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BIOMEDICAL APPLICATIONS

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Contents (continued)

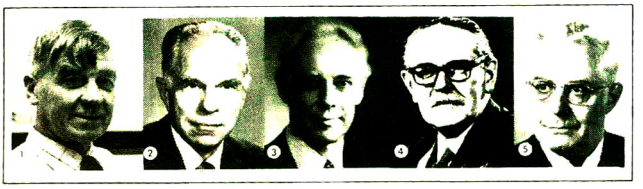
75 Years of Chromatography

A Historical Dialogue

L. S. ETTRE *and* A. ZLATKIS (Editors).

Journal of Chromatography Library - Volume 17

On the occasion of the 75th anniversary of the invention of chromatography, this book compiles the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates). In their contributions to this volume, these pioneers review the events which influenced them to enter the field; explain the background of their inventions; summarize their activities and results during their professional lives; and discuss their interactions with other scientists and other disciplines.



This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated.
The final chapter is devoted to "Those who are no longer with us".

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(*Nobel Prize laureates)

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Biomedical Applications

CHROMBIO. 345

HIGHLY SENSITIVE ASSAY FOR TYROSINE HYDROXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received February 27th, 1979)

SUMMARY

A highly sensitive assay for tyrosine hydroxylase (TH) activity by high-performance liquid chromatography (HPLC) with amperometric detection was devised based on the rapid isolation of enzymatically formed DOPA by a double-column procedure, the columns fitted together sequentially (the top column of Amberlite CG-50 and the bottom column of aluminium oxide). DOPA was adsorbed on the second aluminium oxide column, then eluted with 0.5 *M* hydrochloric acid, and assayed by HPLC with amperometric detection. D-Tyrosine was used for the control. α-Methyldopa was added to the incubation mixture as an internal standard after incubation. This assay was more sensitive than radioassays and 5 pmol of DOPA formed enzymatically could be measured in the presence of saturating concentrations of tyrosine and 6-methyltetrahydropterin. The TH activity in 2 mg of human putamen could be easily measured, and this method was found to be particularly suitable for the assay of TH activity in a small number of nuclei from animal and human brain.

INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.14.16.2) is a monooxygenase which catalyzes the formation of DOPA from L-tyrosine in peripheral and central catecholaminergic neurons and chromaffin cells of the adrenal medulla [1]; its assay is frequently required for physiological and pathological studies. Because the enzyme activity is extremely low, only radioassays [1—3] have been suitable for its measurement, especially in the brain. We have recently devised a sensitive fluorometric assay of TH activity which is widely applicable to any crude tissues, including human brain [4]. In this method, DOPA formed enzymatically from L-tyrosine was isolated rapidly from interfering substances by a sequential double-column procedure (the top column of Amberlite CG-50 and the bottom column of aluminium oxide) and was assayed by an improved hydroxyindole method. The limit of sensitivity was 100 pmol DOPA. Although

this fluorometric method was the first non-isotopic assay for TH widely applicable to any crude tissues, recent progress in the neurosciences requires an extremely sensitive method which permits the assay of TH activity in less than milligram quantities of brain nuclei obtained by punching techniques. This has been somewhat difficult even by using the most sensitive radioassays with carrier-free radioactive tyrosine as substrate.

In this study we have combined the simple and specific isolation of enzymatically formed DOPA by our double-column procedure [4] with the highly sensitive assay of DOPA by high-performance liquid chromatography (HPLC) with amperometric detection [5—7]. α-Methyldopa as an internal standard was added to each sample after TH incubation [8]. Both the double columns and the high-performance liquid chromatography (HPLC) permitted nearly complete isolation of DOPA, and thus the blank values became very low. The only interfering substance is endogenous DOPA in crude tissues and non-enzymatically formed DOPA from both L- and D-tyrosine, and this blank value can be completely cancelled by the control with D-tyrosine. Use of α-methyldopa as an internal standard made the assay very accurate. TH activity in less than 1 mg of a brain nucleus could be assayed by this method. An assay method for TH by HPLC with amperometric detection was first reported by Blank and Pike [9], but our present method is simpler and much more sensitive than their method.

EXPERIMENTAL

Materials

L-Tyrosine, D-tyrosine, and 2-mercaptoethanol were obtained from Wako Chemical Company (Osaka, Japan); 6-methyl-5,6,7,8-tetrahydropterin was from Calbiochem (Los Angeles, Calif., U.S.A.); α-methyldopa was from Sigma (St. Louis, Mo., U.S.A.); catalase was from Boehringer (Mannheim, G.F.R.); Amberlite CG-50 was from Rohm and Haas (Philadelphia, Pa., U.S.A.); and aluminium oxide was from Merck (Darmstadt, G.F.R.). All other chemicals used were of analytical grade.

Rat brain stem (including medulla oblongata, pons and midbrain) was dissected. Human putamen was dissected at autopsy from a patient without a history of neurological disorders. The brains were homogenized in 4 volumes of 0.25 *M* sucrose in a glass Potter homogenizer.

6-Methyl-5,6,7,8-tetrahydropterin was used as cofactor; the 10 m*M* solution was prepared in 1.0 *M* 2-mercaptoethanol and stored at −20°, protected from light and prepared once a week. The molar concentration of 6-methyltetrahydropterin was estimated from the extinction coefficient, 18,500 M^{-1} cm^{-1} at 264 nm in 2 *M* HCl. Amberlite CG-50 and aluminium oxide were treated as described previously [4].

Experimental procedures

All experimental procedures were carried out on the scale of one-fifth of the fluorometric procedures previously reported [4]. The standard incubation mixture consisted of the following components in a total volume of 100 μl (final concentrations in parentheses): 10 μl of 1 *M* acetate buffer pH 6.0 (0.2

M), 20 μl of 1 m*M* L-tyrosine in 0.01 *M* HCl (0.2 m*M*), 10 μl of 10 m*M* 6-methyl-5,6,7,8-tetrahydropterin (1 m*M*) in 1 *M* 2-mercaptoethanol (100 m*M*), 30 μl of 0.25 *M* sucrose (75 m*M*) containing enzyme, 10 μl of 1 mg/ml catalase (10 μg/100 μl) or 10 μl of 10 m*M* ferrous ammonium sulfate (1 m*M*), and water. For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine and 50 pmol or 100 pmol of DOPA were added to another blank incubation as an internal standard for DOPA.

Incubation was done at 37° for 10 min, and the reaction was stopped with 600 μl of 0.5 *M* perchloric acid containing 50 pmol or 100 pmol of α-methyldopa as an internal standard in an ice-bath. After 10 min, 20 μl of 0.2 *M* EDTA and 300 μl of 1 *M* potassium carbonate were added to adjust the pH to 8.0—8.5, and the mixture was centrifuged at 1600 *g* for 10 min at 4°. The clear supernatant was passed through the double columns, the upper column, containing 200 μl of Amberlite CG-50 (12.5 cm × 0.5 cm I.D..), and the bottom column, containing 100 mg of aluminium oxide (12.5 cm × 0.4 cm I.D.), fitted together sequentially. The effluent through both columns was discarded. Both columns were washed once with 1.5 ml of water, and the washings were discarded, DOPA and α-methyldopa were passed through the first Amberlite column and adsorbed on the second aluminium oxide column, which was separated and washed with 1.5 ml of water twice, and with 100 μl of 0.5 *M* HCl once. DOPA and α-methyldopa were eluted with 200 μl of 0.5 *M* HCl.

A 100-μl aliquot of the eluate was injected into the high-performance liquid chromatograph (Yanaco L-2000) with an Yanaco VMD-100 voltammetric detector and a column (25 cm × 0.4 cm I.D.) packed with Yanapak ODS (particle size 5 μm) (Yanagimoto Manufacturing Co., Fushimi-ku, Kyoto, Japan). The mobile phase was a 0.1 *M* potassium phosphate buffer (pH 3.5) with a flow-rate of 0.6 ml/min; the detector potential was set at 0.8 V against the Ag/AgCl electrode. Under these conditions the retention times were: solvent front, 1.8 min; DOPA, 3.8 min; and α-methyldopa, 5.5 min.

The DOPA formed enzymatically by TH was calculated by the equation

$$\frac{R\,(\mathrm{L}) - R\,(\mathrm{D})}{R\,(\mathrm{D}+\mathrm{S}) - R\,(\mathrm{D})} \times 50\ \text{pmol (or 100 pmol)}$$

where R is the ratio of peak heights (peak height of DOPA/peak height of α-methyldopa), R (L) being that from the L-tyrosine incubation, R (D) from the D-tyrosine incubation, and R (D + S) that of D-tyrosine plus DOPA (internal standard, 50 pmol or 100 pmol).

RESULTS

The voltammetric detector system provides high sensitivity for catechol compounds. Therefore DOPA, the product of TH reaction, can be assayed in the column eluate with extremely high sensitivity. Fig. 1 is the calibration curve showing the linear response of the peak height of the voltammetric detector for the amounts of DOPA injected from 500 fmol to 5 nmol.

The chromatographic pattern of the TH reaction with the homogenate of

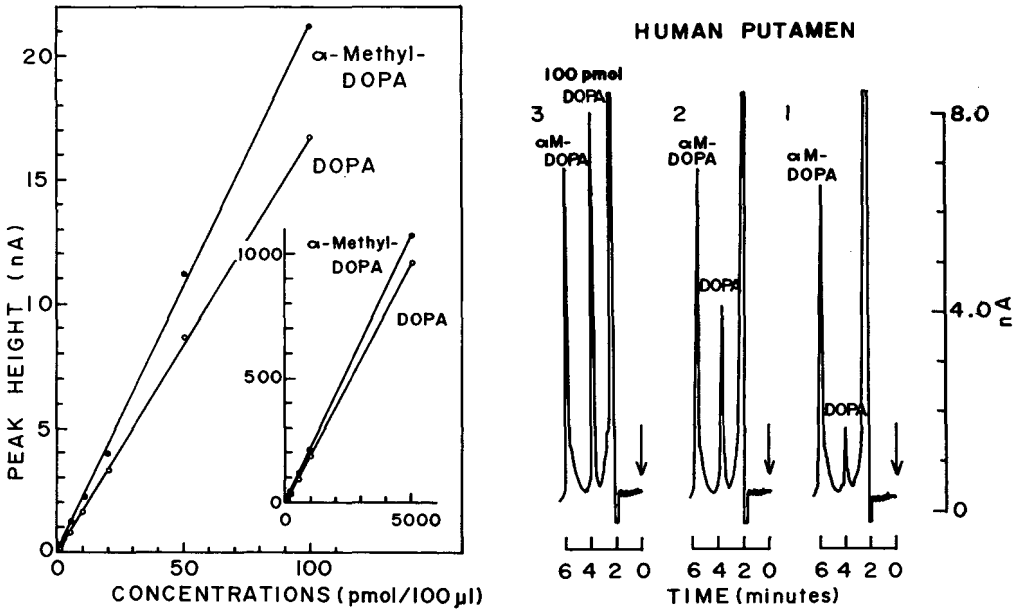

Fig. 1. Standard curves of DOPA and α-methyldopa in HPLC with voltammetric detection for the peak height. One hundred microliters of a sample containing various amounts (500 fmol to 5 nmol) of DOPA and α-methyldopa were injected into the column and detected by a voltammetric detector. The conditions are described in *Experimental procedures.*

Fig. 2. HPLC elution pattern of tyrosine hydroxylase incubation mixtures with the homogenate of human putamen as enzyme. The conditions are described in *Experimental procedures.* The incubation mixture contained 2 mg of human putamen and 10 µg of catalase. (1) Blank incubation with D-tyrosine. (2) Experimental incubation with L-tyrosine. (3) 100 pmol of DOPA were added as an internal standard to a blank incubation with D-tyrosine. 100 pmol of α-methyldopa (αM-DOPA) were added to each sample after incubation. Formation of 37.3 pmol of DOPA from L-tyrosine during 10 min incubation at 37° was calculated from the charts.

