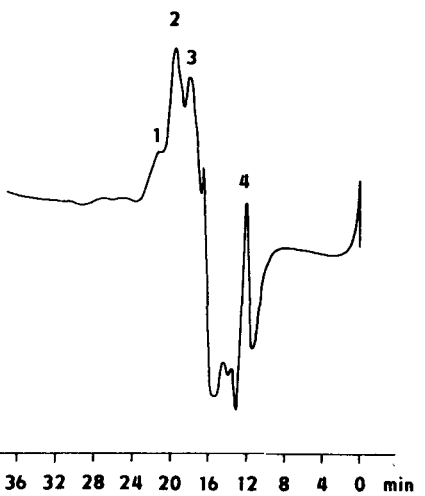

Fig. 2. Separation of polypeptide chains present in collagen type I preparation (10 µl injected). Peaks of intermediate mobility between α and γ are (according to polyacrylamide gel electrophoresis) β fractions (dimers of non-specified α-chains). 1 = Degradation products; 2 = $\alpha_1(I)$; 3 = $\alpha_2(I)$; 4 = γ = $[\alpha_1(I)]_2\alpha_2$.

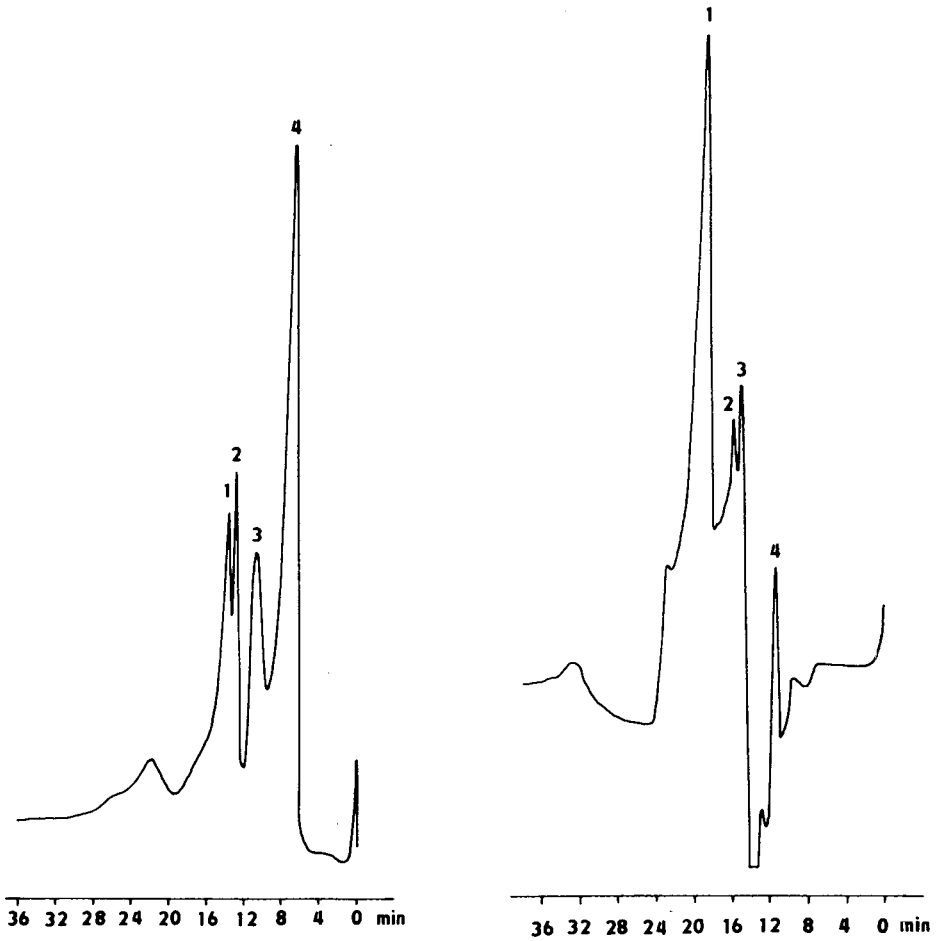

Fig. 3. Separation of polypeptide chains present in collagen type III preparation (2 μl injected). 1 = α_1(I); 2 = α_2(I); 3 = $[\alpha_1(I)]_2\alpha_2$; 4 = γ = $[\alpha_1(III)]_3$.

Fig. 4. Separation of polypeptide chains present in collagen type III preparation after performic acid treatment (2 μl injected). 1 = α_1(III); 2 = α_1(I); 3 = α_2(I); 4 = $[\alpha_1(I)]_2\alpha_2$.

phoretically from $(\alpha_1)_2\alpha_2$ trimer (Fig. 3). After performic acid treatment a pronounced peak of α_1(III) appeared with a mobility lower than both alphas of type I collagen (Fig. 4). Also in mixtures of type I and type III collagen it was possible to distinguish between all three types of α-chains.

Similarly, differences in mobility were also seen in trimers, i.e. native type I collagen. $(\alpha_1)_2\alpha_2$ could be distinguished from type III collagen molecule $[\alpha_1(III)]_3$. For unknown reasons, however, the mobility of $[\alpha_1(III)]_3$ is the highest of all peaks seen while $[\alpha_1(I)]_2\alpha_2$ is the peak to follow. This is just the opposite order of retention to that observed with single α-chains where the order of elution was α_2, α_1(I), α_1(III). Similar relations within dimers (β-chains) were not investigated as these were virtually absent in our pepsin-treated insoluble collagen.

It also appears worth mentioning that in all situations type III collagen preparations were heavily contaminated with collagen type I chains while preparations of collagen type I were devoid of substantial contamination with type III collagen. It was also observed that the contaminating collagen type I α-chains in type III collagen were never in the expected $\alpha_1 : \alpha_2$ 2:1 ratio. Instead, a higher proportion of α_2-chains was consistently seen (Fig. 3). Whether this reflects a higher affinity of α_2-chains for the $[\alpha_1(III)]_3$ molecule or whether this reflects some alternative in assembling individual α-chains into collagen molecules is certainly difficult to decide. The first alternative seems more plausible as long as the other explanation does not fit the generally accepted image about the α-chain composition of individual collagen types.

Obviously, several mechanisms rule this separation. One of these is the molecular-sieve effect that categorizes time periods in which trimers, dimers and eventually monomeric α-chains elute from the column. This mechanism is, however, not the only one as separation of two categories of trimers — $[\alpha_1(I)]_2\alpha_2$ and $[\alpha_1(III)]_3$ — was achieved as well as the separation of three different α-chains. Possible reasons which can be accounted for in separations of molecules with equal molecular weights can be summarized as follows:

(1) Different molecular shapes would lead to different hydrodynamic volumes of separated molecules and thus the elution in three, more or less resolved, α-chain peaks in gel permeation chromatography will result even if there is no other mechanism operative. Calibration of the Separon HEMA Glc column with linear polydextran standards (Pharmacia, Uppsala, Sweden) in 0.05 *M* Tris · HCl + 2 *M* urea shows that this is the main separating mechanism.

(2) Adsorption or partition interactions may be contributing to the resolution obtained by gel permeation chromatography. Hydrophobic adsorption properties of the non-modified Separon HEMA matrix [14] were largely suppressed by the covalent coating [5] with glucose in the sorbent Separon HEMA Glc used in this work. However, some remaining adsorption of hydrophobic domains of collagen α-chains in the eluent used cannot be completely disregarded.

(3) Possible weak selective affinity of collagen chains to the glucose-coated macroporous adsorbent causing respective retention differences of otherwise similar molecules.

It appears that the gel permeation chromatography is primarily controlling the separation; the other mechanisms mentioned most probably contributed to better resolution in this particular case. A detailed study of the role of these interaction principles in fast and highly efficient chromatographic separations of collagen polypeptide chains is being carried out.

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CHROMBIO. 737

Note

Gas—liquid chromatographic determination of pipotiazine in plasma of psychiatric patients

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Pipotiazine palmitate [dimethylsulphamido-3-{(hydroxyethyl-4-piperidino)-3-propyl}-10-phenothiazine palmitate] is a neuroleptic drug of the phenothiazine family (Fig. 1, IA). It is used as an efficacious antipsychotic agent in the

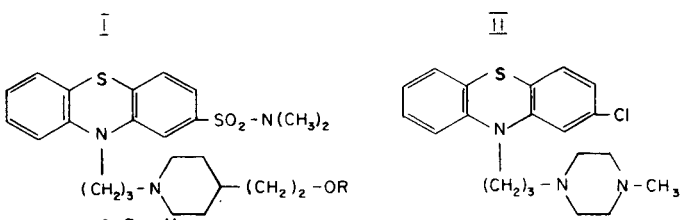

Fig. 1. Structural formulae of pipotiazine (IA), pipotiazine palmitate (IB) and prochlorperazine (II).

treatment of schizophrenia [1—6]. It is available in the long-acting preparation (in sesame oil) as an ester of palmitic acid (Fig. 1, IB) which is intended for intramuscular injection. Another available neuroleptic of this series, viz. pipo-

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tiazine undecylenate, results from the esterification of pipotiazine by undecylenic acid. The principles of depot preparations are (1) slow release of the molecule from the injection site, (2) gradual enzymatic hydrolysis of the ester bond, (3) slow metabolism and (4) its high concentration in the brain.

Recently, we have also found that pipotiazine palmitate is at least as effective or in some cases even superior to fluphenazine enanthate, which is another long-acting injectable neuroleptic in maintaining chronic hospitalized schizophrenic patients [7]. Quantitation of pipotiazine after therapeutic doses requires an analytical method sensitive enough to measure low concentrations in plasma. There is only one study, which describes pipotiazine pharmacokinetics after oral and intravenous administration of tritiated pipotiazine in man. This ratioisotope labelling method was adopted in order to achieve the required sensitivity and accuracy [8]. To our knowledge this is the first report on the specific measurement of pipotiazine in plasma of schizophrenic patients under intramuscular treatment of unlabelled pipotiazine palmitate.

The present study describes a sensitive and specific technique for pipotiazine determination in plasma by electron-capture gas—liquid chromatography (GLC) following acylation of the hydroxyl group with pentafluoropropionyl imidazole (PFPI). The method was applied to quantitative determinations in the plasma of patients treated with pipotiazine palmitate.

EXPERIMENTAL

Reagents

The following reagents were used: Spectroanalyzed grade toluene, isobutyl alcohol and methanol, analytical grade triethylamine (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and a specially purified grade of pentafluoropropionyl imidazole (PFPI) (Pierce Chemicals, Rockford, IL, U.S.A.). The inorganic reagents were made up in doubly distilled water.

Standards

Pipotiazine base and pipotiazine palmitate for chromatographic standards as well as pipotiazine palmitate formulation for intramuscular administration to patients were supplied by Poulenc (Montreal, Canada). Prochlorperazine was used as an internal standard for GLC (Fig. 1, II).

Gas—liquid chromatography

A Hewlett-Packard Model 5830A gas chromatograph equipped with a ^{63}Ni (15 mCi) electron-capture detector (ECD) was used in this study; the instrument was linked to a digital integrator (HP 18850A). The stationary phase was 1% OV-17 on high-performance Chromosorb W (100—120 mesh) packed into a 1.8-m coiled glass column (I.D. 3.5 mm; O.D. 6 mm). The column was conditioned at 275°C for 24 h with an argon—methane (19:1) carrier gas flow-rate of 50 ml/min. The column and the injection port were operated at 265°C and the detector at 300°C. The flow-rate of carrier gas was 50 ml/min. Under these conditions, the relative retention time of pipotiazine to the internal standard prochlorperazine was 1.51 (Fig. 2B).

Extraction procedure

To 3 ml of plasma in a 13-ml conical glass-stoppered centrifuge tube were added 50 μl of the methanolic solution of prochlorperazine (100 μg/ml) as the internal standard. The sample was made alkaline by the addition of 0.5 ml of 5% sodium hydroxide and extracted with 5 ml of toluene—isobutyl alcohol (90:10) by shaking for 15 min in a mechanical shaker. After centrifuging at 600 *g* for 10 min, the organic layer was transferred to another 13-ml tube and the aqueous layer was re-extracted with 5 ml of the same solvent mixture for 15 min. After centrifuging, the organic phases were combined in the previous tube and the aqueous layer was discarded. The organic layer was back-extracted with 2 ml of 0.05 *N* hydrochloric acid for 15 min. The sample was centrifuged as before and the organic layer was removed and discarded. The sample was made alkaline with 0.2 ml of 5% sodium hydroxide and extracted with 2 ml of toluene for 15 min, then centrifuged. The aqueous layer was rejected. The organic layer was transferred into a 3-ml conical glass-stoppered tube and evaporated to dryness at 60°C with a slow stream of nitrogen. The walls of the tube were rinsed with 0.2 ml of methanol by vibrating with a Vortex mixer for 1 min. The solution was evaporated to dryness as before. To the residue were added 50 μl of a solution of 2% (v/v) PFPI in toluene and 20 μl of 0.1 *M* triethylamine in toluene. The mixture was vortexed for 1 min and then heated for 1 h at 65°C. To the mixture were added 100 μl of 0.1 *M* phosphate buffer (pH 6.0) for hydrolyzing the excess of PFPI. The mixture was again vortexed for 1 min, centrifuged at 600 *g* for 3 min and about 2—3 μl of the toluene phase were injected into the gas chromatograph.