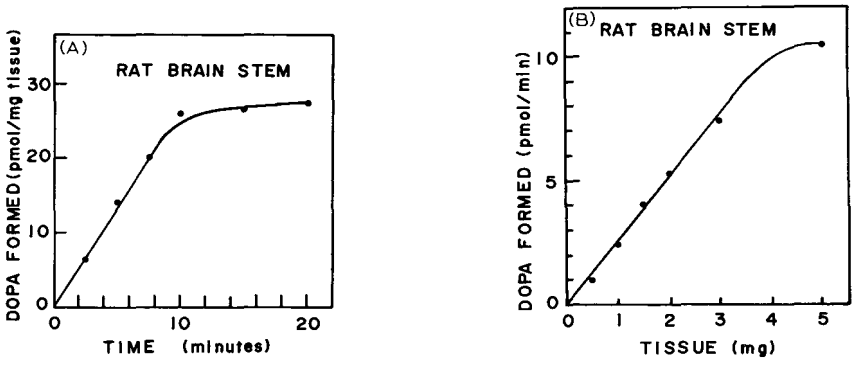

Fig. 3. (A) The rate of DOPA formation using an homogenate of rat brain stem as enzyme at 37°. Standard incubation system containing 10 µg of catalase was used as described under *Experimental procedures.* (B) Tyrosine hydroxylase activity in homogenates of rat brain stem as a function of enzyme concentration. The standard incubation system with 10 µg catalase was used and incubation was carried out for 10 min at 37°.

human putamen is shown in Fig. 2. The peak of DOPA in the blank incubation may be mainly due to its non-enzymatic formation.

With rat brain stem homogenate as enzyme in the presence of 10 μg catalase, the reaction proceeded linearly with time for 10 min at 37° (Fig. 3A). The reaction rate was linear up to 3 mg of tissue (Fig. 3B).

The presence of either catalase or Fe^{2+} ion was required for maximum activity. As shown in Fig. 4, 10 μg of catalase in the standard incubation system with rat brain homogenate gave maximum activity. Fe^{2+} ion was more effective for the stimulation of TH activity, especially for the assay in human brain homogenates. As shown in Fig. 5, the activity in the homogenate of 2 mg human putamen was stimulated by catalase only slightly, but 1—2 m*M* Fe^{2+} ion activated TH activity about three-fold.

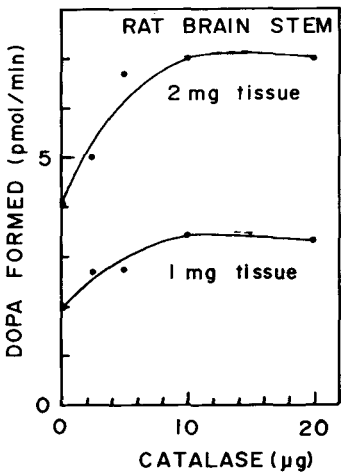

Fig. 4. Effects of catalase on tyrosine hydroxylase activity in the homogenate of rat brain stem. The standard incubation system was used with various concentrations of catalase and with the homogenate containing 1 mg or 2 mg tissue as enzyme. Incubation was carried out for 10 min at 37°.

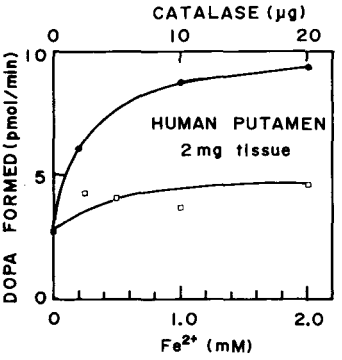

Fig. 5. Effects of Fe^{2+} ion (•) and catalase (□) on tyrosine hydroxylase activity in the homogenate of human putamen. The standard incubation system was used with various concentrations of Fe^{2+} ion or catalase and with the homogenate containing 2 mg tissue as enzyme. Incubation was carried out for 10 min at 37°.

DISCUSSION

This assay of TH with the preliminary isolation of DOPA formed from tyrosine by a double-column procedure and subsequent assay by HPLC with a voltammetric detector has many advantages.

First, it is highly sensitive. The limit of sensitivity was about 5 pmol of DOPA formed enzymatically. The sensitivity was found to be even higher than that of various radioassays, in which the limit is about 10 pmol of DOPA formed even if the substrate concentration is reduced to increase the specific radioactivity of labelled tyrosine, and the V_{max} cannot be obtained. TH

activity could be assayed with less than 1 mg of a brain nucleus. With such a high sensitivity for DOPA, the sensitivity of the TH assay is determined solely by the blank value. In the present method DOPA could be completely and rapidly separated by the combination of the double-column procedure and HPLC. Therefore, the blank value with D-tyrosine, which is not the substrate for TH at all [1], derives either from DOPA formed by the non-enzymatic reaction or from DOPA contained in a crude enzyme preparation. Since endogenous DOPA in crude brain tissue is very low (less than 250 pmol per g tissue [10]), the blank is considered to be mainly derived from non-enzymatically formed DOPA. In this assay catalase gave a lower blank than Fe^{2+} ion, but Fe^{2+} ion was more or less essential for the assay of human brain enzyme. This confirms our previous results [4, 11].

Secondly, the method is simple and rapid, and the total time of the assay was 3 h.

Thirdly, it is economical since labelled substrate and a liquid scintillation spectrometer are not needed.

Fourthly, the incubation can be done in optimal conditions under saturating concentrations of L-tyrosine and a pterin cofactor; thus the V_{max} can be obtained. It is not necessary to measure tyrosine in a crude enzyme preparation to calculate the specific radioactivity of tyrosine as in radioassay.

Blank and Pike [9] first reported an assay of TH activity using HPLC with electrochemical detection. They used 4 nmol of dihydroxybenzylamine as internal standard and a batch method of aluminium oxide for the preliminary isolation of DOPA prior to HPLC. Our method is more sensitive and simpler. The recovery of dihydroxybenzylamine used in their study was found to be low. In contrast, α-methyldopa used as an internal standard in our study proved to be an excellent internal standard, because it showed a good recovery and was well separated from DOPA.

ACKNOWLEDGEMENTS

This work was supported in part by a grant to T.N. from the Ministry of Education, Japan, which is gratefully acknowledged. The authors also gratefully acknowledge the expert assistance in the mechanical aspects of the Yanaco HPLC—voltammetric detector system from Yanagimoto Manufacturing Co. (Fushimi-ku, Kyoto, Japan).

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CHROMBIO. 342

EXCRETION PATTERN OF 3β-HYDROXYSTEROIDS IN PATIENTS WITH ADRENAL TUMOR, CUSHING'S DISEASE AND 21-HYDROXYLASE DEFICIENCY, AND IN PREGNANCY, USING THIN-LAYER CHROMATOGRAPHY AND COLOR DEVELOPMENT OF 3β-HYDROXYSTEROIDS WITH 3β-HYDROXYSTEROID OXIDASE

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(Received February 6th, 1979)

SUMMARY

The fractional assay is described of 3β-hydroxysteroids in various patients by thin-layer chromatography and color development using an enzyme that reacts specifically with some 3β-hydroxysteroids. Together with dehydroepiandrosterone, androst-5-ene-3β,17β-diol and 16α-hydroxydehydroepiandrosterone can be detected, but their concentrations differ with each disease. An unknown fraction, a more polar 3β-hydroxysteroid than 16α-hydroxydehydroepiandrosterone, is also detected in moderate amounts in patients with adrenal tumor (18.3 mg/day), 21-hydroxylase deficiency (3.2 to 1.2 mg/day), and Cushing's syndrome (0.9—2.3 mg/day, as pregn-5-ene-triol).

INTRODUCTION

Dehydroepiandrosterone (DHEA*) was considered to be a main urinary 3β-hydroxysteroid in normal subjects, but once the excretion of some other 3β-hydroxysteroids was found in adrenal disease and in infant or pregnant urine, the presence of other 3β-hydroxysteroids such as 16α-hydroxy-dehydroepiandrosterone (16-OH-DHEA) and androst-5-ene-3β,17β-diol in normal subjects was demonstrated by many investigators [1—6].

The methods used so far for detecting steroids by color development are the Zimmermann reaction for 17-ketosteroids, the Porter—Silber reaction for 17-hydroxycorticosteroids, the Kober reaction for estrogens, sulfuric acid reaction

*Non-standard abbreviations used: DHEA, 3β-hydroxy-androst-5-en-17-one; 16α-OH-DHEA, 3β,16α-dihydroxy-androst-5-en-17-one; A-diol, androst-5-ene-3β,17β-diol; A-triol, androst-5-ene-3β,16α,17β-triol.

for pregnanediol or triol, and the Pincus reaction using antimony trichloride, after paper or thin-layer chromatographic (TLC) separation, etc.

In this paper, a new method for the fractional determination of 3β-hydroxysteroids using TLC and an enzyme that reacts specifically with some 3β-hydroxysteroids [7] is described, using urine samples from patients with adrenal tumor, Cushing's syndrome, and 21-hydroxylase deficiency, and from pregnant women.

MATERIALS

The TLC plates used in this study were Kieselgel 60 F245 (Merck), is heated at 110° for 30 min before use. Analytical-grade organic solvents were used; β-glucuronidase (bacterial powder from *E. coli*, EC 3.2.1.31) and all steroids were purchased from Sigma, St. Louis, Mo., U.S.A.

The enzyme reagent for color development of 3β-hydroxysteroids on thin-layer plates was prepared by dissolving 2 mg of 4-aminoantipyrine and 15 mg of phenol in 20 ml of 0.1 *M* phosphate buffer (pH 7.0) containing, per 20 ml, 10 U of 3β-hydroxysteroid oxidase from *B. sterolicum* (Kyowa Hakko Co., Machida-shi, Tokyo, Japan), 100 U of peroxidase (EC 1.11.1.7) and 0.02 ml of the surfactant Triton X-100.

METHODS

Preparation of sample

Pipette 10 ml of urine (in cases of nephrosis, wash with petroleum ether twice to remove cholesterol) into a 40 ml tube and adjust to pH 6.5 using bromthymol blue paper as indicator. Add 1 ml of β-glucuronidase (1,000,000 Fishman units/l), 1 ml of 0.5 *M* phosphate buffer (pH 6.5) and a few drops of chloroform to the tube and mix well. Incubate the mixture for 24 h at 37°, then adjust to pH 1 with 6 *M* HCl and saturate with 5 g of sodium chloride. Shake the solution with 20 ml of ethyl acetate for 5 min. After centrifugation, discard the urine layer and keep the ethyl acetate layer for another 24 h at 37° to achieve complete solvolysis of the sample. Wash the ethyl acetate layer successively with 2 ml of NaOH (80 g/l), with concd. Na_2CO_3, and water. After centrifugal separation, transfer 15 ml of the ethyl acetate extract to a tube. Evaporate the ethyl acetate aliquots.

Thin-layer chromatography

To the dry residue, a few drops of chloroform are added, and the sample is applied to a thin-layer plate with marker dye (sudan III and isatine) and standards. The plate is developed in the solvent mixture ethyl acetate—benzene (1:1, v/v) for 40—60 min at 20°, the distance of the front from the origin being 15—16 cm.

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

Place the thin-layer plate on a heater at 37° (or over a water-bath at 40°), and spray with enzyme reagent. Incubate for 30 min so that a pink-colored zone is visible. Quantitative densitometric scanning at 500 nm can also be per-

formed for high concentrations of 3β-hydroxysteroids in the sample. The instrument used for the assay was a dual-wavelength TLC scanner CS-910 (Shimadzu).

RESULTS

Specificity of 3β-hydroxysteroid oxidase

The specificity of 3β-hydroxysteroid oxidase was tested with a series of steroids in solution and on thin-layer plates, using 25 μg of steroids per tube. The data are shown in Table I. The R_F values of steroids on thin-layer plates are presented in Table II.

TABLE I

SPECIFICITY OF 3β-HYDROXYSTEROID OXIDASE

Compound	Specificity	
	In solution (%)	On TLC (%)
Dehydroepiandrosterone	100	100
Epiandrosterone	100	65
Androst-5-ene-3β,17β-diol	100	57
16α-Hydroxy-dehydroepi-androsterone	44	39
Pregnenolone	75	20
Cholesterol	65	<20
Androsta-5,16-dien-3β-ol	43	<20
5α-Androst-16-en-3β-ol	5	N.D.*
5β-Pregnane-3β,20α-diol	1	N.D.
3α-Hydroxysteroids	0	N.D.
Estradiol	0	N.D.
Estriol	0	N.D.

*N.D., Not detectable.

TABLE II

R_F VALUES OF STEROIDS ON THIN-LAYER PLATES

Solvent system: ethyl acetate—benzene (1:1).