Calibration curve

To 3 ml of heparinized blank human plasma in 13-ml glass-stoppered centrifuge tubes were added 10, 15, 20, 25 and 30 μl of methanolic solution of pipotiazine (45 μg/ml) and 50 μl of prochlorperazine solution in methanol (100 μg/ml). The samples were then carried through the complete extraction procedure described above. Quantitation was achieved using the ratio of the peak area of pipotiazine to that of the internal standard prochlorperazine. Peak area ratios were plotted against weight to obtain the calibration curve, which was linear up to 60 ng pipotiazine on injection.

Human studies

Five out of forty chronic ambulatory schizophrenics participating in a large-scale double-blind clinical study of pipotiazine palmitate vs. fluphenazine decanoate were the subjects of this experiment. One of the aims of the study was to establish the effective prophylactic dose of antipsychotic medication for these two drugs. The initial dose of the medication was 12.5 mg of pipotiazine palmitate for one month intramuscularly and then the second injection was in an increment of 12.5 mg. Adjustments during the course of treatment were based on the requirements of each patient and considering the pre-study requirements of neuroleptic of each patient. At the end of the last monthly injection 10-ml blood samples were drawn by venipuncture of the antecubital vein before the next dose. Samples were immediately centrifuged; the plasma was aspirated into a second aluminium foil-wrapped tube and was deep-frozen at

−20°C until analysis. This procedure was followed in order to minimize contact time with the evacuated tube's tip which causes contamination of the sample.

RESULTS AND DISCUSSION

Selectivity

Analytical studies indicate that extracts from blank human plasma do not show peaks that could interfere with the quantitative determination of pipotiazine. This is exemplified by a typical chromatogram resulting from blank plasma and plasma with added pipotiazine (Fig. 2) carried through the extraction procedure. However, under the present GLC conditions, pipotiazine could not be resolved from its chemically and pharmacologically related neuroleptic fluphenazine.

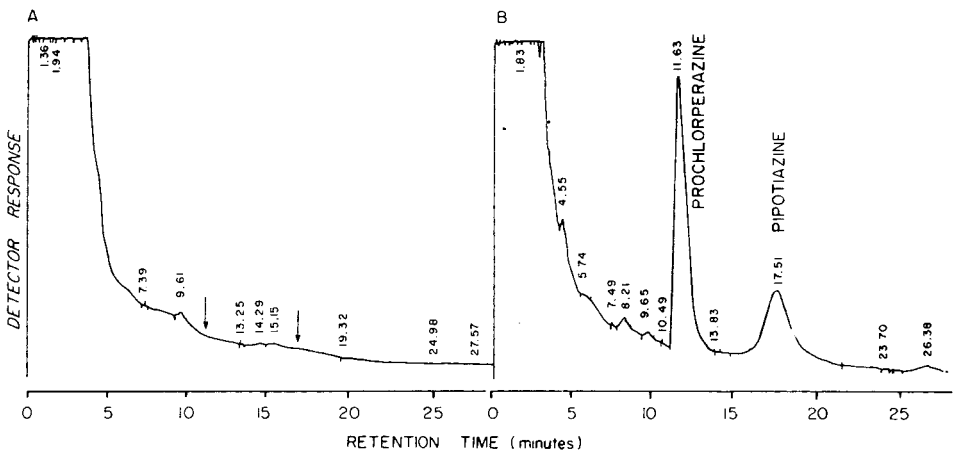

Fig. 2. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma. The arrows show the absence of signals at the retention times of prochlorperazine (internal) standard) and pipotiazine. (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the internal standard prochlorperazine and pipotiazine.

Recovery studies

The absolute recoveries of pipotiazine from spiked plasma were determined using the same internal standardization method as described previously. The peak area ratio of pipotiazine and the internal standard prochlorperazine was used as the index of detector performance and overall efficiency of the analytical procedure. Quadruplicate plasma samples, spiked with pipotiazine at different concentrations were analysed and the results are presented in Table I.

Sensitivity

The method was sensitive enough to measure pipotiazine down to a level of 10 ng/ml of plasma. The lower detection limit was fixed to the minimum response of the ECD to pipotiazine with peak areas up to 40,000 counts at an attenuation of X 128.

TABLE I

ABSOLUTE RECOVERY OF PIPOTIAZINE FROM PLASMA BY GLC

Pipotiazine added (μg/ml)	Pipotiazine recovered (μg/ml)	Absolute recovery* (%)	Coefficient of variation (%)
0.050	0.042	84.00	9.41
0.100	0.083	83.00	6.08
0.150	0.121	80.67	8.72
0.225	0.176	78.22	2.36
0.300	0.230	76.67	5.20
0.375	0.275	73.33	7.63
0.450	0.319	70.89	4.14

*Each value is the mean of four determinations. Mean absolute percentage recovery in plasma is 78.11 ± 4.87%.

Application of the method to human studies

The plasma levels of pipotiazine in psychiatric patients are presented in Table II. The plasma concentrations of pipotiazine measured in patients after a monthly intramuscular injection show a marked individual variation. However, these patients did not receive a uniform dose and had different periods of treatment. The therapeutic levels of pipotiazine are not yet well defined. This method can be useful in clinical therapeutic monitoring of patients.

TABLE II

PLASMA LEVELS OF PIPOTIAZINE IN PATIENTS RECEIVING PIPOTIAZINE PALMITATE THERAPY

Patient No.	Duration of pipotiazine palmitate therapy (weeks)	Last dose of pipotiazine palmitate (mg)	Plasma level of pipotiazine (ng/ml)
1	18	25	28.7
2	19	112.5	18.1
3	22	87.5	39.3
4	27	50	57.9
5	20	112.5	45.5

ACKNOWLEDGEMENTS

The authors express their gratitude to Poulenc, Montreal, Canada for providing them with Piportil$_{L4}$® (pipotiazine palmitate: intramuscular formulation) for clinical use and authentic standards of pipotiazine, pipotiazine palmitate and prochlorperazine for laboratory use. They also acknowledge the technical assistance of Miss Angelique Bordeleau.

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Note

Sensitive assay for pseudoephedrine and its metabolite, norpseudoephedrine in plasma and urine using gas—liquid chromatography with electron-capture detection

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Different techniques have been used to determine pseudoephedrine (PS) and its major metabolite, norpseudoephedrine (NPS) in biological fluids. These techniques include gas—liquid chromatography (GLC) with electron-capture [1—4] and nitrogen-specific [5] detection, radioimmunoassay [6, 7] and high-performance liquid chromatography (HPLC) [8]. However, these methods either have low sensitivity [1, 2] or require large plasma sample sizes [3—6]. Some of the above methods are applicable only to plasma determination [1, 4, 6, 7] and one, only to urine determination [8].

This paper describes a sensitive and specific electron-capture GLC assay of PS and NPS in plasma and urine. The assay is a modification of that described by Lin et al. [2]. However, the toxic extraction solvent, benzene is replaced by toluene and the derivatisation reagent, heptafluorobutyric anhydride by trifluoroacetic anhydride (TFAA). Also a different internal standard was used. More importantly the sensitivity has been increased so that concentrations as low as 50 ng/ml for both PS and NPS in plasma and urine can be detected readily from a 100-μl sample.

EXPERIMENTAL

Reagents and chemicals

Toluene Distal grade (Fisons, Loughborough, Great Britain), 0.01 *M* hydrochloric acid, 4 *M* sodium hydroxide, TFAA (Pierce and Warriner, Chester, Great Britain), pseudoephedrine (Sigma, Poole, Great Britain), norpseudo-

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ephedrine hydrochloride and the internal standard (IS), N-benzylethanolamine (both from Aldrich, Gillingham, Great Britain) were used.

All aqueous solutions were prepared using glass-distilled water unless otherwise stated.

Instrumentation

A gas chromatograph (Model F30, Perkin-Elmer, Beaconsfield, Great Britain), modified to allow samples to be injected from an automatic sampler (Model 7670A, Hewlett-Packard, Winnersh, Great Britain) was used with a pulse-modulated ^{63}Ni electron-capture detector. A coiled glass column (1.8 m × 4 mm I.D.) packed with GP 2% SP-2510-DA on 100—120 mesh Supelcoport (Supelco, Rayleigh, Great Britain) was used isothermally at 121°C. The column was conditioned at 280°C for 24 h (carrier gas, 50 ml/min, was used for the last 22 h only) and silylated if necessary before use. The injection port temperature was 170°C and the detector 263°C. The carrier gas was methane—argon (5:95, v/v) flowing at 90 ml/min. The pulse repetition frequency was 2 kHz, nominal.

The chromatograms, the chromatographic peak areas, the retention times and the area ratios of the PS or NPS peak to the IS peak were calculated and recorded by a data system (Spectra-Physics, St. Albans, Great Britain) which consisted of a central processor (SP 4000), a data interface (SP 4020) and a printer/plotter (SP 4050).

The calibration curve was obtained by the method of internal standardisation. The peak area ratios of PS to IS or NPS to IS in the standard sets were plotted against the standard concentrations. The best straight line was calculated using the least squares linear regression method (Texas Instruments SR-51-II calculator).

Sample preparation

Standards of PS and NPS were prepared by adding the appropriate volume of an aqueous stock solution (1 mg/ml) to blank (i.e. drug-free) plasma or urine to give final concentrations of each compound of 50, 100, 200, 500 and 1000 ng/ml.

To each plasma or urine standard (100 μl) in a 10-ml Sovirel tube (V.A. Howe, London, Great Britain) were added 100 μl of N-benzylethanolamine aqueous solution (IS, 2.5 μg/ml). The mixture was made alkaline with 100 μl of 4 *M* sodium hydroxide solution and toluene (1 ml) was added. The tube was screw-capped, mixed for 30 min along its long axis at 25 oscillations/min and then centrifuged at 1000 *g* for 10 min. As much toluene as possible was taken into a 10-ml BC24/C14T conical centrifuge tube (Quickfit and Quartz, Corning, Stone, Great Britain) using a pasture pipette (Kullman Glass, Whitby, Great Britain). After adding 50 μl TFAA, the tube was vortex-mixed, stoppered with a glass stopper and then left standing at room temperature for 4 h. The toluene layer was washed with 3 ml of ice-cold 0.01 *M* hydrochloric acid and then transferred to a microvial (Hewlett-Packard). The vial was capped and 2 μl of the toluene layer were injected into the gas chromatograph for analysis. Care was taken to ensure that no aqueous layer was transferred with the toluene at any step. The plasma or urine samples were analysed in the same way as the standards. Where necessary the urine samples were diluted with water and the corresponding standards made up in water.

Validation

An estimate of the assay precision for PS and NPS was obtained by carrying out replicate analyses of the standards over the range of 50—1000 ng/ml.

RESULTS

Symmetrical, adequately resolved peaks were obtained for PS, NPS and IS after derivatisation with TFAA (Figs. 1 and 2). The retention times for the TFAA derivatives of PS (PS-TFAA), IS (IS-TFAA) and NPS (NPS-TFAA) were 574, 770, and 1316 sec respectively. Although other substances extracted by the method appeared in the chromatograms, the data system used had no problems in identification and quantitation of the peaks of interest. The results for the method precision and the calibration curves for PS and NPS are summarized in Tables I and II. Both calibration curves were linear over the concentration range 50—1000 ng/ml.