Compound	R_F
Cholesterol	0.49
Androsta-5,16-dien-3β-ol	0.45
Pregnenolone	0.39
Dehydroepiandrosterone	0.36
Epiandrosterone	0.35
5β-Pregnane-3β,20α-diol	0.30
Androst-5-en-3β,17β-diol	0.28
16α-Hydroxy-dehydroepi-androsterone	0.22
Androst-5-en-3β,16α,17β-triol	0.08
Marker dyes:	
Sudan III	0.63
Isatine	0.31

The excretion patterns of 3β-hydroxysteroids in a patient with adrenal tumor (sample 1), 21-hydroxylase deficiency in an adult (sample 2) and in a child (sample 3), in pregnancy (samples 4—6), Cushing's syndrome (samples 7 and 10), in a normal subject (sample 8) and after administration of ACTH (sample 9) are shown in Fig. 1. The densitometric scanning patterns of the thin-layer plates are shown in Fig. 2. The percentage of each steroid fraction is presented in terms of peak area, not corrected with reactivity of 3β-hydroxysteroid oxidase for each steroid, in Table III.

Precision

In five repeated assays on thin-layer plates using a standard solution containing 10 μg of DHEA, 10 μg of androst-5-en-3β,17β-diol and 10 μg of 16-α-

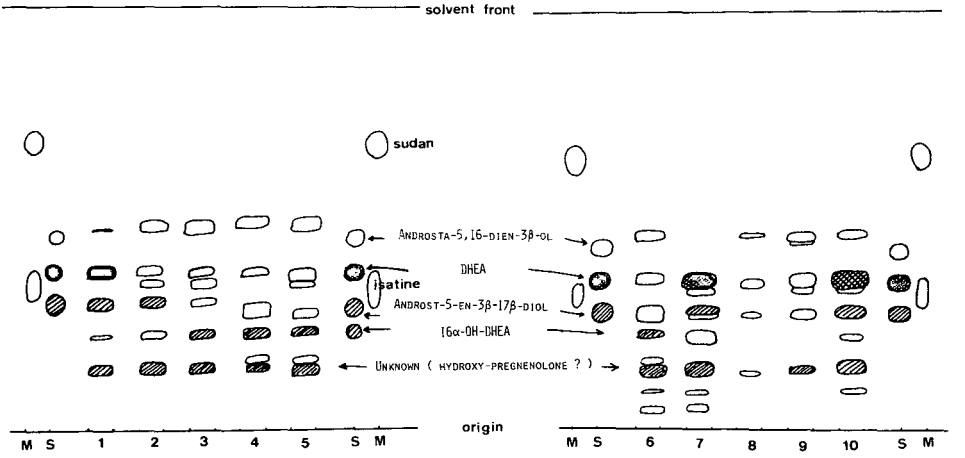

Fig. 1. TLC of 3β-hydroxysteroids. The numbered samples are from: 1, adrenal tumor; 2, 21-hydroxylase deficiency (22-year-old female); 3, 21-hydroxylase deficiency (6-year-old female); 4, pregnancy; 5, pregnancy; 6, pregnancy; 7, Cushing's syndrome; 8, a normal subject; 9, ACTH administration; 10, Cushing's syndrome. M is a marker dye of sudan III (R_F = 0.63) and isatine (R_F = 0.31); S is a mixture of standards of androsta-5,16-dien-3β-ol, dehydroepiandrosterone, and androst-5-en-3β,17β-diol.

TABLE III

PERCENTAGE OF EACH 3β-HYDROXYSTEROID FRACTION IN VARIOUS DISEASES

	% fraction				
	DHEA	A-diol	16-OH-DHEA	Unknown	A-triol
Adrenal tumor (Sample 1)	64.6	16.9	2.2	12.1	4.2
21-Hydroxylase deficiency					
Sample 2	12.3	14.0	11.4	49.7	13.0
Sample 3	15.5	3.2	24.7	48.2	8.3
Pregnancy (Sample 6)	16.2	12.8	10.7	30.3	29.9
Cushing's disease					
Sample 7	44.3	12.5	10.5	17.7	15.0
Sample 10	44.2	17.7	13.2	19.4	5.5

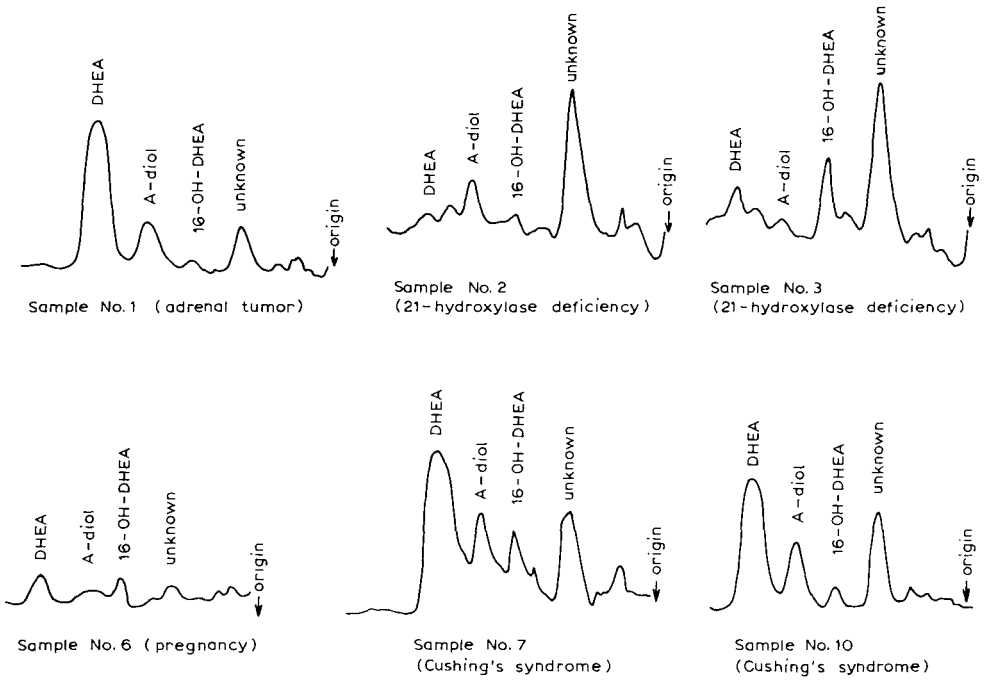

Fig. 2. Densitometric scanning pattern of patients 1, 2, 3, 6, 7 and 10 using a dual-wavelength TLC scanner CS-910 at 500 nm. Samples 2 and 3 are amplified × 2.

OH-DHEA, the percentages of each fraction (mean ± S.D.) obtained from peak areas were 50.75% ± 1.48 for DHEA, 29.00% ± 0.71 for androst-5-en-3β,17β-diol, and 20.25% ± 1.48 for 16α-OH-DHEA. The values for each fraction remained fairly constant up to 20 μg of each steroid per one TLC application.

In five repeated assays using a sample from a patient with Cushing's syndrome, the CV was 4.2% for the DHEA fraction and 10.3% for 16α-OH-DHEA.

DISCUSSION

16-Hydroxylation, which is said to inactivate of biologically active steroids, can be seen for DHEA in all samples, especially in a high percentage in pregnancy and Cushing's syndrome. Although 16α-OH-DHEA is a 17-ketosteroid, the Zimmermann chromogen was not obtained with *m*-dinitrobenzene because there is no active methylene group at position 16.

A moderate amount of an unknown 3β-hydroxysteroid with an R_F of 0.14 was observed in all samples. This unknown steroid seems to be a hydroxypregnenolone derivative (such as pregn-5-ene-3β,17α,20α-triol identified by Hirschmann and Hirschmann [8]) on the basis of the mass number of 334 obtained by mass spectrometry.

The excretion of 3β-hydroxysteroids in the last stage of pregnancy was 2—6 mg/day; most of them were more polar 3β-hydroxysteroids than DHEA itself, as shown in Fig. 3 and Table III.

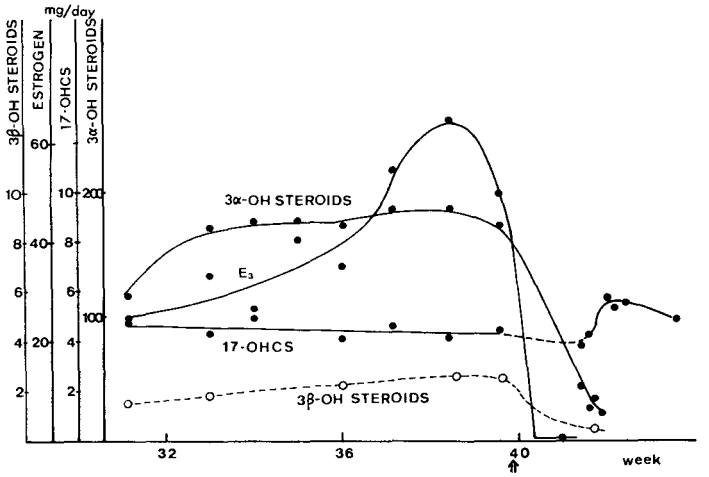

Fig. 3. Excretion of 3β-hydroxysteroids in pregnancy. 3β-Hydroxysteroids were determined by the method described in ref. 7. 17-OHCS was determined by the Porter—Silber reaction after hydrolysis using β-glucuronidase. Estrogen was determined by the Kober—Ittrich method. 3α-Hydroxysteroids were determined after hydrolysis with β-glucuronidase using "Sterognost-3α" (purchases from Nyegaard and Co.).

In this paper, the fractional determination of 3β-hydroxysteroids is shown to be useful for the diagnosis of some adrenal diseases.

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CHROMBIO. 339

DETERMINATION OF SULFINPYRAZONE AND TWO OF ITS METABOLITES IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY AND SELECTIVE DETECTION

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SUMMARY

A selective and sensitive gas chromatographic method for simultaneous determination of sulfinpyrazone and two of its metabolites (the *para*-hydroxylated metabolite and the sulfone metabolite) in biological fluids using alkali flame ionization detection (AFID), electron capture detection (ECD) and mass fragmentographic detection is described. The compounds are extracted from the samples, methylated and separated on 2% OV-17 or 3% OV-225 columns. Phenylbutazone is used as internal standard. Standard curves are linear. The coefficient of variation at 10 μg/ml of sulfinpyrazone in plasma was shown to be 1.8% (AFID), and the detection limits were 0.1 μg/ml (AFID) and 10 ng/ml (ECD). Mass spectra of the methylated compounds are shown and serum concentration curves after oral administration of 100 mg sulfinpyrazone to two persons are determined together with the excreted amounts of drug and metabolites.

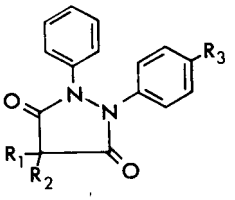
INTRODUCTION

Sulfinpyrazone, 1,2-diphenyl-4-[2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione, Anturan® (I, Table I), which was originally introduced as a uricosuric agent in the treatment of gout, has been shown to suppress platelet function presumably by competitive inhibition of platelet prostaglandin synthetase (fatty acid cyclooxygenase) [1]. Studies [2] have demonstrated that sulfinpyrazone is an effective drug in the management of certain thromboembolic conditions. Recently, a controlled clinical trial has shown that sulfinpyrazone reduces the annual death-rate after myocardial infarction with 46% compared to placebo [3].

Sulfinpyrazone is mainly excreted unchanged and as a monoglucuronide (VI) in urine. In addition two minor metabolites have been identified, namely the *p*-hydroxymetabolite (III) and the sulfone metabolite (IV). These two

TABLE I

COMPOUNDS INVESTIGATED



No.	Compound	R_1	R_2	R_3
I	Sulfinpyrazone	Ph—S(=O)—CH_2CH_2—	H	H
II	Phenylbutazone	$CH_3CH_2CH_2CH_2$—	H	H
III	G 32642	Ph—S(=O)—CH_2CH_2—	H	OH
IV	G 31442	Ph—SO_2—CH_2CH_2—	H	H
V	GP 52097	Ph—S(=O)—CH_2CH_2—	OH	H
VI	I-glucuronide	Ph—S(=O)—CH_2CH_2—	Glu	H

metabolites together with a third uncertain metabolite (V) represent only about 12% of the urinary excretion products from a single oral dose of 200 mg [^{14}C] sulfinpyrazone [4]. For the determination of sulfinpyrazone in biological fluids, Burns et al. [5] described a spectrophotometric assay. The limited sensitivity and poor selectivity of this method have resulted in development of several high-performance liquid chromatography (HPLC) methods [6—8]. Until recently [9] no gas chromatographic (GC) method has been published. The present report presents a selective and sensitive GC method for simultaneous determination of sulfinpyrazone and two of its metabolites in human plasma and urine.