A plasma profile from a healthy fasting male volunteer after taking two 60-mg pseudoephedrine hydrochloride tablets is shown in Fig. 3. Absorption of PS was rapid and NPS was not detected in the plasma samples from this study. The plasma half-life of PS was calculated as 4.3 h which was within the range found

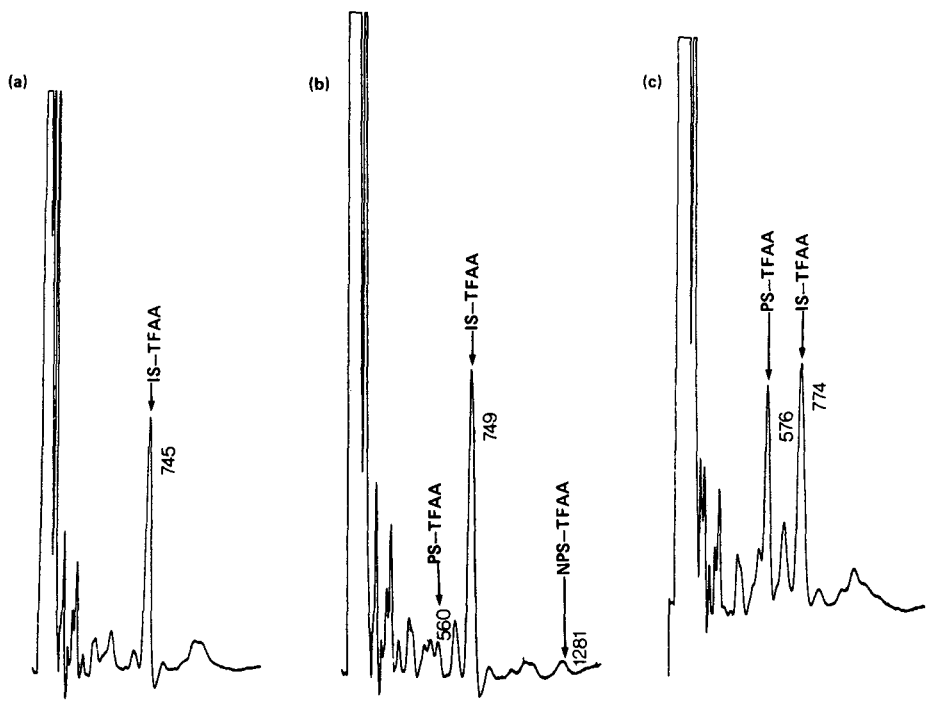

Fig. 1. Typical chromatograms of human plasma after extraction and derivatisation with TFAA. (a) Blank plasma containing the internal standard, N-benzylethanolamine (IS, 2.5 μg/ml). (b) Plasma standard containing pseudoephedrine (PS, 50 ng/ml), norpseudoephedrine (NPS, 50 ng/ml) and IS (2.5 μg/ml). (c) Plasma sample containing PS (461 ng/ml) and IS (2.5 μg/ml). Time in sec.

TABLE I

THE ASSAY PRECISION WHEN PSEUDOEPHEDRINE REPLICATE STANDARDS IN PLASMA OR URINE WERE ANALYSED

	Pseudoephedrine concentration (ng/ml)											
	In plasma						In urine					
	0	50	100	200	500	1000	0	50	100	200	500	1000
No. of replicates	6	6	6	6	6	6	6	5	5	6	5	4
Mean value of peak area ratio	0	0.047	0.092	0.25	0.605	1.233	0	0.043	0.121	0.241	0.643	1.295
Standard deviation	0	0.0087	0.0117	0.0213	0.0439	0.1139	0	0.0060	0.0173	0.0424	0.0473	0.1133
Percentage of standard deviation (%)	0	18.7	12.7	8.5	7.3	9.2	0	13.9	14.3	17.6	7.3	8.8
Correlation coefficient				0.9997						0.9999		
Slope value				0.00124						0.00131		
Intercept				−0.0123						−0.0124		

TABLE II

THE ASSAY PRECISION WHEN NORPSEUDOEPHEDRINE REPLICATE STANDARDS IN PLASMA OR URINE WERE ANALYSED

	Norpseudoephedrine concentration (ng/ml)											
	In plasma						In urine					
	0	50	100	200	500	1000	0	50	100	200	500	1000
No of replicates	6	6	6	6	6	6	6	6	5	6	5	4
Mean value of peak area ratio	0	0.065	0.119	0.258	0.597	1.203	0	0.058	0.126	0.260	0.572	1.128
Standard deviation	0	0.0117	0.0146	0.0159	0.0335	0.1103	0	0.0098	0.0071	0.037	0.0598	0.1048
Percentage of standard deviation (%)	0	18.0	12.3	6.2	5.6	9.2	0	16.9	5.6	14.5	10.4	9.3
Correlation coefficient				0.9999						0.9995		
Slope value				0.00120						0.00112		
Intercept				0.0043						0.0118		

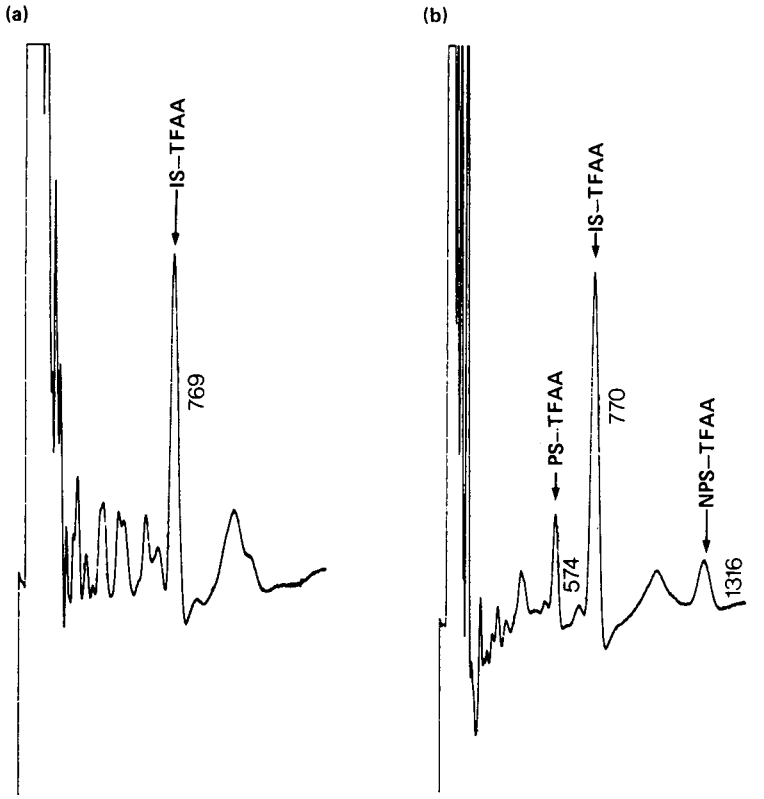

Fig. 2. Typical chromatograms of human urine after extraction and derivatisation with TFAA. (a) Blank urine containing the internal standard, N-benzylethanolamine (IS, 2.5 μg/ml). (b) Urine standard containing pseudoephedrine (PS, 200 ng/ml), norpseudoephedrine (NPS, 200 ng/ml) and IS (2.5 μg/ml).

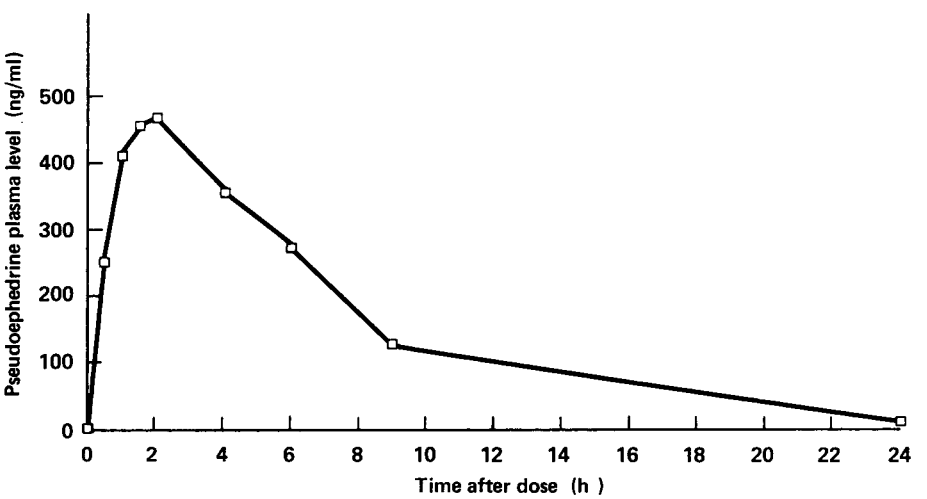

Fig. 3. Plasma profile of pseudoephedrine from a healthy male volunteer after taking two 60-mg pseudoephedrine hydrochloride tablets.

by Kuntzman et al. [6]. The plasma level at 24 h was approaching the sensitivity limit of the assay (about 20 ng/ml).

Cumulative urinary excretion of PS and NPS is given in Table III. In the 0—24-h urine, 43.4% of the dose was excreted as PS and 1.2% as NPS. The rate of urinary excretion of PS is consistent with the calculated plasma half-life in this subject.

TABLE III

EXCRETION OF PSEUDOEPHEDRINE AND NORPSEUDOEPHEDRINE IN URINE FOLLOWING A 120-mg ORAL DOSE OF PSEUDOEPHEDRINE HYDROCHLORIDE

Urine excretion time after dose (h)	Urine pH	Urine volume (ml)	Total amount excreted (mg)	
			Pseudoephedrine	Norpseudoephedrine
0	6.5	—	0	0
0—1.5	6.0	89	8.12	0.07
1.5—6	6.0	342	19.28	0.31
6—9	6.8	234	4.40	0.15
9—24	6.5	618	10.90	0.52

CONCLUSION

The described method is useful for bioavailability and pharmacokinetic studies as the extraction is simple and rapid, the sensitivity is good and only a small sample size is required. Other substances present in plasma and urine are extracted but when chromatographed under the described conditions, they were adequately resolved from the peaks of interest on the chromatograms, so that reliable quantitation for both PS and NPS was obtained. This assay provides good sensitivity and precision for monitoring levels of PS in plasma and PS and NPS in urine after a single therapeutic dose of PS.

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Note

Determination of tofisopam in serum by high-performance liquid chromatography

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Tofisopam (Grandaxin^{R}, 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-2,3-benzodiazepine) is a new type of tranquillizer valuable for the relief of anxiety and tension in a wide range of emotional disorders. There is no convenient method available for the determination of tofisopam in biological fluids.

On the other hand, several papers have already been published on the determination of diazepams, compounds chemically closely related to tofisopam, and its metabolites [1—3]. High-performance liquid chromatographic determination of various benzodiazepines and their derivatives [4—7] proved to be fast and reproducible.

In the present paper we describe a technique for the determination of tofisopam in human serum using high-performance liquid chromatography.

EXPERIMENTAL

Materials

Samples of tofisopam and internal standard [1-(3,4-dimethoxyphenyl)-4,5-

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dimethyl-7,8-dimethoxy-2,3-benzodiazepine] (EGYT-2964)* were products of the Preparative Laboratory of EGYT Pharmacochemical Works (Budapest, Hungary). All other chemicals were of analytical grade.

Chloroform used for extraction was purified in the following way: 100 ml of 1 mol/l hydrochloric acid were added to 1 l of chloroform and shaken for 30 min. The phases were separated, then the organic phase was shaken with 100 ml of 1 mol/l sodium hydroxide for another 30 min, followed by extraction with 2 × 200 ml of distilled water. The washed chloroform was dehydrated by filtration through Whatman phase-separation paper.

Liquid chromatography

Chromatography was performed on a 25 × 0.21 cm LiChrosorb Si-60 (5 μm) column. The sample (20 μl) was applied by a Rheodyne 7120 injection valve.

The column was eluted isocratically, by *n*-heptane—isopropanol—methanol (70:10:1) at a flow-rate of 1 ml/min. The solvent was pumped by an Altex Type 110 pump, back pressure 35 bar. The effluent was monitored by a Spectro-Monitor II of Laboratory Data Control at 311 nm, where tofisopam shows maximum absorbance.

Standard solutions

Tofisopam was dissolved in methanol (1 mg/ml); this solution was diluted ten-fold with distilled water. The internal standard was dissolved in distilled water (100 μg/ml).

Serum standards

Serum standards for calibration were prepared by adding 0—40 μl of the 100 μg/ml tofisopam solution to 5.0 ml of human serum in extraction tubes (16 × 150 mm). To each mixture 20 μl of the internal standard solution were added.

In this way a series of serum standards was prepared containing 0—800 ng/ml tofisopam and 200 ng/ml internal standard each.

Extraction

The serum standards and samples (5 ml) were deproteinized by the addition of 10 ml of methanol containing 0.1 mol/l hydrochloric acid, and 5 ml of 1 mol/l hydrochloric acid. The precipitate was removed by centrifugation for 30 min at 1000 *g*.

The supernatants were transferred by syringe to test-tubes and evaporated to dryness over a water-bath at 60°C in a Rotavapor (Büchi) vacuum evaporator. The dry residue was dissolved in 2 ml of 0.1 mol/l hydrochloric acid and shaken with 10 ml of chloroform for 30 min in a shaking machine. The pH of the water phase was adjusted to 10 by the dropwise addition of 1 mol/l sodium hydroxide. The pH was checked using indicator paper. After adjustment of the pH, shaking was continued for another 10 min. The organic phase was withdrawn by syringe, and the alkaline water phase shaken with 5 ml of chloroform for 10 min. The organic phases were combined and washed with 5

*This compound is available on request from the Preparative Laboratory of EGYT Pharmacochemical Works.

ml of 5% sodium carbonate solution. The organic phase was filtered through Whatman phase-separation paper and evaporated to dryness in a stream of air at room temperature. The dry residue was washed into 0.3 × 3 cm conical test-tubes with 2 × 0.25 ml of methanol and dried again.