MATERIALS AND METHODS

Apparatus

A Varian Model 2100 gas chromatograph equipped with a specific nitrogen-sensitive detector (AFID) and a ^{63}Ni electron capture detector (ECD) was used. The GC column for alkali flame ionization detection was a 100 cm × 2 mm I.D. U-shaped glass column filled with 2% OV-17 on Gas-Chrom Q (Pierce, Rockford, Ill., U.S.A.). The column was conditioned for 24 h at 300° with a nitrogen flow-rate of 30 ml/min and silylated at 200° by injecting 5 μl of Silyl-8 (Pierce) ten times. Operating conditions: injector temperature 280°, detector temperature 300°, column temperature 200° and carrier gas (nitrogen) flow-rate 30 ml/min. Air and hydrogen flow-rates for the alkali flame ionization detector were adjusted according to the manual. A number of chromato-

grams were recorded with different injector temperatures (230—300°). In the analysis of the sulfone metabolite the column temperature was raised to 280°.

For electron capture detection a 150 cm × 2 mm I.D. U-shaped glass column filled with 3% OV-225 on Gas-Chrom Q (Pierce) was used. The column was conditioned at 290° for 2 h with nitrogen flow. Operating conditions: injector temperature 270°, column temperature 210°, detector temperature 290° and carrier gas (oxygen-free nitrogen) flow-rate 40 ml/min.

Mass spectra were recorded with a Jeol D 100 mass spectrometer in connection with a gas chromatograph (Jeol, JGC-20K), a three-channel multiple ion detector unit and the Jeol mass data system. The GC column was a 100-cm glass column (3% OV-17) and the carrier gas was helium.

Standards and reagents

Sulfinpyrazone, phenylbutazone and sulfinpyrazone metabolites (G 31442 and G 32642) were obtained as pure crystalline compounds from Ciba-Geigy (Copenhagen, Denmark). Standard solutions of 5 and 100 μg/ml of sulfinpyrazone and metabolites in methanol and internal standard solutions of 1 mg/ml and 20 μg/ml of phenylbutazone in methanol were prepared and kept at 4°. Dichloromethane, methanol, ethyl acetate, toluene and methyl iodide were of analytical grade obtained from Merck (Darmstadt, G.F.R.). The methyl iodide was distilled before use. Dimethylacetamide and tetramethylammonium hydroxide (TMAOH), 20% in methanol, were obtained from EGA-Chemie (Steinheim, G.F.R.). The derivatization reagent (TMAOH solution) was prepared immediately before use by mixing 1 ml of 2% tetramethylammonium hydroxide in methanol with 8 ml of dimethylacetamide.

Procedure

One ml of sample (urine, serum or plasma), to which were added 25 μl of the internal standard solution (1 mg/ml for alkali flame ionization detection and 20 μg/ml for electron capture detection) and 1 ml of 2 *N* hydrochloric acid, was extracted with 6 ml of dichloromethane. An amount of 5.5 ml of the organic phase was transferred into a new tube and back extracted into 4 ml of 1 *N* sodium hydroxide; 3.5 ml of the aqueous phase were then transferred to a third centrifuge tube, acidified with 0.5 ml of 10 *N* hydrochloric acid and extracted with 6 ml of dichloromethane.

After centrifugation 5.5 ml of the organic phase was transferred to a tapered centrifuge tube and evaporated to dryness at 45° under a gentle stream of nitrogen. The remanence was dissolved in 100 μl of the TMAOH solution by vortex mixing for 10 sec. Methyl iodide 50 μl was added, the tube was shaken for 10 sec and left at room temperature for 10 min. Then 0.5 ml water and 1 ml dichloromethane were added, the tube was vortex mixed for about 30 sec and after centrifugation the organic phase was transferred to a centrifuge tube and evaporated to dryness at 45° under nitrogen flow. For alkali flame ionization detection the remanence was dissolved in 100 μl of ethyl acetate of which 1 μl was injected onto the gas chromatograph. For electron capture detection 200 μl of toluene and 1 ml of a hot saturated silver sulfate solution was added. The tube was shaken and after centrifugation 1—2 μl of the organic phase was injected.

Preparation of standard curves

Known amounts of sulfinpyrazone, the *p*-hydroxymetabolite or sulfone metabolite were added to plasma or urine and the samples were treated as described under *Procedure*. Standard curves were constructed by plotting the ratio of the peak heights of the derivatized sulfinpyrazone or *p*-hydroxymetabolite to that of the derivatized phenylbutazone against the concentration of sulfinpyrazone or the *p*-hydroxymetabolite. In constructing the standard curve for the sulfone metabolite, the peak height was plotted against the concentration.

Experiments in humans

To each of two healthy male volunteers, body weight 79 kg (AK) and 68 kg (PJ) a 100-mg Anturan® tablet was given orally. Blood samples were drawn at certain intervals (0—16 h) and the 24-h urine was collected. Serum samples and urine samples were analysed according to *Procedure*. The urine samples were tentatively treated with β-glucuronidase at pH 5 for 24 h at 37° and reanalysed.

RESULTS AND DISCUSSION

Gas chromatography

Sulfinpyrazone and its metabolites could not be chromatographed directly. Sulfinpyrazone showed more than one peak and the metabolites no peaks.

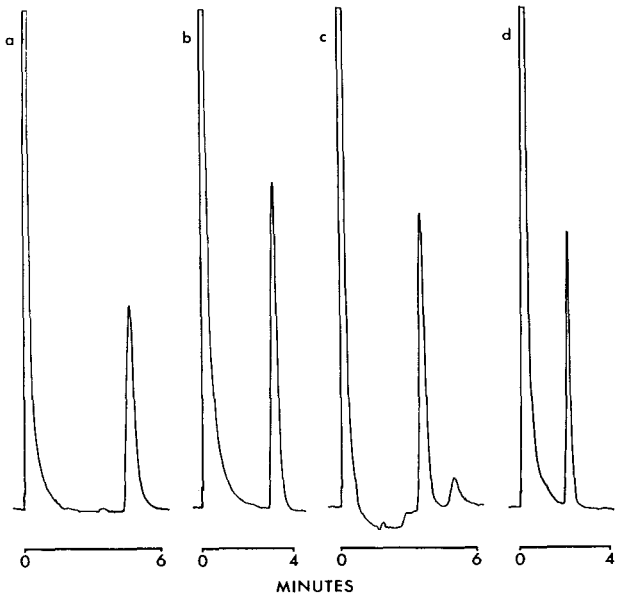


Fig. 1. Gas chromatograms with alkali flame ionization detection of (a) phenylbutazone (100 ng), (b) methylphenylbutazone (100 ng), (c) sulfinpyrazone (500 ng) and (d) methylsulfinpyrazone (100 ng). Column, 100 cm 2% OV-17, column temperature 200°, injector temperature 270°. Attenuation 64 × 10^{-13} A/mV.

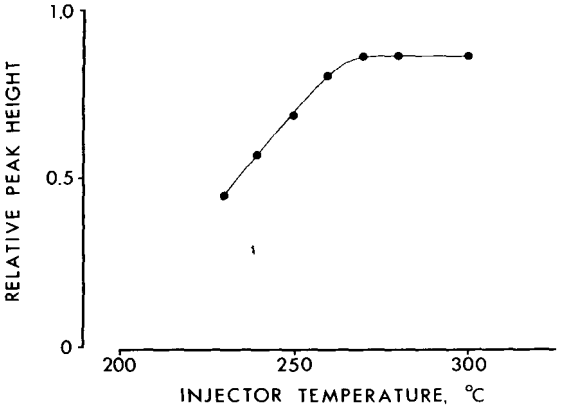


Fig. 2. Influence of injector temperature on the peak height of methylsulfinpyrazone. The ratios of the peak heights of methylsulfinpyrazone to methylphenylbutazone (50 ng of each) are plotted against the injector temperature.

Underivatized phenylbutazone gave a single peak. Methylation of the compounds gave derivatives with excellent GC properties when the injector port temperature was kept above 270° (Fig. 1). In contrast to methylated phenylbutazone, methylated sulfinpyrazone seems to degrade in the injector port as the peak shape and peak height improve with increasing injector temperature. At temperatures above 270° the degradation appears to be complete (Fig. 2). In order to analyse the methylated sulfone metabolite it was necessary to elevate the column temperature to 280° to reduce the retention time. Retention times for the methylated compounds in question are listed in Table II.

TABLE II

RETENTION TIMES FOR THE METHYL DERIVATIVES (min)

Compound	100 cm 2% OV-17, 200°	150 cm 3% OV-225, 210°
Sulfinpyrazone	2.1	3.1
Phenylbutazone	3.2 (4.7)*	4.1 (5.9)*
p-Hydroxy metabolite	5.6	7.5
sulfone metabolite	7.0**	35

*Underivatized phenylbutazone.
**Column temperature 280°.

Extraction and derivatization

Single extraction of acidified plasma blanks followed by derivatization gave a peak of variable size with the same retention time as methylated phenylbutazone together with a peak with a retention time of about 30 min. Back extraction into alkali with subsequent acidification, re-extraction and deriva-

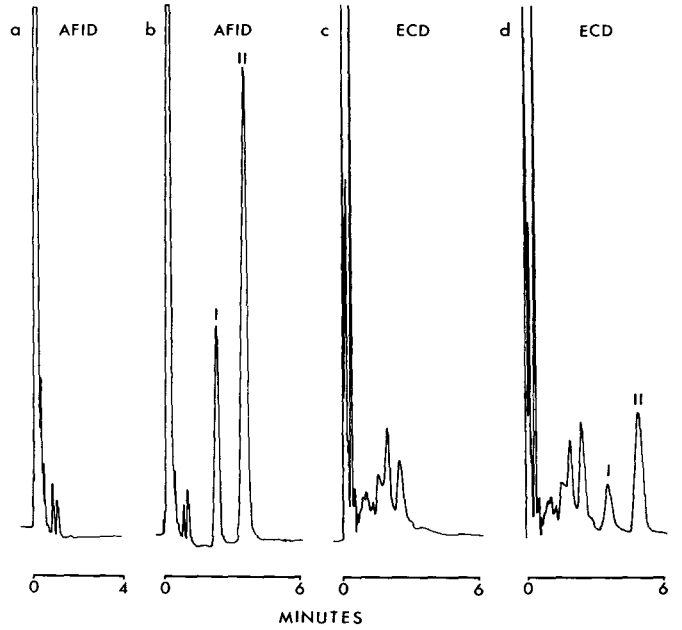


Fig. 3. Gas chromatograms with electron capture (ECD) and alkali flame ionization detection (AFID) of derivatized plasma extracts. a and c, plasma blanks; b, plasma containing sulfinpyrazone (I) (10 μg/ml) and phenylbutazone (II) (25 μg/mg); d, plasma containing sulfinpyrazone (I) (100 ng/ml) and phenylbutazone (II) (500 ng/ml). Conditions as described under Materials and methods.

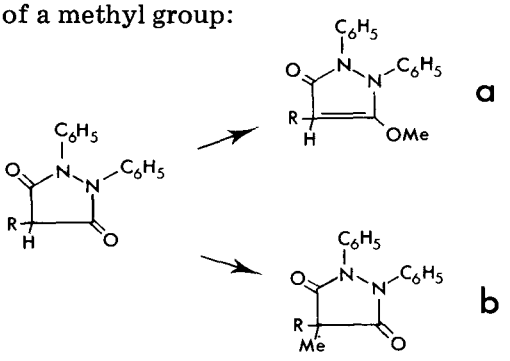
tization resulted in disappearance of the disturbing peaks. Fig. 3 shows gas chromatograms of plasma blanks together with plasma samples spiked with both sulfinpyrazone and phenylbutazone.

The method chosen for alkylation of the compounds has been applied in butylation of theophylline [10]. The reaction scheme for compounds with active hydrogen atoms is shown below:

$$HA + (CH_3)_4N^+ OH^- \rightarrow (CH_3)_4N^+ A^- \xrightarrow{RI} R{-}A$$

The reaction is based on formation of an ion pair between the quaternary ammonium ion and the appropriate anion which in turn reacts with an alkyl iodide to form an alkylated compound.

Two locations in the 3,5-dioxopyrazolidine ring structure are available for introduction of a methyl group:



O-Alkylation of malonic ester type compounds has to our knowledge not been found, so compound b is probably more easily formed than compound a. The structure has not been confirmed.

The reaction proceeds quickly with approximately 100% yield and the only disadvantage is the use of dimethylacetamide as solvent. This solvent has to be completely removed before injection as its presence will cause large tailing of the solvent peak when using a nitrogen sensitive detector. Other solvents such as acetonitrile, methanol, dichloromethane and ethyl acetate were tried without success as yields were poor. However, evaporation to dryness at 45° under nitrogen could be achieved within 30 min without loss of methylated compounds. In case of electron capture detection it was necessary to treat the remanence with silver sulfate in order to minimize solvent tailing. Attempts to methylate sulfinpyrazone with etheral diazomethane was not successful as more than one GC peak appeared. In course of preparation of the present analytical method Rosenfeld et al. [9] have described a GC method for the determination of sulfinpyrazone based on extractive methylation and normal flame ionization or mass spectrometric detection. In our preliminary experiments this extractive methylation technique was tried but analytical yields were in our hands not satisfactory.

Recovery and standard curves

For sulfinpyrazone and phenylbutazone no trace of underivatized compounds was found in the gas chromatograms after methylation of pure substances. Presumably all compounds mentioned are methylated quantitatively. When peak heights of derivatized pure compounds were compared to the peak heights of the same amounts of compounds carried through the complete sample preparation procedure, the following recoveries were found: 94.4% ± 4.8 (sulfinpyrazone), 93.8% ± 3.2 (phenylbutazone), 89.7% ±6.8 (the *p*-hydroxymetabolite) and 93.8 ± 8.2 (the sulfone metabolite). The values are corrected for loss of solvents during the extraction procedure.

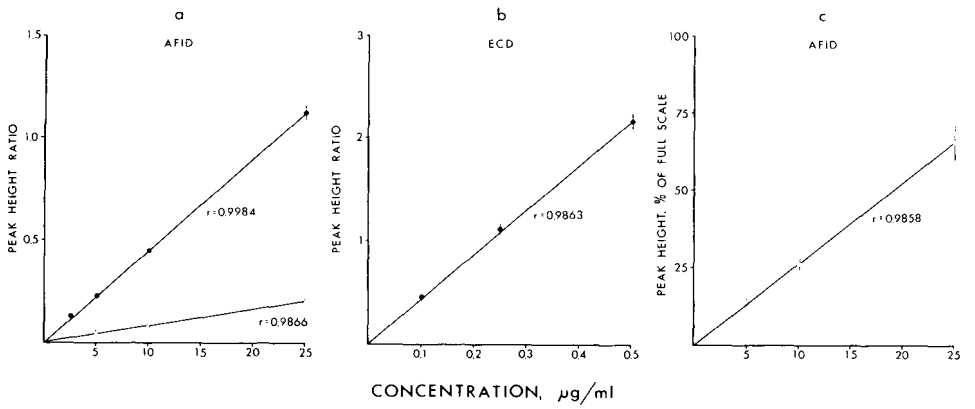


Fig. 4. Standard curves for determination of sulfinpyrazone (●——●), the *p*-hydroxy metabolites (G 32642) (△——△) and sulfone metabolite (G 31442) (○——○). Peak height ratio refers to the ratio between the peak heights of derivatized drug or metabolite and the derivatized internal standard (phenylbutazone).

A linear relationship between detector response and plasma concentration was found for sulfinpyrazone and its two metabolites (Fig. 4). As no suitable internal standard was available in the determination of the sulfone metabolite the peak height versus concentration was used as standard curve. For sulfinpyrazone the minimum detectable concentration was about 100 ng/ml sample using AFID and 10 ng/ml using ECD.

The analytical precision of the method depends on the concentration of drug. For sulfinpyrazone, the coefficient of variation was found to be 6.0% (n = 4) using electron capture detection of 100 ng/ml plasma samples and 1.8% (n = 6) using alkali flame ionization detection of 10 μg/ml plasma samples.

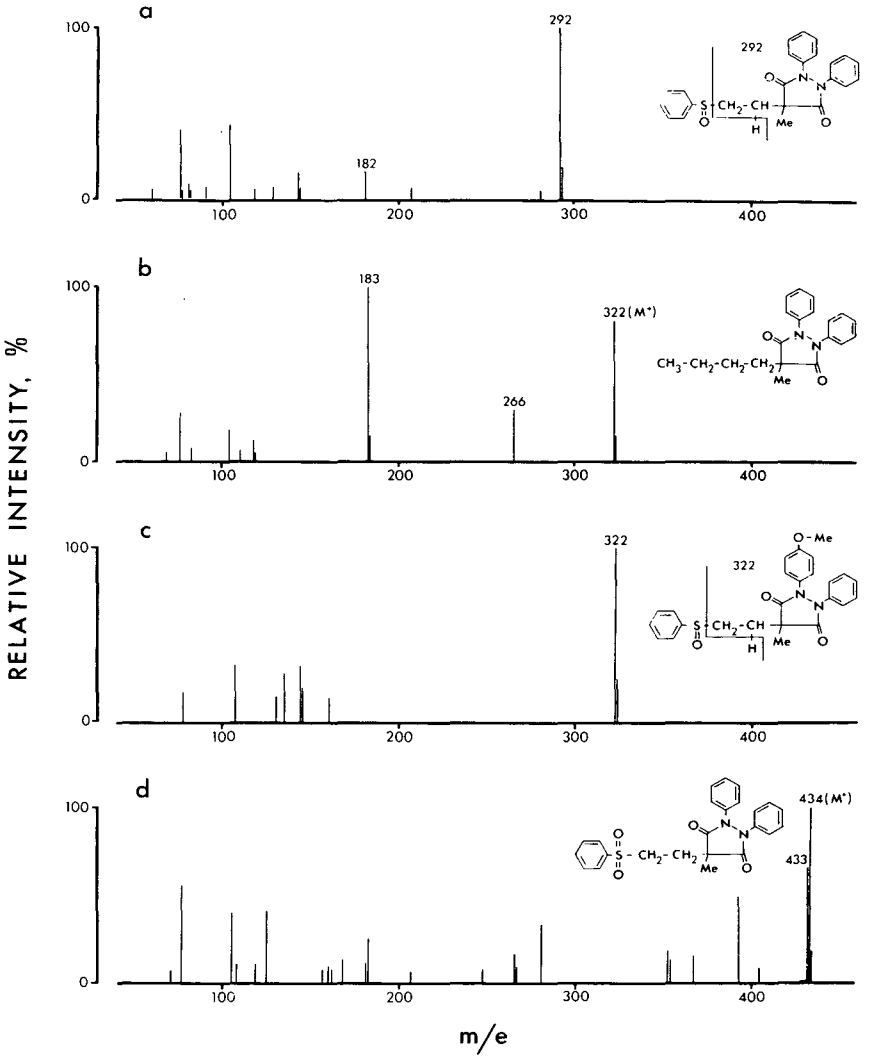


Fig. 5. GC—MS (26 eV) of the methyl derivatives of (a) sulfinpyrazone, (b) phenylbutazone, (c) p-hydroxy metabolite (G 32642) and (d) sulfone metabolite (G 31442).

Mass spectrometry

Mass spectrometry (MS), ionization energy 10 eV or 26 eV, using direct inlet of the methylated sulfinpyrazone gave a molecular ion (*m/e* = 418) of 14 mass units above the molecular weight of sulfinpyrazone showing substitution of a single methyl group in the sulfinpyrazone molecule. Combined gas chromatography—mass spectrometry (GC—MS) at 26 eV ionization energy did not show this ion but a loss of 126 mass units from the methylated sulfinpyrazone indicating elimination of the fragment [C_6H_5SOH] (Fig. 5a). GC—MS of methylated phenylbutazone (Fig. 5b) showed no degradation. The same cleavage as that of methylsulfinpyrazone with splitting off of the fragment [C_6H_5SOH] also takes place during GC-MS of the methylated-hydroxy metabolite. Fig. 5c shows substitution of the phenolic hydrogen atom as well as in the pyrazolidine ring structure. GC—MS of the sulfone metabolite (Fig. 5d) showed introduction of one methyl group. The high-intensity molecular ion together with the long retention time indicate thermal stability of this compound.

The loss of [C_6H_5SOH] seen in the mass spectra of methylated sulfinpyrazone and the methylated *p*-hydroxy metabolite is due to a thermal cleavage, which probably takes place in the gas chromatograph. Sulfoxides are usually not thermally stable compounds. They undergo 1,2-elimination [11] reactions at elevated temperature resulting in formation of alkenes:

$$R-\underset{\underset{O}{\|}}{S}-CH_2-CH_2-R' \xrightarrow{\Delta} [RSOH] + CH_2 = CH-R'$$

When sulfinpyrazone undergoes this elimination reaction a compound with a structure very similar to that of phenylbutazone is formed. The two compounds are however easily separated on 2% OV-17 or 3% OV-225 columns.

GC—MS of methylsulfinpyrazone shows a base peak of *m/e* 292, an ion which is not present in the mass spectrum of the methylphenylbutazone. The high-intensity molecular ion of this compound (*m/e* 322) is present as base peak in the mass spectrum of the methyl derivative of the *p*-hydroxy metabolite. The presence of these two ions makes it possible simultaneously to determine sulfinpyrazone and its hydroxylated metabolite using multiple ion detection with only two channels. Injection of 0.1—50 ng of the methyl derivatives of sulfinpyrazone and the *p*-hydroxy metabolite together with a fixed amount of methylated phenylbutazone (50 ng) gave linear standard curves for both methylated sulfinpyrazone (r = 0.996) and the methylated *p*-hydroxy metabolite (r = 0.987). Single extraction from acidified plasma samples was found to be satisfactory as ions of *m/e* 292 and *m/e* 322 from the impurities in plasma did not interfere (Fig. 6). This improves the capacity and speed of the method. With a proper internal standard, determination of the sulfone metabolite is possible with probably just as good a sensitivity using the molecular ion of the methylated sulfone metabolite.

Since both the selectivity and the sensitivity of the mass fragmentographic detection method is superior to that of electron capture detection, the mass fragmentographic method is suitable for the determination of trace amounts of the substances.

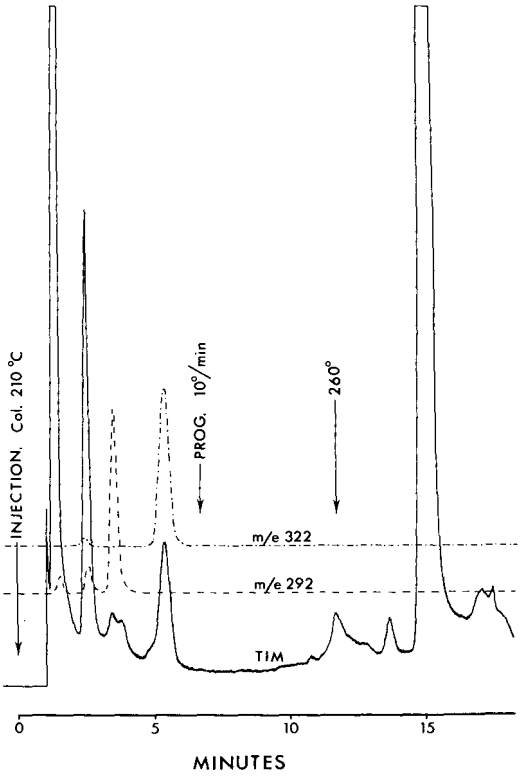


Fig. 6. Total ion monitoring (TIM) and selected ion monitoring of a derivatized extract of plasma containing sulfinpyrazone and phenylbutazone (single extraction). The mass spectrometer was set to detect the base peak in the mass spectra of methylsulfinpyrazone (*m/e* 292) and the molecular ion of methylphenylbutazone (*m/e* 322).