The dry residue was dissolved in 0.2 ml of mobile phase.

RESULTS AND DISCUSSION

By changing the polarity of the eluent we could adjust the retention time of tofisopam to 10 min. According to the elution pattern the tofisopam preparation contains a second component with a retention time of 12 min. This compound represents about 12% of the total and is thought to be a conformer of tofisopam [8].

An established way for checking extraction is the application of an internal standard. We used EGYT-2964 for this, since it has very similar structure and extraction properties to tofisopam yet is well separated from it. The retention

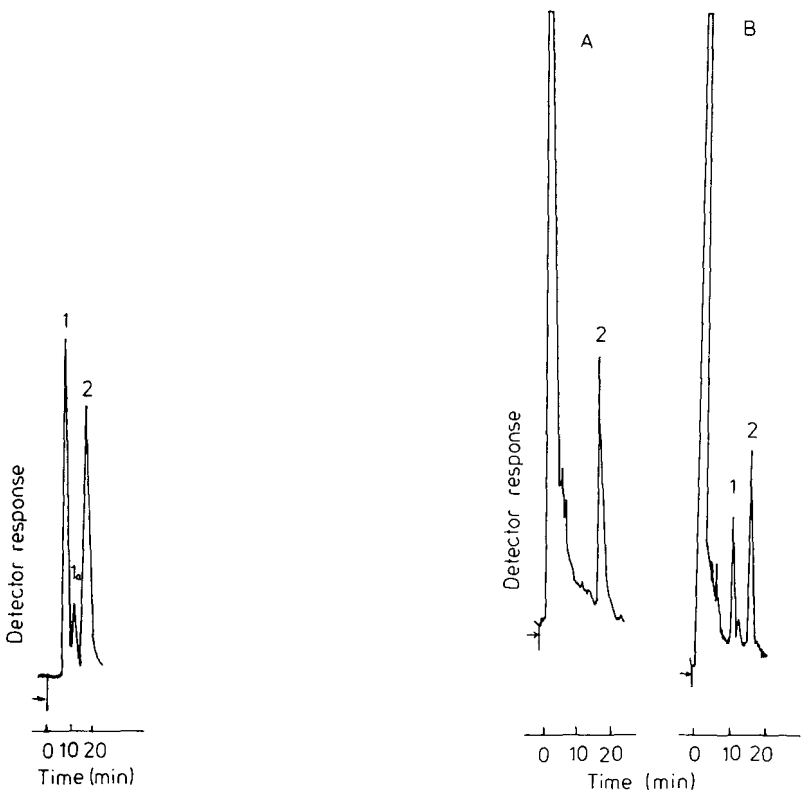

Fig. 1. Separation of tofisopam and internal standard. 1, 150 ng of tofisopam; 1a, tofisopam conformer; 2, 200 ng of internal standard. For separation conditions see Methods.

Fig. 2. Chromatograms of human serum extracts before (A) and after (B) tofisopam treatment. In each case 5 ml of serum were extracted. (A) Serum extract obtained from blood withdrawn before tofisopam treatment; (B) serum extract obtained from blood withdrawn 30 min after tofisopam treatment (100 mg, orally). 1 = Tofisopam; 2 = internal standard.

time of the internal standard was 17 min. A typical chromatogram of the mixture of tofisopam and internal standard is shown in Fig. 1.

A crucial aspect of quantitative techniques is the linearity of the calibration curve. We found that there was a linear reationship between the amount of tofisopam and the detector response expressed in peak height up to 800 ng of tofisopam injected. Likewise, a linear calibration curve was obtained after the extraction of different amounts of tofisopam and constant amounts (400 ng/ml) of EGYT-2964 from serum. The relationship between the peak heights of tofisopam and internal standard could be described by the equation $Y = 0.0054 + 0.0063X$ ($r^2 = 0.9906$) where Y is the ratio of the peak heights of tofisopam and internal standard, and X is the concentration of tofisopam (ng/ml) in serum. The drug concentration in the serum sample was calculated from this formula.

In human serum extracts tofisopam and internal standard separate well from other components extracted by the above technique (Fig. 2). The metabolic products of tofisopam do not interfere; they are eluted either before tofisopam or are strongly bound to the column.

Other contaminants are not retained; therefore, after elution of the internal standard the system is ready for the next sample. Thus the analysis of one sample takes about 20 min.

According to the extraction scheme presented, the efficiency in the concentration range 40—800 ng/ml was 34.6% ± 2.5 (S.D.) with a coefficient of variation of 7.2%. The extraction efficiency of the internal standard was 38.2% ± 0.5 (S.D.), the coefficient of variation 1.3%.

In preliminary pharmacokinetic experiments we could follow changes in the tofisopam concentration of human serum when Grandaxin^R was administered orally to healthy human volunteers (Table I). Since the aim of the method was

TABLE I

PLASMA TOFISOPAM CONCENTRATIONS FOLLOWING ORAL ADMINISTRATION OF 100 mg OF TOFISOPAM IN TABLET FORM

Concentrations are expressed in ng/ml tofisopam.

Subject No.	Time/h			
	0.5	1.0	1.5	2.0
1	<30	96	133	90
2	128	220	46	0
3	35	78	81	47
4	90	220	143	99
5	111	76	76	69
6	257	263	41	49
7	30	64	41	47
8	58	170	70	41
9	93	24	41	30
Mean	89	112	74	50
S.D.	±71	±80	±37	±27

the pharmacokinetic study of the drug, interference in the assay by other drugs was not tested. These pharmacokinetic studies are in progress and will be presented later.

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Note

Determination of xylazine in plasma using high-performance liquid chromatography

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Xylazine hydrochloride (Rompun Bay, 1470) is a sedative analgesic drug which was first evaluated extensively in Germany. Xylazine [2-(2,6-dimethylphenylamino)-4H-5,6-dihydro-1,3-thiazine] was first synthesized in 1962 and since then has been used in many animal species including man. Its sedative properties have been used to good advantage in horses, cattle, sheep, goats, dogs, cats, and many different wild and zoo animals. In the United States, xylazine has been cleared by the FDA for use in horses, dogs and cats, but it has not been cleared for use in food-producing animals.

Assay methods reported for the determination of xylazine [1] in biological fluids include spectrophotometric studies and thin-layer chromatography. But these techniques are slow and not sufficiently sensitive. Moreover, the ionic character and low volatility of xylazine does not permit direct quantitation by gas chromatography.

In the present study, a rapid, sensitive and specific high-performance liquid chromatographic (HPLC) technique was developed for the quantitation of xylazine in plasma and for pharmacokinetic studies.

EXPERIMENTAL

Reagents

Xylazine hydrochloride was purchased from Bayer (Puteaux, France); the reagents were of analytical grade (Prolabo, Paris, France).

Apparatus

The HPLC system consisted of a continuous-flow constant-volume delivery system (Model 6000A; Waters Assoc., Milford, MA, U.S.A.), a U6K universal

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injector (Waters) and a variable-wavelength UV detector (GM 770; Schoeffel Instruments). A stainless-steel column (30 cm × 4 mm I.D.) packed with a stable reversed-phase stationary phase consisting of porous silica beads (mean diameter 10 μm) coated with a chemically bonded monolayer of octadecylsilane (μBondapak C_{18}; Waters) was included with the apparatus.

Standards

A xylazine standard stock solution was prepared containing 1 mg/ml in methanol. The solution was diluted with mobile phase in order to obtain 1, 0.1 and 0.01 μg in a constant injection volume (10 μl). Standard solutions were stored at 4° C.

Drug extraction from plasma samples

Aliquots of plasma (0.5 ml) were pipetted into 21 × 150 mm tubes (Corex; Corning, Corning, NY, U.S.A.). After the addition of 1 μg of internal standard (10 μl of a solution containing 10 mg of doxapram in 100 ml of methanol), 1 ml of buffer solution (0.05 *M* borax, pH 11) was added. The sample was vortexed for 5 sec and then shaken with 10 ml of chloroform for 10 min. After centrifugation at 11,400 *g* for 10 min, the organic phase was isolated by filtration on phase-separating paper (No. 1 PS; Whatman, Clifton, NJ, U.S.A.) and evaporated at 70° C under a nitrogen gas stream. The evaporated extract was redissolved in 100 μl of mobile phase and the entire sample injected into the liquid chromatograph.

Operating conditions

Analysis was performed with a mobile phase permitting ion-pair chromatography by adjusting the pH so that the sample was present in its ionic form (pH 3.5). A strongly ionic counter-ion was chosen with a very lipophilic group attached (heptanesulfonic acid, Pic B7, Waters Assoc.).

The composition of the mobile phase was 2% glacial acetic acid in water—methanol—heptanesulfonic acid (55:45:0.2, v/v). Before use, the mobile phase was degassed by applying vacuum to the solvent reservoir for approximately 5 min. The system was operated at ambient temperature at a flow-rate of 2 ml/min. The UV detector was operated at a wavelength of 225 nm.

The retention times were xylazine 4 min, and doxapram 5.5 min.

Calibration and reproducibility

Known quantities of xylazine chloride in the concentration range 0.1—1 μg were added to blank plasma samples. Calibration curves were constructed by plotting the peak height ratios between xylazine and the internal standard doxapram, versus the amount of xylazine added. The reproducibility of the analytical procedure was checked by determining the calibration curve on four different days. The data were analyzed by analysis of variance and were not found to be significantly different.

RESULTS AND DISCUSSION

The system reported here provides a reliable method for the extraction and quantification of xylazine in plasma. The use of doxapram as internal standard was supported by the fact that its dosage required similar conditions (unpublished observation). Fig. 1 shows chromatograms typical of those obtained for the separation of xylazine and the internal standard, doxapram, following injection of plasma extracts. The linear relationship calculated between the peak height ratio (R) and the concentration (x) of xylazine in plasma up to 1 μg/ml was $R = 4.79x + 0.23$ ($r = 0.998$).

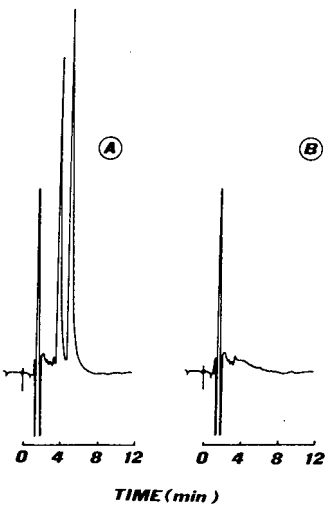

Fig. 1. Chromatograms of sheep plasma extracts (A) containing xylazine (0.4 μg) and doxapram (1 μg) and of blank plasma (B) (0.1 absorbance units full scale).

When standard curves were constructed on four different days over a xylazine concentration range of 0.1—1 μg/ml, an excellent linear relationship was obtained each time. The slopes of the calibration curves were quite reproducible with a coefficient of variation of 4.5%; the coefficients of variation of the different experimental data are between 2.08 and 8.1%.

Recovery of xylazine from plasma was found to be 76.4 ± 3.4% within the concentration range 0.1—2 μg. The proposed assay procedure can be used to estimate levels below 20 ng/ml by using a plasma sample larger than 0.5 ml. The method is sufficiently sensitive to measure pharmacokinetic parameters of xylazine in domestic animals (unpublished data). Moreover, it can be used for the determination of doxapram, for which an HPLC method has not previously been described.

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Note

Quantitation of metolazone in plasma and urine by high-performance liquid chromatography with fluorescence detection

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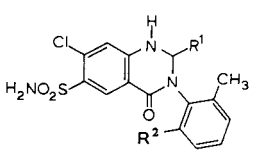
(First received June 30th, 1980; revised manuscript received August 13th, 1980)

Metolazone (Fig. 1, Ia) is a recently introduced diuretic drug. Although a fluorimetric [1] and a high-performance liquid chromatographic (HPLC) [2] assay have been reported for the drug in urine, no method is currently available for metolazone in plasma, with the result that any information about its absorption and pharmacokinetics have been based on measurements of total radioactivity [3].



I (a) $R^1 = CH_3$; $R^2 = H$
(b) $R^1 = (CH_2)_3\,CH_3$; $R^2 = H$
(c) $R^1 = (CH_2)_2\,CH_3$; $R^2 = H$
(d) $R^1 = CH(CH_3)_2$; $R^2 = H$
(e) $R^1 = R^2 = CH_3$

Fig. 1. Structures of metolazone and its analogues.

In conjunction with studies of the pharmacokinetics and absorption of the drug in man a HPLC method utilising a fluorescence detector has been developed to quantify the drug in plasma and urine. The method and some initial results of its application are described in this report.

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EXPERIMENTAL

Apparatus

Chromatography was performed with a component system comprising an Applied Chromatography (Luton, Great Britain) Model 750/03 HPLC pump, Deci-linear gradient programmer, solvent composition optimising unit and a column (10 cm × 5 mm I.D.) fitted with a syringe injector (Shandon Southern Products, Runcorn, Great Britain) and slurry packed with C_8-alkyl silylated silica (5 μm, SAS-Hypersil, Shandon Southern Products) according to the method of Knox et al. [4]. The column was linked to a Perkin-Elmer (Beaconsfield, Great Britain) 3000 fluorescence spectrophotometer fitted with a 100-μl flow cell. The output from the fluorimeter was linked to a 10-mV potentiometric recorder (Servoscribe 1S, Smiths Industries, Cricklewood, Great Britain) and an integrator (Supergrator, Columbia Scientific Industries, Austin, TX, U.S.A.).

Materials

Metolazone (Ia) was obtained from G.D. Searle (High Wycombe, Great Britain) and its four analogues (Fig. 1) were kindly donated by Pennwalt (Rochester, NY, U.S.A.). All other reagents, and solvents which were redistilled before use, were of Analar quality, purchased from Hopkin and Williams (Chadwell Heath, Great Britain). Control plasma and urine samples were obtained from healthy human volunteers.

Selection of fluorimetric conditions and internal standard

The fluorescence spectra of metolazone and its analogues recorded in methanol—water (50:50), exhibited excitation and emission maxima at 230 and 420 nm respectively. The separation of metolazone from plasma components was optimised using flow programming and the metolazone analogues were chromatographed in the selected optimised solvent mixture methanol—water (35:65). This showed the 2-isopropyl analogue (Id) to be a suitable internal standard for a quantitative assay.

Plasma assay procedure

A 0.05-ml aliquot of a solution (1.25 μg/ml) of the internal standard (Id) in methanol—water (50:50) was added to plasma (2 ml) in an acid-washed amber glass tube. The plasma was then extracted with hexane (2 ml) on a partitioning extractor for 5 min, centrifuged and the hexane layer was discarded. Saturated potassium dihydrogen phosphate solution (2 ml) was added, mixed and the acidified plasma was extracted with chloroform (4 ml), centrifuged, the chloroform layer was transferred to an acid-washed amber glass Quick-Fit tube (MF 23/5) and evaporated to dryness under a nitrogen stream.

The residue was dissolved in 50 μl methanol—water (40:60) and 10-μl aliquots were analysed on a column of SAS-Hypersil (5-μm; 10 cm × 5 mm I.D.) with methanol—water (35:65) mobile phase at a flow-rate of about 2.3 ml/min, pressure 80—100 bar, with the fluorimeter set at 230 nm (excitation) and 420 nm (emission). The peak height ratio of metolazone and the internal standard, and the metolazone concentration in unknown samples were determined by the integrator. The calibration curve and the assessment

of the accuracy and precision of the assay method were obtained by the analysis of plasma samples containing added metolazone.

Urine assay procedure

A 0.05-ml aliquot of a solution (11 μg/ml) of the internal standard (Id), in methanol—water (50:50) was added to the urine (0.5 ml) in an acid-washed amber glass tube. Saturated potassium dihydrogen phosphate solution (0.5 ml) was added, mixed, and the acidified urine was extracted with chloroform (1 ml). The chloroform extract was separated by centrifugation and evaporated to dryness. The residue was dissolved in 0.5 ml methanol—water (40:60) and 0.01-ml aliquots were analysed as described above for the plasma extracts.

RESULTS AND DISCUSSION

The chromatographic conditions provided an efficient resolution of metolazone and the internal standard from the endogenous plasma and urine components as shown in Figs. 2 and 3, although a small peak with a retention time close to that of metolazone was observed. The slurry packed SAS-Hypersil reversed-phase columns (10 cm × 5 mm I.D.) gave 4200—5500 theoretical plates for metolazone and the internal standard which had retention times of about 4 and 8 min respectively. Column performance deteriorated gradually during the analysis of plasma extracts, but the high efficiency could be regained by replacing the ballotini beads and re-packing the first 2—3 mm of the column with a concentrated slurry of the stationary phase. However, after the analysis of some 150—200 plasma samples column performance could not be improved by this technique and a new column was prepared.

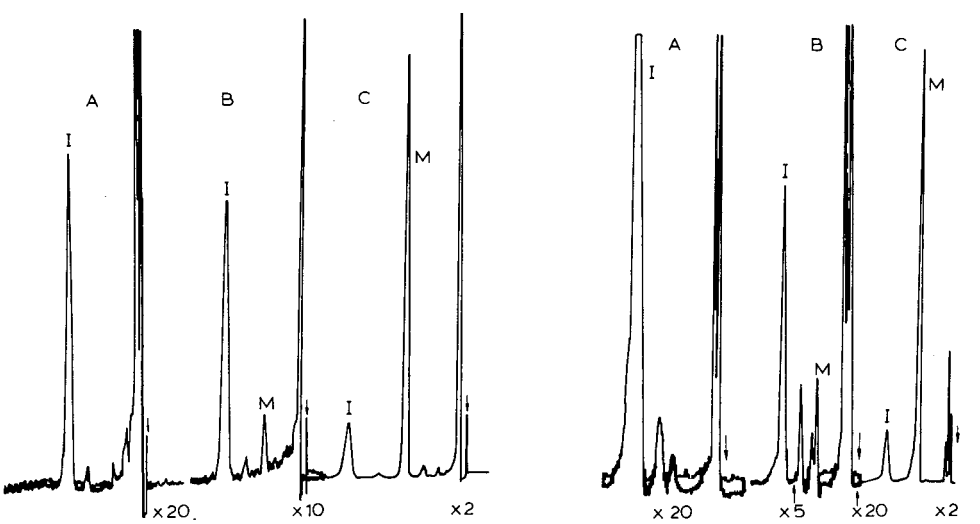

Fig. 2. Chromatograms of extracts from plasma samples containing (A) 0, (B) 3 and (C) 124 ng/ml of metolazone. M = Metolazone; I = internal standard.

Fig. 3. Chromatograms of extracts of urine samples containing (A) 0, (B) 50, and (C) 5000 ng/ml of metolazone. M = Metolazone; I = internal standard.

There was a linear correlation between the metolazone concentration in plasma and the metolazone/internal standard peak height ratio over the concentration range 0—200 ng/ml. The analysis of quality control plasma samples showed a satisfactory level of accuracy and precision (Table I). Plasma levels of 4.7 ng/ml could be accurately measured with a between-assay coefficient of variation of 8.1%. The limit of detection was about 1 ng/ml with a 2-ml plasma sample.

TABLE I

BETWEEN-ASSAY ACCURACY AND PRECISION OF PLASMA METOLAZONE ASSAY

Metolazone concentration (ng/ml)			C.V. (%)	Recovery (%)
Theory	Measured	*n*		
107	105	2	—	98.1
51.4	52.8	4	1.1	103
25.7	25.0	6	1.4	97.2
10.3	10.7	5	4.1	104
4.7	4.89	3	8.1	104
			Mean	101 ± 3.3% (S.D.)

There was a linear correlation between the metolazone concentration in urine and the metolazone internal standard peak height ratio over the concentration range 0—5000 ng/ml. Analysis of quality control urine samples showed a satisfactory level of accuracy and precision (Table II), and 50 ng/ml concentrations could be measured with a between-assay coefficient of variation of 7.8%. The limit of detection was about 5 ng/ml with a 0.5-ml urine sample.

Assay interference by metolazone metabolites was not specifically investigated. However, some 80% of the administered compound is excreted unchanged by man [3], and the known metabolites of metolazone are more polar than the drug (Pennwalt Corporation, unpublished data) and would be expected to elute in the solvent front region of the chromatogram. Thus assay interference by metabolites in human samples seems unlikely.

TABLE II

BETWEEN-ASSAY ACCURACY AND PRECISION OF URINARY METOLAZONE ASSAY

Metolazone concentration (ng/ml)			C.V. (%)	Recovery (%)
Theory	Measured	*n*		
5000	5070	4	2.4	101
2000	1979	3	0.4	99.0
50	51.8	5	7.8	104
			Mean	101 ± 2.5% (S.D.)

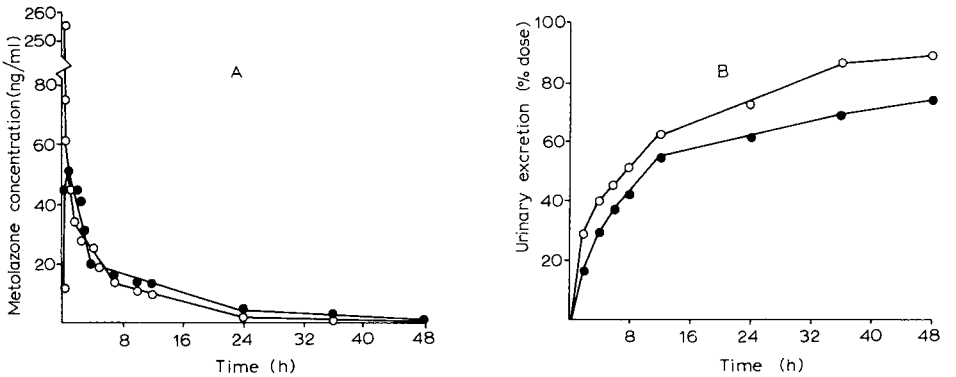

Fig. 4. Plasma levels (A) and urinary excretion (B) of metolazone in a healthy man following single 5-mg intravenous (○) and oral (●) doses of the drug.

The method allowed the measurement of plasma levels and urinary excretion of metolazone in man for up to 48 h after single intravenous or oral solution doses containing 5 mg of metolazone, as shown in Fig. 4.

Metolazone is structurally related to the thiazide diuretics, e.g. hydroflumethiazide, hydrochlorothiazide, and some of these might interfere in the assay during the analysis of samples from patients receiving metolazone in combination with other drugs. The specificity of the assay would therefore have to be determined relative to the co-administered drugs.

Until recently the most common analytical methods for the quantitation of the thiazide drugs have been fluorimetry [1, 5] and gas chromatography [6—8]. However, direct fluorimetry may lack specificity, and all these drugs must be derivatized prior to gas chromatographic analysis, which makes these assays more time consuming. The combination of fluorescence detection and HPLC described in this report therefore offers a new sensitive, specific and simpler quantitative method for this class of diuretic drugs in biological fluids. Applied to metolazone this technique has provided an assay some 50—100 times more sensitive than previously published assays [1, 2] and allowed its accurate and precise determination in plasma for the first time. It seems probable that this assay procedure will also be applicable to the determination of metolazone in whole blood.

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Note

Improved microdetermination of gentamicin and sisomicin in serum by high-performance liquid chromatography with ultraviolet detection

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A high-performance liquid chromatographic (HPLC) method for the determination of gentamicin in serum was previously reported [1]. In the present paper we describe a modified procedure, using an internal standard, as well as the application of the method to the determination of sisomicin in serum. To our knowledge no other chromatographic determination of sisomicin in serum has been reported. The HPLC determination of sisomicin is compared with a microbiological assay. An in vivo experiment in which the sisomicin concentration is determined following intramuscular administration of the drug is reported.

MATERIALS AND METHODS

Chemicals and reagents

The water used was demineralized. 1-Fluoro-2,4-dinitrobenzene (FDNB), tris(hydroxymethyl)aminomethane (Tris) and acetic acid were p.a. grade from Merck (Darmstadt, G.F.R.). Acetonitrile, "zur Synthese", was also from Merck.

The preparations for injections — Garamycin® (Essex, Heist-op-den-Berg, Belgium), Obracin® (Eli Lilly, St.-Cloud, France) and Extramycin® (Bayer, Leverkusen, G.F.R.) — contained the equivalents of 40 g/l gentamicin, 40 g/l tobramycin and 50 g/l sisomicin, respectively. The gentamicin components C_1, C_{1a} and C_2 were obtained as their sulphate salts (by courtesy of Schering, Bloomsfield, NJ, U.S.A.). Sisomicin sulphate, lot Pt 489 851, standard substance was by courtesy of Bayer Nederland (Mijdrecht, The Netherlands). All antibiotic concentrations were calculated relative to potency.

Pooled human serum from ambulant patients was frozen and stored at −18°C within three days of collection.

Stoppered polypropylene centrifuge tubes of 1.5 ml capacity, and ampoules of 0.5 ml capacity were also used.

Chromatographic conditions

The chromatographic instrumentation was described previously [1].