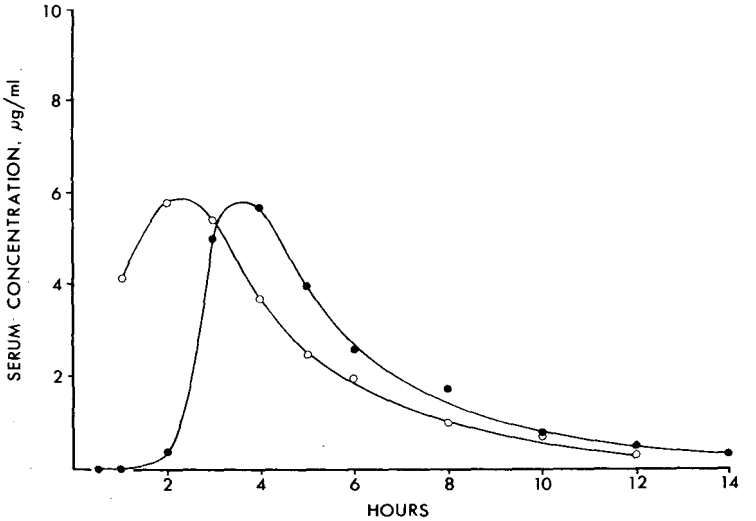


Fig. 7. Sulfinpyrazone in serum (alkali flame ionization detection) after oral administration of 100 mg of the drug to two volunteers. ○——○: AK; ●——●: PJ.

TABLE III

AMOUNTS (mg) OF UNCHANGED DRUG AND METABOLITES EXCRETED IN URINE IN 24 H AFTER ORAL ADMINISTRATION OF 100 mg OF SULFINPYRAZONE

Compound	PJ	AK
Sulfinpyrazone	36.1	57.3
p-Hydroxy metabolite	7.3	9.0
Sulfone metabolite	2.5	2.4

Fig. 7 shows the serum concentration curves obtained from the human experiments. No measureable amounts of metabolites were found in serum using alkali flame ionization detection. The excreted amounts of sulfinpyrazone and two of its metabolites in the 24-h urine are listed in Table III. Treatment of the urine with β-glucuronidase did not increase the measured amounts.

A number of commonly used acidic drugs including barbiturates, weak analgesics and diuretics did not interfere with the determination.

The finding that β-glucuronidase is not able to increase the measured amounts of sulfinpyrazone in urine is consistent with the earlier work of Dieterle et al. [4], who found that glucuronic acid is conjugated to sulfinpyrazone via a C—C bond, which cannot be cleaved by β-glucuronidase. This metabolite is not measured by our method.

At present only limited information is available about plasma levels of sulfinpyrazone during therapy. A convincing correlation between the dose of sulfinpyrazone and inhibition of platelet function, measured by [^{14}C]serotonine release, has been reported [1]. The plasma levels in patients with transient ischemic attacks receiving 800 mg per day reported by Rosenfeld et al. [9] showed a considerable scatter within the range of 4—29 μg/ml. Furthermore, the presence of active metabolites, which seem to be equipotent with the parent molecule in inhibiting the platelet release reaction [12], has been suggested to cause the biphasic effect of sulfinpyrazone in rabbits in vivo reported by Buchanan et al. [13]. These data call for more detailed investigations of the metabolism and pharmacokinetics of sulfinpyrazone in man.

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SIMULTANEOUS DETERMINATION OF GRISEOFULVIN AND 6-DESMETHYLGRISEOFULVIN IN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

Two methods have been developed for the simultaneous determination of griseofulvin and its major metabolite 6-desmethylgriseofulvin in plasma using electron-capture gas chromatography. The first method was based on the quantitative reversion of the 6-desmethyl metabolite to griseofulvin by diazomethane. Plasma extract was chromatographed both before and after treatment with diazomethane, the former being the measure of griseofulvin and the latter representing the sum of the two compounds. In the second method, plasma extract was treated with diazobutane and griseofulvin and the butylated metabolite were separated by gas chromatography. The sensitivity for griseofulvin was 20 ng/ml by both methods and that for the metabolite was 20 ng/ml and 40 ng/ml by the first and the second method, respectively. The concentrations of the metabolite as well as griseofulvin were determined in dog and human plasma after oral administration of griseofulvin.

INTRODUCTION

Griseofulvin is an orally active antifungal agent used widely in clinical practice. The concentration of this drug in blood has been measured by spectrofluorometry [1, 2], electron-capture gas chromatography [3] and, more recently, high-performance liquid chromatography [4, 5]. In all of these methods, the concentration of unmetabolized griseofulvin has been the object of determination. Since griseofulvin is metabolized extensively to 6-desmethylgriseofulvin in rabbits [6], dogs [7] and man [8, 9] and excreted in urine almost exclusively as the 6-desmethyl metabolite and its conjugate, the de-

termination of 6-desmethylgriseofulvin in blood in addition to the unchanged drug should be useful in biopharmaceutical studies. This report describes gas chromatographic (GC) methods of determining griseofulvin and 6-desmethylgriseofulvin simultaneously in blood plasma.

EXPERIMENTAL

Chemicals

Griseofulvin was purchased from Nihon Kayaku (Tokyo, Japan). Tablets each containing 125 mg of griseofulvin, were a commercial preparation (Yamanouchi Pharmaceutical, Tokyo, Japan). 6-Desmethylgriseofulvin was isolated from urine of a male beagle dog, which received orally a total of 12 g of griseofulvin, according to the method of Harris and Riegelman [7]. The compound was recrystallized from ethanol and had a melting point of 276—278° (decomp., lit. 279—281° [7]). The IR spectrum agreed with that reported in the literature [7]. The elemental analysis and NMR spectrum also supported the structure.

Indomethacin methyl ester, used as the internal standard, was prepared from indomethacin (Sumitomo Chemical, Osaka, Japan). A 0.5—1-ml aliquot of dichloromethane containing 1 mg of indomethacin was mixed with 3 ml of ethereal diazomethane and left at room temperature for 5 min. After evaporation of the reagent with slight warming, the residue was dissolved in benzene to a concentration of 100 μg/ml. This solution was stable for at least one month at room temperature. At the time of assay, an aliquot was diluted with methanol to give a concentration of 1.25 μg/ml. Ethereal solution of diazomethane was prepared from *p*-toluenesulfonyl-N-methyl-N-nitrosamide (Tokyo Kasei, Tokyo, Japan) using a specialized equipment Diazald® Kit (Aldrich, Milwaukee, Wisc., U.S.A.). Ethereal solution of diazobutane was prepared simply as follows. To 0.8 g of N-butyl-N-nitrosourea (Nakarai, Kyoto, Japan), dissolved in 8 ml of diethyl ether and cooled to 0°, was added 3 g of KOH pellets. After 10 min of occasional shaking, the diethyl ether layer was transferred to another glass tube containing 2 g of KOH pellets and was used for derivatization. All other reagents used were commercial preparations and of analytical grade.

Instrumentation

A Hewlett-Packard Model 5710A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The column was a glass tube (110 cm × 1.8 mm I.D.) packed with 1.5% OV-225 on 100—200 mesh Gas-Chrom Q (Nihon Chromato Works, Tokyo, Japan). The temperatures were 300° for the injector, 275° for the column, and 300° for the detector. The carrier gas, argon—methane (95:5), was dried over molecular sieve and passed at a flow-rate of 30 ml/min. Mass spectra were obtained with a Hitachi RMU-6MG combined gas chromatograph—mass spectrometer under the following conditions: electron energy 20 eV, emission current 80 μA, ion source temperature 160°, accelerating voltage 1.4 kV.

Simultaneous determination of griseofulvin and 6-desmethylgriseofulvin

Method 1. 6-Desmethylgriseofulvin in the plasma extract was converted to and assayed as griseofulvin after griseofulvin itself had been assayed with the same extract. To 0.5 ml of plasma were added 1.5 ml of 0.1 *N* hydrochloric acid and 0.2 ml of methanol containing 250 ng of indomethacin methyl ester. The mixture was extracted with 5 ml of diethyl ether and, after centrifugation, the diethyl ether layer was taken to dryness at 40—50°. The residue was dissolved in 0.1 ml of benzene and 1—2 μl was injected into the gas chromatograph column for the determination of the unchanged drug. The remaining benzene solution was then treated with 0.2 ml of ethereal diazomethane and, after leaving for 5 min at room temperature, evaporated. This treatment converted the 6-desmethyl metabolite quantitatively to griseofulvin [8]. After reconstituting the residue in 0.1 ml of benzene, 1—2 μl was chromatographed for the determination of the unchanged drug plus its metabolite. At a column temperature of 275°, the retention times of the internal standard and griseofulvin were 3 min and 3.8 min, respectively.

Alternatively, the diethyl ether layer was divided into two parts and each was evaporated to dryness. The residue from one part was taken up in 50 μl of benzene and that from the other half was treated with 0.1 ml of ethereal diazomethane, followed by evaporation after 5 min.

The amount of griseofulvin as well as griseofulvin plus its metabolite was calculated by measuring the peak height ratio of griseofulvin and the internal standard and referring to the standard curve. The standard curve was prepared by subjecting to the above procedure 0.5 ml of the drug-free control plasma, to which 10—250 ng of griseofulvin dissolved in 0.2 ml of 20% methanol had been added. The peak height ratio of griseofulvin and the internal standard was plotted against the concentration of griseofulvin. When the concentrations of the two compounds were high, the plasma samples were appropriately diluted with distilled water.

Method 2. Extraction of the two compounds from plasma was performed as in Method 1. After evaporation of the diethyl ether layer, 0.2 ml of etheral diazobutane was added to the residue. The mixture was left at room temperature for 5 min, the reagent evaporated and the residue dissolved in 0.1 ml of benzene. A 1—2-μl volume of the solution was injected into the column. At a column temperature of 275°, the retention times of the internal standard, griseofulvin and butylated 6-desmethylgriseofulvin were 3.0 min, 3.8 min and 5.2 min, respectively. Ratios of the peak height of griseofulvin as well as the butylated metabolite to that of the internal standard were measured. Standard curves were prepared by analyzing drug-free control plasma spiked with 10—250 ng each of the two compounds per 0.5 ml according to the above procedure.

Extraction recoveries

To 0.5 ml of control dog plasma were added 200 ng of griseofulvin or the metabolite dissolved in 0.2 ml of 20% methanol. After adjusting the pH between 0.5 and 10 by the addition of 0.1—1.0 *N* HCl or 0.1—1.0 *N* NaOH and bringing the volume to 2 ml, the mixture was extracted with 5 ml of diethyl ether, followed by centrifugation. A 2.5-ml aliquot of the diethyl ether layer

was mixed with 200 ng of the internal standard dissolved in 0.5 ml of diethyl ether and analyzed for each compound by Method 1. The extraction recoveries were calculated by comparing the peak height ratios with those obtained when 100 ng of each compound, dissolved in 2.5 ml of diethyl ether, was mixed with 200 ng of the internal standard and processed without the extraction procedure.

Animal and human studies

Three male beagle dogs, weighing 8—12 kg, were fasted for 21 h and given 125 mg of griseofulvin orally as a tablet. Similarly, three male volunteers, aged 38—44 years, received 250 mg of the drug after overnight fasting. Blood samples were obtained by venipuncture with heparinized syringes and immediately centrifuged. Plasma samples were stored frozen until analyzed.

RESULTS AND DISCUSSION

Extraction of griseofulvin and the metabolite from plasma

6-Desmethylgriseofulvin differed from griseofulvin in its extractability from plasma. While the metabolite was extracted quantitatively with diethyl ether below pH 4, it was extracted less efficiently at higher pH values and remained totally unextracted above pH 8. Similar results have previously been reported for the metabolite in urine by Rowland and Riegelman [10]. Extraction of griseofulvin, on the other hand, was quantitative at any pH value (Fig. 1).

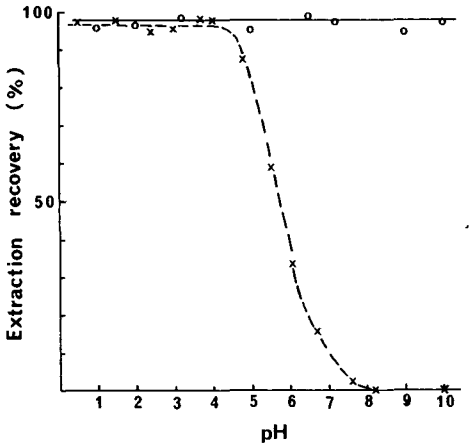

Fig. 1. Effect of pH on the extraction of griseofulvin (O——O) and 6-desmethylgriseofulvin (X- - -X) from plasma.

Evaluation of the methods

In the preliminary study in which GC properties of 6-desmethylgriseofulvin were examined using various columns and operating conditions, it was found that this metabolite does not produce a well-defined chromatographic peak when analyzed without derivatization, as reported by Schwarz et al.