The mobile phase was prepared by mixing 300 ml of water (filtered through a 0.2-μm filter) with 700 ml of acetonitrile (filtered through a 0.2-μm filter) and 1 ml of acetic acid, and deaerated ultrasonically. The flow-rate was 3.0 ml/min. A μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) was used (Waters Assoc., Milford, MA, U.S.A.). Chromatography was performed at room temperature. Ultraviolet detection was made at 365 nm. The detector signal was recorded at two different attenuation settings.

Procedures

Procedure A: derivatization of aminoglycosides. Dispense into a centrifuge tube 50 μl of serum or aqueous aminoglycoside solution; add 50 μl of a solution containing 20 g/l Tris in water, also containing the equivalent of 160 mg/l tobramycin (the internal standard), and vortex. Add 200 μl of acetonitrile and vortex. Centrifuge, in the case of serum samples, at 2500 *g* for 5 min. Transfer 200 μl of the supernatant into an ampoule, add 20 μl of FDNB in acetonitrile (170 g/l) and close the ampoule. Place in a water-bath at 80°C for 45 min. Inject 150 μl into the chromatograph.

Procedure B: in vivo experiment and bioassay comparison study. A healthy 70-kg volunteer received 75 mg of sisomicin by intramuscular injection. Blood samples were collected at regular time intervals, and the serum was separated and stored at −18°C. Sisomicin standards in the range 0—4 mg/l were prepared by adding appropriate quantities of aqueous solutions of sisomicin sulphate standard substance to pooled serum. Standards and serum samples were analyzed in duplicate in one run according to procedure A.

The serum samples, obtained from the in vivo experiment, were also analyzed in duplicate by a microbiological assay, against the same sisomicin standard substance. This bioassay was carried out by the National Institute of Public Health (RIV), Department of Chemotherapy, Utrecht, The Netherlands, using an agar well diffusion technique. The antibiotic medium was DST (Oxoid, Basingstoke, Great Britain). The test organism was *Staphylococcus aureus* Alkmaar, a strain resistant to penicillin, cefalothin, sulfonamide antibiotics, chloramphenicol and streptomycin but susceptible to aminoglycoside antibiotics. The seeded agar was incubated overnight at 37°C and the diameter of the zones of growth inhibition was measured. Zone sizes were plotted against the log values of the concentrations of the standards to obtain a straight line.

RESULTS AND DISCUSSION

The aminoglycoside antibiotics sisomicin and tobramycin and the three main components of the gentamicin complex (gentamicin C_1, C_{1a} and C_2) are closely related (see Table I). These aminoglycosides have the same number of amino

TABLE I

RETENTION OF 2,4-DINITROPHENYL DERIVATIVES OF AMINOGLYCOSIDES RELATIVE TO GENTAMICIN C_{1a}, AND FUNCTIONAL GROUPS

	$RRT_{C_{1a}}$*	Expected No. of derivatized amino groups	No. of hydroxyl groups	No. of methyl groups
Tobramycin	0.47	5	5	0
Sisomicin	0.97	5	3	2
Gentamicin C_{1a}	1	5	3	2
Gentamicin C_2	1.15	5	3	3
Gentamicin C_1	1.15	5	3	4

*Retention time relative to gentamycin C_{1a}.

groups, and there is evidence that all five amino groups of these antibiotics are derivatized by FDNB [1]. Tobramycin has two more hydroxyl groups than the other substances; consequently the tobramycin—dinitrophenyl derivative is the most polar of these derivatives, and has the shortest retention time in this reversed-phase system. The influence of the number of methyl groups on the retention behaviour is much less than the influence of the number of hydroxyl groups. The gentamicin C_2 derivative contains one more methyl group (C—C bond) than the gentamicin C_{1a} derivative. This gives rise to a slightly longer retention time for the gentamicin C_2 derivative due to the less polar nature of the more methylated derivative. In the gentamicin C_1 derivative, one further methyl group is present (N—C bond), but no further increase in retention time was observed.

Sisomicin is a dehydro analog of gentamicin C_{1a}. The retention behaviour of the derivatives of these two compounds is very similar.

With the chromatographic system used, the tobramycin derivative is not completely resolved from peaks near the solvent front. At a high concentration of tobramycin (160 mg/l) the contribution of the blank to the peak height of the tobramycin derivative was found to be only 4% (see Fig. 1). By monitoring the effluent of the column at two different attenuation settings, tobramycin at this high concentration could be used as internal standard in the determination of gentamicin and sisomicin in serum.

Recovery

Recovery of sisomicin from the deproteinization step was estimated by spiking blank serum samples with the same amount of aminoglycoside before and after the addition of acetonitrile; the same procedure was followed for determining the recovery of tobramycin.

The recovery of sisomicin, measured at 4 mg/l, was 84% (S.D. = 6%; n = 6). The recoveries of gentamicin C_{1a} and gentamicin C_1+C_2, measured under the same conditions, were determined previously and found to be 83% and 84% respectively [1]. The recovery of tobramycin, measured at 160 mg/l, was 64% (S.D. = 2%; n = 6).

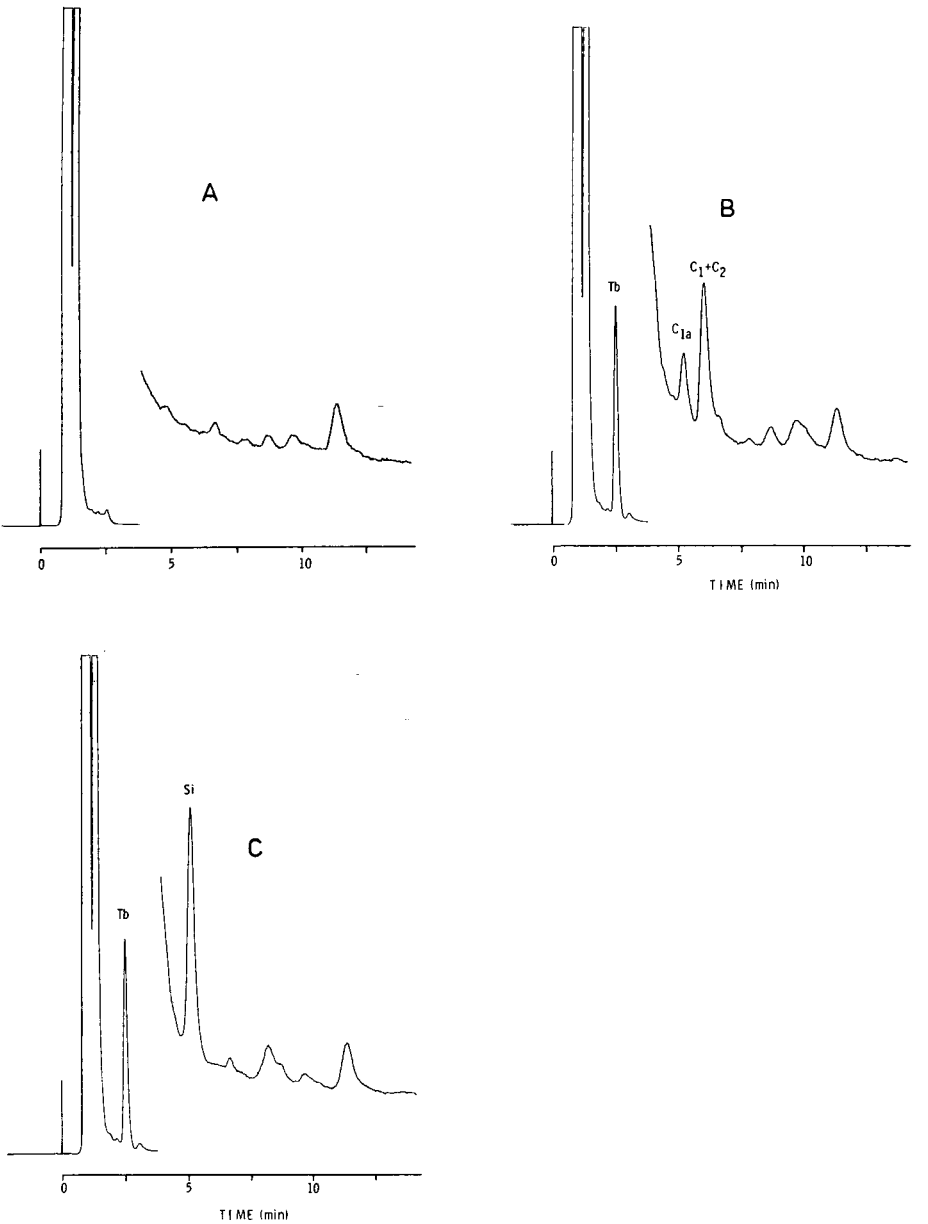

Fig. 1. HPLC of serum samples. Detector settings: 0—3.8 min, 1.0 a.u.f.s.; 3.8—14 min, 0.01 a.u.f.s. Chromatogram A was obtained from 50 μl of blank serum. No internal standard was added. Chromatogram B was obtained from 50 μl of blank serum, spiked to a concentration of 4 mg/l gentamicin with tobramycin as internal standard added. Chromatogram C was obtained from 50 μl of blank serum, spiked to a concentration of 4 mg/l sisomicin with tobramycin as internal standard added. Tb = tobramycin derivative; Si = sisomicin derivative; C_{1a} = gentamicin C_{1a} derivative; C_1+C_2 = gentamicin C_1 and gentamicin C_2 derivatives (not separated).

Other authors also report low recoveries if serum containing a high concentration of tobramycin is deproteinized with acetonitrile [2]. However, as tobramycin is always added at the same concentration, this lower recovery still permits the use of 160 mg/l tobramycin as the internal standard, because the recovery was found to be sufficiently reproducible.

Precision, linearity and sensitivity

Sera were spiked with different quantities of gentamicin, or sisomicin, to obtain concentrations ranging from 0.5 to 16 mg/l, and were analyzed in one run according to procedure A. The results are summarized in Tables II, III and IV. As is apparent from the data provided, the calibration curves are straight lines in the concentration range studied. The 95 per cent confidence interval of the intercept of the calibration line for sisomicin includes zero, so this calibration line passes through the origin. The calibration lines for gentamicin C_{1a} and gentamicin C_1+C_2 have intercepts that do not pass through the origin at this level of confidence. However, the intercepts are small and can be neglected for all practical purposes. The sensitivity of the method is sufficient to allow the determination of sisomicin and gentamicin at concentrations in, and well below, the therapeutic range using 50-μl serum samples.

TABLE II

PEAK HEIGHT RATIOS (PHR) OF DERIVATIZED GENTAMICIN COMPONENTS TO INTERNAL STANDARD (TOBRAMYCIN) AND COEFFICIENTS OF VARIATION (C.V.) OBTAINED WITH SERUM SAMPLES (50 μl) CONTAINING 0.5—16 mg/l GENTAMICIN

Analyses at each concentration were performed in six-fold.

Gentamicin C_{1a}				Gentamicin C_1 and C_2		
Concentration (mg/l)	PHR*	PHR/concentration	C.V. (%)	PHR*	PHR/concentration	C.V. (%)
0.5	0.055	0.111	10	0.110	0.220	2
1	0.095	0.095	4	0.214	0.214	3
2	0.192	0.096	2	0.417	0.209	3
4	0.384	0.096	1	0.850	0.213	2
8	0.745	0.093	1	1.666	0.208	1
16	1.462	0.091	3	3.268	0.204	2

*Peak heights of the tobramycin and gentamicin derivatives based on detector settings of 1.0 a.u.f.s. and 0.01 a.u.f.s., respectively.

In vivo experiment and bioassay comparison study

For the HPLC assay, the working calibration curve obtained from the standard sera gave an intercept with a 95 per cent confidence interval that included the origin, so the working calibration curve was recalculated forcing this line through the origin [3]. The concentrations in the serum samples, obtained in the in vivo experiment, were calculated from this calibration curve. The serum concentration—time curve for sisomicin, obtained by the HPLC assay, is shown in Fig. 2. The comparison of the values obtained from the

TABLE III

PEAK HEIGHT RATIOS (PHR) OF DERIVATIZED SISOMICIN TO INTERNAL STANDARD (TOBRAMYCIN) AND COEFFICIENTS OF VARIATION (C.V.) OBTAINED WITH SERUM SAMPLES (50 μl) CONTAINING 0.5—16 mg/l SISOMICIN

Analyses at each concentration were performed in six-fold.

Concentration (mg/l)	PHR*	PHR/concentration	C.V. (%)
0.5	0.162	0.324	9
1	0.291	0.291	4
2	0.539	0.270	4
4	1.052	0.263	3
8	2.109	0.264	4
12	3.283	0.272	5
16	4.255	0.266	2

*Peak heights of the tobramycin and sisomicin derivatives based on detector settings of 1.0 a.u.f.s. and 0.01 a.u.f.s., respectively.