[3]. The phenol function of the metabolite had to be derivatized for successful chromatography. Trimethylsilylation, which was used by Kabasakalian et al. [11] to measure the metabolite in human urine, was not quite satisfactory since the chromatographic peak tended to be accompanied by tailing and separation from endogenous material in plasma was sometimes difficult. Acylation of the phenol group, particularly propionylation, produced a derivative with a fine chromatographic property and which was separated from griseofulvin and endogenous material using a column of 3% OV-105. The drawback of the propionylated metabolite was its much lower sensitivity towards electron-capture detection compared with the parent drug and its comparative instability which necessitated careful handling during analysis.

The most satisfactory derivatives in terms of stability, chromatographic properties and simplicity in preparation were obtained by alkylation. 6-Desmethylgriseofulvin was alkylated quantitatively to its methyl and butyl derivatives by the methods described in Experimental. This was confirmed as follows: (1) When the alkylation reaction mixture containing the metabolite (20 μg) and diazomethane or diazobutane was examined by thin-layer chromatography using a silica gel plate (Merck, Darmstadt, G.F.R., silica gel 60 F_{254}) and a solvent system chloroform—diethyl ether—acetone—acetic acid (65:20:15:0.5), the metabolite, detectable by short-wave UV light at R_F 0.37, disappeared within 1 min and was completely replaced by new compounds which appeared at R_F values of 0.59 and 0.71, respectively. The former agreed with griseofulvin in its R_F value. The latter was supposedly a butylated analogue of the drug. (2) GC examination of the reaction mixture revealed that, after treatment with diazomethane, the metabolite gives a well-shaped peak at the same retention time as griseofulvin, while the metabolite itself does not give rise to any discernible peak. An equimolar quantity of the drug and the metabolite (10—250 ng), added either to the alkylation reaction mixture or to control plasma and processed as in Method 1, produced exactly the same peak height demonstrating the quantitative methylation of the latter. After treatment with diazobutane, the metabolite gave a sharp peak at a retention time different from that of the parent drug. The mass spectrum of this peak, obtained by GC—mass spectrometry, showed a molecular ion at *m/e* 394 in agreement with the supposed structure, while griseofulvin itself showed a molecular ion at *m/e* 352. The peak height of the butylated metabolite was about 40% less than that from equimolar griseofulvin under the conditions described in Method 2.

The chromatograms obtained from a control dog plasma to which both compounds had been added at a concentration of 200 ng/ml are shown in Fig. 2. As is evident, the peak height of griseofulvin relative to that of the internal standard was doubled when the plasma extract was treated with diazomethane in Method 1. Treatment with diazobutane resulted in appearance of a new peak due to the butylated metabolite. Indomethacin methyl ester as an internal standard, in combination with an OV-225 column, gave excellent separation of the peaks of interest with minimum tailing. Drug-free control plasma gave no interfering peaks either in Method 1 or in Method 2.

The standard curves are shown in Fig. 3. Linear response was obtained for griseofulvin over the concentration range of 20—500 ng/ml plasma in

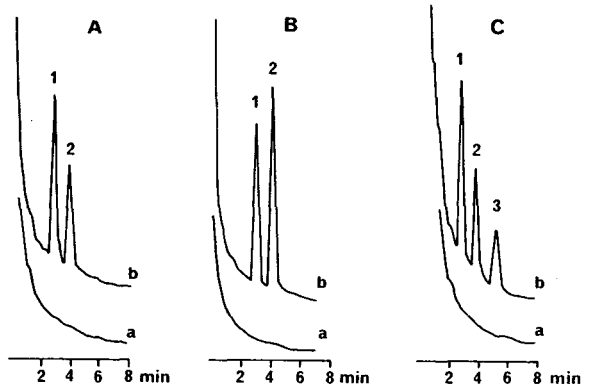

Fig. 2. Chromatograms of control dog plasma (a) and dog plasma spiked with 200 ng/ml each of griseofulvin and 6-desmethylgriseofulvin (b). A, Without derivatization (Method 1); B, after treatment with diazomethane (Method 1); C, after treatment with diazobutane (Method 2). Peaks: 1, internal standard; 2, griseofulvin; 3, butylated 6-desmethylgriseofulvin.

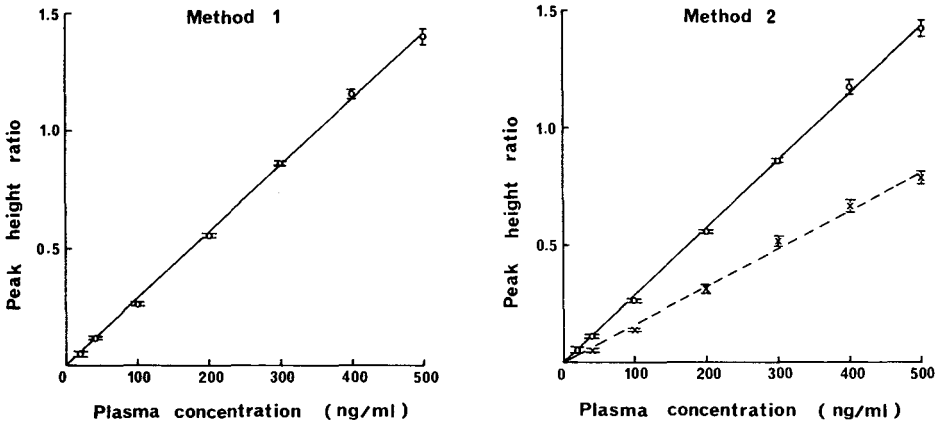

Fig. 3. Standard curve for the determination of griseofulvin (O—O) and 6-desmethylgriseofulvin (X- - -X) in plasma. Each point represents the mean ± S.E.M. from four experiments.

both methods using a 0.5-ml sample. The sensitivity for the metabolite in the second method was slightly lower than that for the parent drug and linear response was obtained over the range of 40—500 ng/ml. The standard curve prepared from 6-desmethylgriseofulvin in the first method was exactly the same as that prepared from griseofulvin. Moreover, by analyzing plasma samples which contained graded amounts of the drug (20—400 ng/ml) in the presence of a fixed amount of the metabolite (200 ng/ml) or plasma samples which contained graded amounts of the metabolite (20—400 ng/ml) in the presence of a fixed amount of the drug (200 ng/ml), it was confirmed that the amount of the drug detected is not affected by the metabolite present when treatment with diazomethane is omitted and the amount detected after diazomethane treatment is exactly equal to that predicted from quantitative conversion of the metabolite.

The advantage of the first method is that it is applicable even when the standard sample of the 6-desmethyl metabolite is not available, while the second method, being a simpler one-step procedure, is preferred when the metabolite is available.

Application of the methods

The two compounds in plasma were determined after oral administration of griseofulvin to dogs and man. All the plasma samples were assayed by both Method 1 and Method 2. The results, obtained by Method 2, are shown in Figs. 4 and 5. An example of a chromatogram is shown in Fig. 6. The plasma concentration of griseofulvin in man was comparable to that of the metabolite throughout the sampling period, which agreed with the result reported by Lin et al. [9] using the ^{14}C-labelled drug. In contrast, the concentration of the metabolite was more than 6 times higher than that of the parent drug

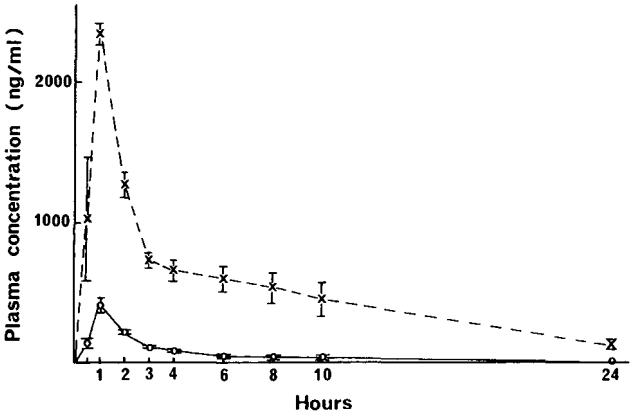

Fig. 4. Plasma concentration of griseofulvin (O—O) and 6-desmethylgriseofulvin (x- - -x) in dogs after oral administration of 125 mg of griseofulvin. Each point represents the mean ± S.E.M. from three experiments.

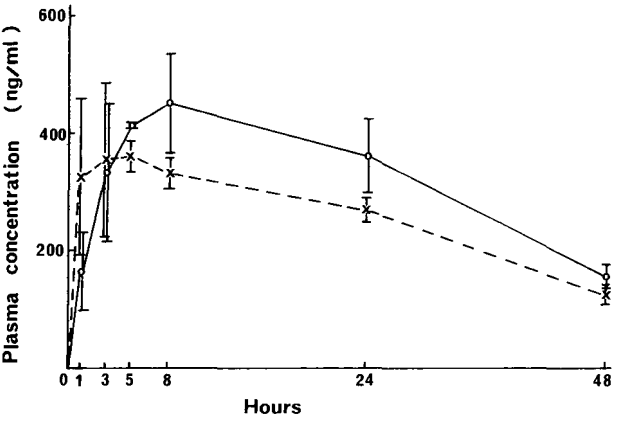

Fig. 5. Plasma concentration of griseofulvin (O——O) and 6-desmethylgriseofulvin (x- - -x) in man after oral administration of 250 mg of griseofulvin. Each point represents the mean ± S.E.M. from three experiments.

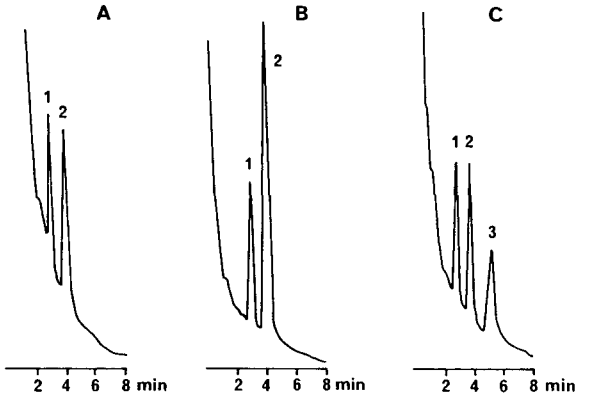

Fig. 6. Chromatograms of a plasma sample obtained from a volunteer 5 h after oral administration of 250 mg of griseofulvin. A, Without derivatization (Method 1); B, after treatment with diazomethane (Method 1); C, after treatment with diazobutane (Method 2). Peaks: 1, internal standard; 2, griseofulvin; 3, butylated 6-desmethylgriseofulvin.

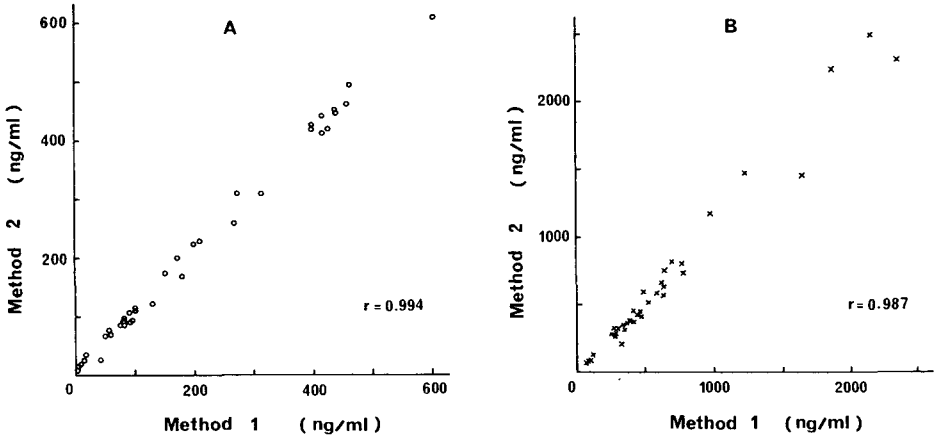

Fig. 7. Correlation between values obtained by Method 1 and Method 2. A, Griseofulvin; B, 6-desmethylgriseofulvin.

in dogs. This observation supports the conclusion reached by Harris and Riegelman [7] on the basis of pharmacokinetic studies that the metabolism of the drug is much faster in dogs than in man. The values obtained for the same samples by the two methods agreed quite well. This is shown in Fig. 7, in which all the data obtained in dogs and man by Method 2 are plotted against the corresponding data obtained by Method 1. The correlation coefficients for the parent drug and the metabolite were 0.994 and 0.987, respectively.