TABLE IV

STANDARD CURVES FOR AMINOGLYCOSIDES IN SERUM

Aminoglycoside	Range (mg/l)	n*	Equation**		
			Slope	Intercept ± 95 per cent confidence interval	r
Gentamicin C_{1a}	0.5—16	36	0.091	0.012 ± 0.008	0.9993
Gentamicin C_1+C_2	0.5—16	36	0.204	0.017 ± 0.014	0.9996
Sisomicin	0.5—16	42	0.267	0.011 ± 0.037	0.9986

*Number of determinations.
**Estimated by linear least-squares regression analysis.

determinations by the HPLC method and the microbiological method is also shown in Fig. 2. The correlation found is comparable, or better, than those reported in other studies in which chromatographic assays for aminoglycoside antibiotics in human serum are compared to a microbiological assay [4—9].

An elimination half-life of 1.8 h for sisomicin was estimated from the serum concentration—time curve. Doenicke et al. [10], summarizing the results of several investigators, found a half-life of 2 h for sisomicin in healthy subjects.

Advantages of the proposed method

The determination of gentamicin in serum previously reported [1] requires six transfers of accurately measured volumes of liquids. By introducing an internal standard only two accurately measured liquid transfers are necessary. However, as most of these transfers are performed using repeating dispensers and an autosampler injection device, this advantage is comparatively small. In our opinion, the main advantage of internal standardisation is here that a check

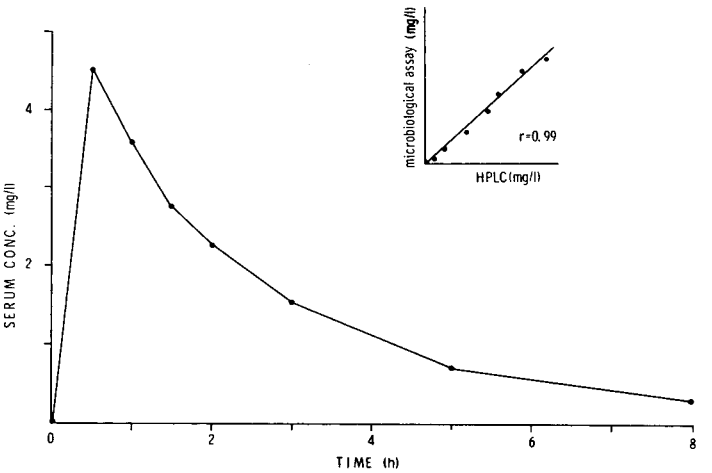

Fig. 2. Serum concentration of sisomicin as a function of time obtained in a volunteer following an intramuscular injection of 1.07 mg/kg body weight. Each value is the mean of two HPLC determinations. Inset: least linear-squares regression analysis of the sisomicin concentrations in the sera from the in vivo experiment, determined by HPLC and microbiological assay. Equation: $Y = -0.04\ (\pm\ 0.24) + 0.91\ (\pm\ 0.10)\ X$ (8 data pairs), where X = result of the HPLC assay (mean of duplicate determinations) and Y = result of the microbiological assay (mean of duplicate determinations); number between brackets: 95 per cent confidence interval.

of the peak heights of the internal standard (which should remain constant in all the chromatograms) provides a means of spotting irregularities due to incorrect sample handling, or fluctuations in the derivatization conditions and/or chromatographic conditions, thereby improving the reliability of the results.

Sisomicin, as compared to gentamicin, is more easily determined by chromatographic methods. Gentamicin consists of three main components, and the ratios between these components may vary, making calibrations in chromatographic procedures more cumbersome [1, 4]. On the other hand, sisomicin consists of one major component, and calibrations can be made against a reliable standard substance.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. A.W.M. Indemans for helpful discussions, Ms. A. Rutgers for the antimicrobial assays, and Mr. J. Teeuwsen and Mr. J.S. Blauw for technical support. Generous support has been provided by Bayer Nederland, Schering, and Eli Lilly Corporations by providing specimens of aminoglycoside antibiotics.

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CHROMBIO. 727

Note

Quantitative thin-layer chromatographic determination of ticrynafen in plasma of the dog

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(First received May 30th, 1980; revised manuscript received September 12th, 1980)

Ticrynafen (I) is a potent diuretic agent with uricosuric properties [1]. It is metabolized by two pathways as shown in Fig. 1 [2]. The first pathway gives a secondary alcohol (II) produced by reduction. This metabolite is then partially transformed into the corresponding methoxy derivative (III). The second metabolic pathway consists of oxidative cleavage at the level of the ketone function and produces a dicarboxylic acid (IV).

Ticrynafen and its metabolites have been determined in biological fluids by gas chromatographic (GC) methods [3, 4] and by measurement of the radioactivity of the ^{14}C-labelled drug in animals [2]. Also a thin-layer chromatographic (TLC) method for the determination of ticrynafen has been reported [5].

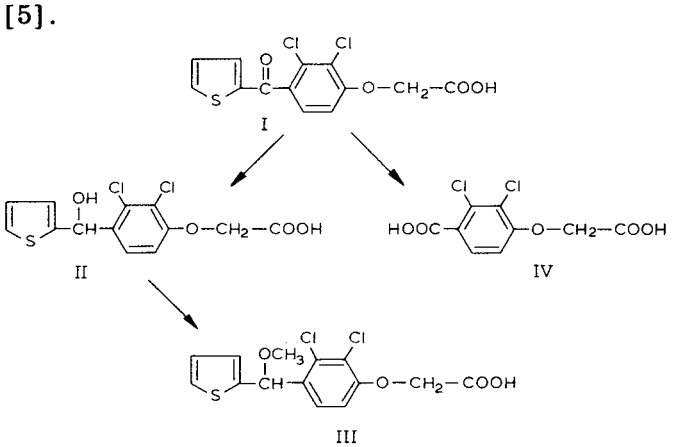

Fig. 1. Proposed pathway of the major metabolites of ticrynafen [2].

This report describes a TLC method for the determination of tricrynafen in plasma of the dog. The method is characterized by a short analysis time, and good accuracy, sensitivity and specificity.

EXPERIMENTAL

Reagents and materials

Ticrynafen and its pharmaceutical preparation, Diflurex tablets, were obtained from Anphar (Chilly-Mazarin, France). The other chemicals were of analytical reagent grade (Merck, Darmstadt, G.F.R.). Commercial silica-gel K6F TLC plates 20 cm × 20 cm (Whatman, Clifton, NJ, U.S.A.), layer thickness 0.25 mm, were used. Solutions were spotted on to the TLC plates using micropipettes.

Apparatus

The shaker was an M4020 from Köttermann (Hanigsen, G.F.R.), the centrifuge a "Superspeed" from Sorvall (Newtown, CN, U.S.A.) and the block thermostat a Grant BT3 (Cambridge, Great Britain).

Preparation of plasma standards

Three plasma standards were extracted for every six unknown samples. First, 66.7 mg of ticrynafen were weighed out into a 100-ml volumetric flask and dissolved in 100 ml of methanol. Into 30-ml centrifuge tubes were placed 10, 50 and 75 μl of this solution. The solutions were then evaporated to dryness in a 50°C water-bath under a stream of nitrogen, whereupon 2 ml of control plasma were added to the residues. The three plasma standards prepared in this way contained 3.3, 16.7 and 25.0 μg/ml ticrynafen.

Extraction procedure

To 30-ml centrifuge tubes, each containing 2 ml of plasma sample or standard, were added 1 ml of 23% hydrochloric acid and 20.0 ml of chloroform. The tubes were stoppered with glass stoppers and shaken mechanically for 15 min at 80 cycles/min. After centrifuging for 5 min at 2611 *g*, 15.0 ml of the chloroform layers were transferred to 25-ml conical test-tubes and evaporated to dryness at 50°C in a block thermostat under a stream of nitrogen. The residues were dissolved in 100 μl of absolute methanol.

For TLC, 20 μl of the solutions were applied to the origin of the TLC plate. Nine samples (six unknown and three standards) were spotted on the same TLC plate. The TLC tank was lined with filter paper saturated with the solvent mixture ethyl acetate—acetic acid (95:5) and the system was allowed to equilibrate for 60 min. After placing the TLC plate into the tank the solvent front was allowed to migrate 16 cm from the origin (ca. 50 min). The developed plate was dried for 15 min in a stream of warm air, then heated in an oven for 10 min at 120°C. The spots were located under short-wavelength UV light.

Densitometry

The TLC plate was scanned at 20 mm/min in a direction perpendicular to the direction of development using a Shimadzu dual-wavelength TLC scanner,

Model CS-910, with dual-pen recorder (Philips, Model PM 8222), using the following operating conditions: photometric mode, dual wavelength, λ_s = 300 nm, λ_r = 400 nm; detection mode, reflection; measuring mode, absorbance; stage scanning mode, zigzag; working curve linearizer, channel 1. The speed of the recorder was 20 mm/min. The profile and integration curves were recorded for each spot on the TLC plate.

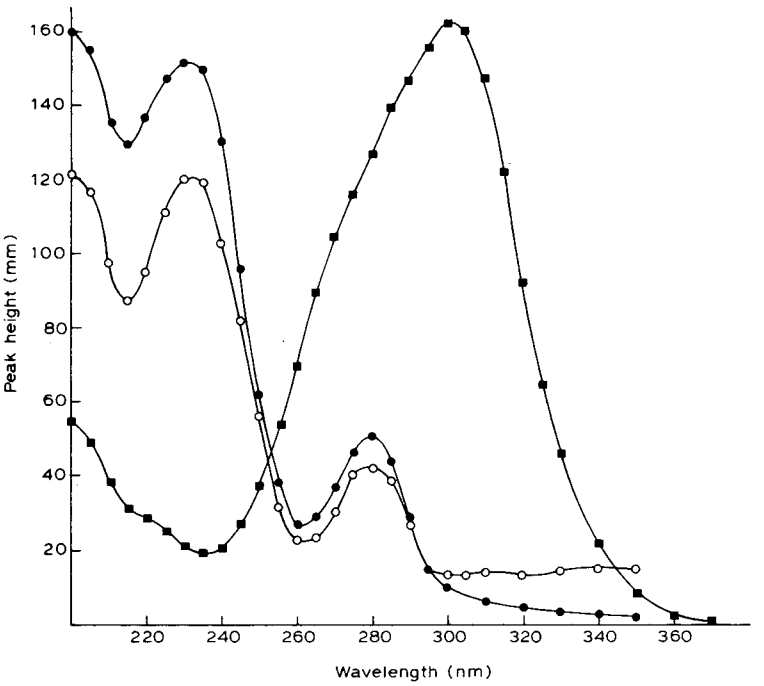

Fig. 2. Ultraviolet absorption spectra of ticrynafen (■), metabolite II (●) and metabolite III (○) obtained by scanning the TLC plate after separation of dog plasma extract.

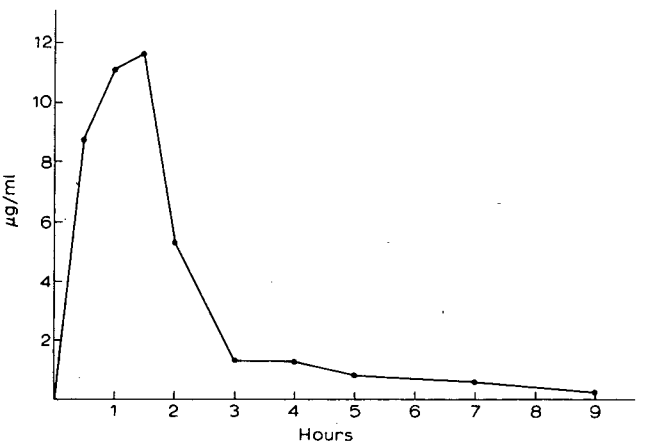

Fig. 3. Plasma level of unchanged drug after oral administration of 46 mg/kg ticrynafen to dog.

RESULTS AND DISCUSSION

The absorption spectrum for the ticrynafen spot was constructed by plotting absorbance at different wavelengths (Fig. 2). The maximum absorbance was at 300 nm. This wavelength was used for the sample side while 400 nm was used for the reference side.

The quantitatively lowest recordable amount is about 0.1 μg/spot (0.33 μg/ml of plasma). Linear responses were obtained up to 7.5 μg/spot.

The relative standard deviations for plasma samples from 3.3 to 25.0 μg/ml in a quintuplicate study on five TLC plates ranged from 3.6 to 13.6%,

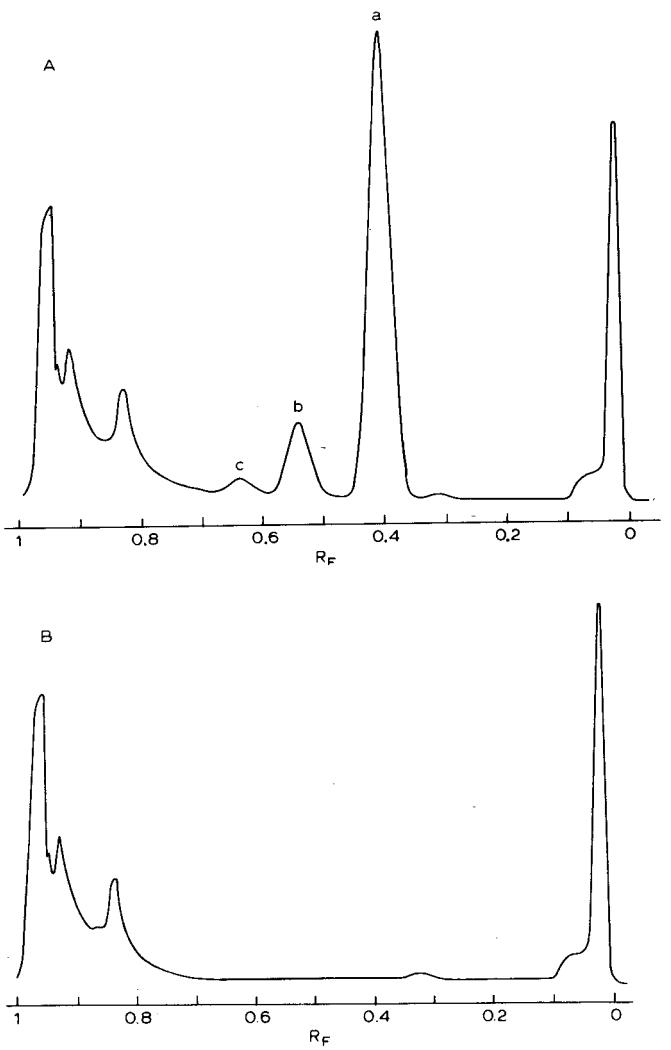

Fig. 4. Typical chromatograms obtained from dog plasma extracts by linear scanning of the TLC plate. (A) Plasma extract from dog treated with ticrynafen. Peaks: a = ticrynafen, b = metabolite II, c = metabolite III. (B) Control plasma.

indicating variabilities among the plates. However, the plasma standards had to be extracted and spotted along with the unknowns on each TLC plate.

Recoveries were 75.3 ± 6.3% (mean ± S.D.) for triplicate plasma standards at 3.3 μg/ml, and 82.3 ± 1.1% for triplicate plasma standards at 16.7 μg/ml.

The accuracy of the assay was tested by determining six unknown spiked plasma samples. The average percentage difference between the observed and the theoretical concentrations for plasma samples from 1.0 to 7.5 μg/spot was 5.2%, indicating a good accuracy.

The method was used for the measurement of plasma ticrynafen concentrations in dog. Prior to dosing the dog was fasted for 12 h. The peak plasma of the ticrynafen concentration, 11.7 μg/ml, occurred at 90 min (Fig. 3). These values agree with the ranges reported earlier [5].

In addition to the spot corresponding to ticrynafen, spots which might be metabolites II and III were also detected (Fig. 4); this assumption is based on data in the literature [2].

The assay of control plasma samples showed no background peak in the area of ticrynafen and metabolites. Using the solvent mixture ethyl acetate—acetic acid (95:5) a good separation of ticrynafen and metabolites was achieved, while the interferences of biological origin proved to be present at the solvent front as well as in the region of lower R_F values (Fig. 4).

Advantages of our method when compared with the TLC method [5] are rapid determination, higher sensitivity and the possible determination of metabolites. A major advantage over the gas chromatographic methods [3, 4] is the possibility of scanning the spots on the TLC plate directly in the UV range. Thus the absorption spectrum and comparison with the known absorption spectra for a positive identification could be achieved. Also extracted substances can be chromatographed without any derivatization.

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Vol. 222, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

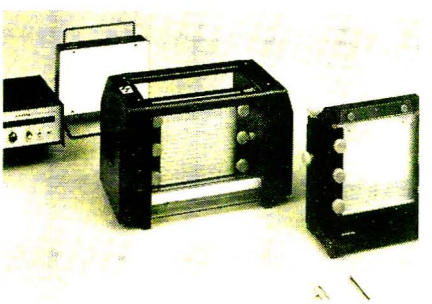
NEWS SECTION

APPARATUS

N-1532

ELECTROPHORESIS SYSTEM

CAMAG has introduced a vertical gel electrophoresis system of modular construction which enables a large number of permutations of this separation technique to be performed. It lends itself both to analytical serial analysis and to clear-cut preparative fractionation by continuous elution procedures. The separation techniques feasible with the CAMAG system include disc and SDS electrophoresis, isoelectric focusing and isotachophoresis. Matrices of polyacrylamide, agarose, starch and granulated gels can be used.



CHEMICALS

N-1525

AGAROSE IEF

Pharmacia Fine Chemicals has introduced Agarose IEF, a highly purified agarose that has been optimized for isoelectric focusing by charge balancing. Gradient drift is said to be reduced to a minimum, in this way giving high resolution, stable pH gradients and trouble free isoelectric focusing.

A complete system for isoelectric focusing with the new Agarose IEF, Pharmalyte^{tm} and accessories is available from Pharmacia together with a booklet describing the properties and applications of Agarose IEF.

N-1520

ULTROGELS

LKB Produkter AB have introduced 5 new types of gels for gel filtration and affinity chromatography. The type AcA 202 is suitable for desalting and gel filtration of low-molecular-weight compounds; types A2/A4/A6 can be used for gel filtration and affinity chromatography of high-molecular-weight compounds, and type HA can be used for adsorption chromatography in cases where gel filtration and ion-exchange chromatography do not give the expected results. All LKB Ultrogels are packed in the swollen form and are instantly ready for use.

N-1537

IEF PROTOCOL

An illustrated step-by-step guide to isoelectric focusing in agarose gels has been issued by the BioProducts Department of Marine Colloids. This guide is based on the use of two new products, Isogel^{tm} agarose and GelBond^{tm} film, and a detailed new protocol developed jointly by Calvin A. Savaris of Harvard Medical School and Richard B. Cook of Marine Colloids.

N-1515

HANDBOOK OF LIPIDS AND SELECTED CARBOHYDRATES

A 103-page handbook of lipids and selected carbohydrates, released by Supelco, Inc., presents lipids, carbohydrates and derivatives, mixtures and kits for GLC and TLC, aflatoxins and mycotoxins and clinical products. Featured in this sixth edition of the handbook are many savings in synthetic lecithins and lysolecithins whose quantities have been increased considerably without an increase in price.

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.

PROCEDURES

N-1513

GEL SCANNING

Varian's Instrument Group has published an article describing gel scanning with UV-Vis spectrophotometers. Gel electrophoresis is widely used for the detection and characterization of biological compounds and for the estimation of their purity. The Varian paper describes this technique, the use of Varian equipment and the spectrophotometric analysis of stained and unstained gels. A number of spectra are included.

N-1522

AGAROSE

The Marine Colloids Division of the FMC Corp. have published new literature on agarose types for use in electrophoresis. A booklet by Guiseley and Renn deals with the properties, the purification and the application possibilities of the various types of agarose isolated from marine sources. A paper by Sun gives a survey of the present status of electrophoresis. Finally, the 1980 Bioproducts catalog is now available from the Marine Colloids Division.

NEW BOOKS

The chromatography of hemoglobin, by W.A. Schroeder and T.H.S. Huisman, Marcel Dekker, New York, Basel, 1980, IX + 255 pp., price SFr. 68.00, ISBN 0-8247-6941-4.

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Radioimmunoassay (Proc. Int. Symp. Recent Progress in Radioimmunoassay of Hormones, Proteins and Enzymes, Gardone Riviera, May 8–10, 1980), edited by A. Albertini, Excerpta Medica, Amsterdam, New York, 1980, ca. 286 pp., price Dfl. 115.00, US$ 56.00, ISBN 0-444-90173-6.

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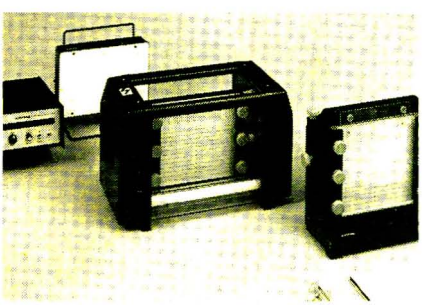

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MEETINGS

XXIXth ANNUAL COLLOQUIUM PROTIDES OF THE BIOLOGICAL FLUIDS

The XXIXth Colloquium Protides of the Biological Fluids will be held May 4–7, 1981, in the Sheraton Hotel, Brussels, Belgium. Topics will be: (A) Isolation and characterization of membrane proteins; (B) Receptor–ligand interaction; (C) Monoclonal proteins as reagent. Further information is available from Dr. Hubert Peeters, Colloquium secretariat, c/o Lipid and Protein Dept., Institute for Medical Biology, Alsembergsesteenweg, B-1180 Brussels, Belgium.

CHROMATOGRAPHY '81

16th INTERNATIONAL SYMPOSIUM ADVANCES IN CHROMATOGRAPHY

The 16th International Symposium on Advances in Chromatography will be held September 28–October 1, 1981 at the Autonomous University of Barcelona in Bellaterra, Spain. The meeting is being organized in cooperation with the Spanish Chromatography Group of the Royal Society of Physics and Chemistry. The symposium will consist of invited and submitted papers on all aspects of chromatography given by leading authorities from throughout the world. Informal discussions will permit the free exchange of ideas on various current questions related to the chromatographic techniques and their applications. Authors intending to submit papers for the symposium will be required to adhere to the following schedule: abstracts (200 words), March 1, 1981 (in Houston); manuscript (4 copies + 1 set original figures or glossy prints), April 15, 1981 (in Houston). Authors accepted for the symposium will be notified by March 15. The papers to be presented at the symposium will be published in the Proceedings and will be available to all delegates at the registration desk. These papers will undergo the normal reviewing procedures of the Journal of Chromatography and only those accepted will appear in the Journal and the Proceedings. An exhibition of the latest instrumentation and books in chromatography and ancillary techniques will be held in an area adjacent to the main lecture hall. Two-day intensive courses of the "hands-on" type will be available on the Friday and Saturday (September 25, 26) preceding the symposium: (a) capillary gas chromatography, (b) high-performance liquid chromatography, (c) gas chromatography–mass spectrometry, (d) high-performance thin-layer chromatography. The symposium will be held in the Aula of the medical school in Bellaterra. Accommodations will be available in a number of hotels which have been reserved for this occasion. Details regarding hotels and the social program for delegates and their guests will be made available at a later date. Further information on the symposium including the technical program, details of short courses, hotel accommodations and available space for the exhibition can be obtained from:

Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.

4th INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND RELATED TECHNIQUES

The 4th International Symposium on Affinity Chromatography and Related Techniques – Theoretical Aspects, Industrial and Biomedical Applications will be held from June 22–26, 1981 in the Conference Centre "De Koningshof" at Veldhoven near Eindhoven, The Netherlands. The scope of the meeting will cover the following topics:

Theoretical Aspects – Ligand/ligate interaction in homogeneous and heterogeneous systems. General theory of electrostatic, hydrophobic and charge-transfer interaction. Theoretical analyses of affinity separations. Matrix structure. Column/bath procedures. (8 plenary lectures).

Polymeric Matrices and Ligand Immobilization – Natural synthetic polymers. Immobilization of ligand molecules. (9 plenary lectures).

Applications – Isolation and purification. Biomedical Applications. Miscellaneous. (17 plenary lectures).

The proceedings of the symposium will be published by Elsevier Scientific Publishing Company in the Analytical Chemistry Symposia Series. The deadline for receiving the final "camera ready" manuscripts will be the day of presentation.

Plenary lectures will be presented by invited speakers. Further details can be obtained from the organizing committee at the following address: Secretariat, Department of Organic Chemistry/Faculty of Science, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands.

PUBLICATION SCHEDULE FOR 1981

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

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	The publication schedule for further issues will be published later.									
Chromatographic Reviews														
Biomedical Applications	221/1	221/2	222/1	222/2	222/3									

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.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

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.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

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STATISTICAL TREATMENT OF EXPERIMENTAL DATA

By J.R. GREEN, *Lecturer in Computational and Statistical Science, University of Liverpool, U.K. and* D. MARGERISON, *Senior Lecturer in Inorganic, Physical and Industrial Chemistry, University of Liverpool, U.K.*

PHYSICAL SCIENCES DATA 2

This book first appeared in 1977. In 1978 a revised reprint was published and in response to demand, further reprints appeared in 1979 and 1980. Intended for researchers wishing to analyse experimental data, this work will also be useful to students of statistics. Statistical methods and concepts are explained and the ideas and reasoning behind statistical methodology clarified. Noteworthy features of the text are numerical worked examples to illustrate formal results, and the treatment of many practical topics which are often omitted from standard texts, for example testing for outliers, stabilization of variances and polynomial regression.

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–CHOICE

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