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DETERMINATION OF 6-MERCAPTOPURINE AND AZATHIOPRINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Using 1-ml plasma samples, levels of 6-mercaptopurine (6MP) as low as 5 ng/ml and azathioprine (AZA) as low as 40 ng/ml can be detected using a high-performance liquid chromatography reversed-phase column procedure following extraction. Both compounds were stable in frozen plasma for seven weeks. AZA stability in blood was temperature dependent; the half-lives of AZA breakdown to 6MP at 37° were 28 and 46 min in blood drawn from two rhesus monkeys. Plasma levels of 6MP were measured in a rhesus monkey following 6MP (1.47 mg/kg) and AZA (3 mg/kg) intravenous administration. 6MP levels were also measured in three renal transplant patients on daily 50- and 100-mg AZA doses. Peak levels (45—75 ng/ml) were reached within an hour and 6MP levels were detected for up to 7 h.

INTRODUCTION

The immunosuppressive agent azathioprine (AZA), 6-(1-methyl-4-nitro-5-imidazolyl) thiopurine, is primarily used as an adjunct for preventing rejection of organ transplantation. It is metabolized in vivo to 6-mercaptopurine (6MP), which is anabolized to the biologically active thioinosinic acid, methylthioinosinic acid and thioguanylic acid [1].

In our attempt to determine the bioavailability and pharmacokinetics of AZA, we endeavored to measure plasma levels of the parent drug and 6MP. A limited number of analytical methods have been developed to measure levels of AZA or 6MP in plasma and urine. Methods employing radioisotopes such as [^{35}S]AZA [2—4] or [^{14}C]AZA [5] with the label in the imidazole moiety, give non-specific information on the absorption, distribution and elimination characteristics of the drug. The fluorescence method of Finkel [6] for measuring serum levels of 6MP was not sensitive enough to measure the low levels found in animals or man after AZA or 6MP administration. The spectro-

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photometric method by Chalmers [7] for measuring urinary AZA and 6MP is also not sufficiently sensitive nor specific for our purpose. Other methods of determining 6MP include gas chromatography (GC) [8] and mass spectrometry (MS) [9]; both methods involve derivatization of 6MP prior to analysis. The gas—liquid chromatographic (GLC) method [8] is not sensitive enough to measure 6MP serum levels following the usual oral doses of AZA given to patients. The GC—MS method [9] reports a limit of detection of 20 ng/ml and yields 18% recovery for the combined extraction, derivatization, drying and measurement procedures.

Most high-performance liquid chromatographic (HPLC) analyses of thiopurines have been limited to determination in tissue extracts [10—14]. De-Miranda et al [15] however, did separate AZA and its metabolites in rat plasma and urine using LC techniques. Day et al. [16] used paired ion HPLC to determine 6MP in plasma. A sensitivity of 0.2 μg of 6MP per ml is reported. In the present work, a sensitive and specific assay for 6MP and AZA is described using HPLC. Both 6MP and AZA can be assayed from a one-ml plasma sample.

EXPERIMENTAL

Materials

6MP monohydrate, AZA and 8-OHMP hemihydrate were generously supplied by Burroughs Wellcome (Research Triangle Park, N.C., U.S.A.). 9-Methylmercaptopurine (9MMP) and 6-methylmercaptopurine (6MMP) were purchased from Heterocyclic Chemical Co. (Harrisonville, Mo., U.S.A.). Dithioerythritol (DTE) was obtained from Sigma (St. Louis, Mo., U.S.A.) and stored at 4°. HPLC grade ethyl acetate was from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Acetonitrile was of nanograde purity (Mallinckrodt, St. Louis, Mo., U.S.A.). Stock solutions of 6MP, 9MMP, 6MMP, and AZA were dissolved in methanol and stored at 4°.

Procedure

One ml of plasma was placed in a 15 mm × 150 mm test tube; 120 ng 9MMP and 500 ng 6MMP were added as internal standards for 6MP and AZA, respectively; 200 μl of 2 *N* hydrochloric acid and 5 ml ethyl acetate were added. The tube was capped and shaken for 10 min using a tube rotator (BBL, Cockeysville, Md., U.S.A.). The tube was centrifuged for 10 min and the organic layer was transferred to another test tube for analysis of AZA. The extraction was repeated with another 5 ml of ethyl acetate. The organic portions were combined together in one tube and 1 ml of 1 *M* sodium acetate buffer, pH 5.1 was added. The sample was shaken for 10 min and centrifuged for 10 min. The ethyl acetate layer was transferred to a nipple tube and evaporated to dryness under nitrogen. The residue was reconstituted with 50 μl of acetonitrile, vortexed for 1 min and centrifuged for 5 min; 15 μl of the sample were injected into the HPLC apparatus for AZA analysis using a μBondapak C_{18} column.

To the plasma left after the two ethyl acetate extractions, 10 μl of a 1% solution of DTE in distilled water were added; 1 ml of 1 *M* sodium acetate

buffer, pH 5.1 and 10 ml ethyl acetate were added. The sample was shaken and centrifuged, each process for 10 min. The organic layer was transferred to a nipple tube and evaporated to dryness under nitrogen. The sample was reconstituted with 50 μl of HPLC buffer used for 6MP analysis (see below), 50 μl of 0.2 *N* sulfuric acid and 100 μl of ethyl acetate. The tube was vortexed for 1 min, centrifuged for 5 min and about 90 μl of the aqueous phase was injected into the HPLC apparatus, using a LiChrosorb column for analysis.

HPLC analysis was performed using a Perkin-Elmer liquid chromatograph Series 2, equipped with a LC-55 Perkin-Elmer spectrophotometer for detection at 325 nm for 6MP and 280 nm for AZA. 6MP was assayed with a LiChrosorb RP-18 column, 10 μm particle size, 25 cm × 4.6 mm I.D. (E. Merck, Darmstadt, G.F.R.) and a Spectrum 921 filter. The eluent is comprised of 1% methanol, 0.5% acetonitrile and 60 mg DTE per liter 0.005 *M* potassium phosphate buffer at pH 4.0; flow-rate 2 ml/min. AZA was analyzed with a μBondapak C_{18} column, 10 μm particle size, 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, Mass., U.S.A.); the eluent is 11% acetonitrile in 0.01 *M* sodium acetate buffer pH 4.0: flow-rate 2 ml/min.

Stability study of 6MP and AZA in plasma

Plasma samples spiked with 45 ng 6MP per ml were frozen and assayed at 0, 2, 8, and 52 days. A similar stability study was carried out for AZA spiked at 0.41 μg/ml plasma.

Stability of AZA in blood

The stability of AZA in blood drawn from two rhesus monkeys was determined at 37°, room temperature and in ice. Blood was spiked with AZA and incubated at the respective temperatures. Aliquots of blood were removed at 0, 30, 60, 120, 180 min and immediately centrifuged. An additional aliquot was removed at 15 min for blood incubated at 37°. The plasma was then assayed for AZA.

Animal studies

Intravenous preparations of AZA at 3 mg/kg and 6MP at 1.47 mg/kg were administered to a rhesus monkey in separate studies. The solution of AZA was prepared by injecting 10 ml water into a 100-mg vial of the drug (pH of final solution = 9.7). The solution of 6MP was prepared by dissolving the powder in sodium hydroxide solution and diluting with saline (pH of final solution = 10). Blood was sampled at 0, 5, 10, 15, 20, 30, 40, 60, 90, 120, 180, 240 min and kept in ice before it was centrifuged. The plasma was frozen and assayed for 6 MP and AZA.

Patient studies

Informed consent was obtained from three renal transplant patients, hospitalized at the Moffitt Hospital, University of California, San Francisco. Two of these patients were receiving daily oral AZA doses of 50 mg while the third was receiving 100-mg daily doses. Eight-ml blood samples were taken 10 times over a 12-h period. The blood samples were immediately placed in

an ice bath until the plasma was separated by centrifugation. Plasma samples were split for analysis of oral steroids in another study and for the AZA and 6MP measurements described here.

RESULTS AND DISCUSSION

It was found necessary to add DTE during the final extraction step and in the HPLC solvent system for 6MP analysis. Following these additions, improved peak heights of 6MP and 9MMP were observed at 325 nm. Consistent peak heights for duplicate injections of the same amount of thiopurine were observed when DTE was used. This suggests that DTE may have a stabilizing effect on the unsubstituted thiols. Bailey et al. [8] have also used DTE as a sulfhydryl-protecting reagent in their extraction procedures for GC analysis of 6MP.

The addition of DTE, however, would convert AZA to 6MP if the unchanged drug was present in the plasma sample. For this reason AZA was separated from 6MP in the initial extraction steps. AZA was then analyzed separately from 6MP. If the amount of AZA in the plasma was less than 100 ng/ml, very little of the drug would be left in the plasma following extraction to be converted to 6MP upon adding DTE.

Sample HPLC chromatograms are depicted in Fig. 1 for 6MP and 8-hydroxymercaptopurine (8-OHMP) (left portion of Fig. 1) and AZA (right portion of Fig. 1). Blank plasma samples are designated as I while chromatograms for plasma with added drug and internal standard are designated II.

Thiouric acid, the chief metabolite formed following oxidation of 6MP by the enzyme xanthine oxidase is very poorly extracted from plasma. It

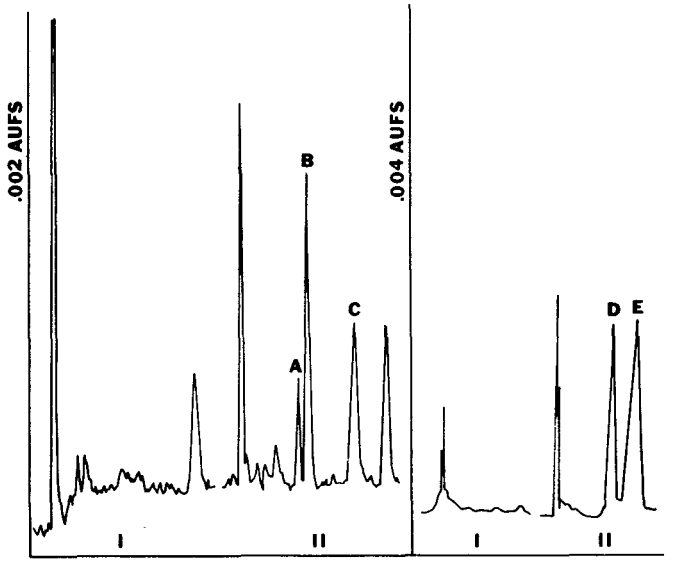

Fig. 1. HPLC chromatograms of plasma samples without (I) and with (II) added drug and internal standard. Numbers in parentheses indicate retention times in min. A = 8-OHMP (5 min); B = 6MP (5½ min); C = 9MMP (8 min); D = 6MMP (6 min); E = AZA (8 min).

elutes at an earlier retention time than 6MP and 8-OHMP after passing through the column.

Over the 6MP concentration range of 10—100 ng/ml, standard curves of peak height ratio (6MP/9MMP) versus 6MP concentration were constructed (slope = 0.0138, intercept = 0.0509, r^2 = 0.9970). The limit of sensitivity for accurate measurement of 6MP is 5 ng/ml. Standard curves for AZA were constructed over a 0.05—0.80 μg/ml plasma concentration range using peak height ratios of AZA to 6MMP (slope = 1.2156, intercept = —0.0094, r^2 = 0.9976). 6MMP was used as the internal standard since it was not detected in the plasma of monkeys dosed with AZA. AZA was recovered to the extent of 68% from spiked plasma samples. The overall recovery of 6MP, however, was considerably less (12%) approximating the recovery previously reported for the CG—MS method [9]. The major loss of drug occurs in the initial extraction steps.

The stability study of 6MP and AZA in plasma showed that samples could be kept frozen for at least 7 weeks without decomposition. As indicated in Table I, an average value of 44 ± 4 ng/ml was measured for samples spiked with 45 ng/ml of 6MP; an average of 0.39 ± 0.02 μg/ml was obtained for AZA samples spiked at 0.41 μg/ml.

Chalmers et al. [17] have shown that AZA is rapidly converted to 6MP at pH 7.35 and 37° in the presence of 1 m*M* glutathione. We attempted to determine whether the breakdown by glutathione in whole blood was significant after the blood was drawn from the animal and before it was spun down. The stability study in blood (see Fig. 2) showed that blood samples kept in ice were relatively more stable compared to those left at room temperature, and even more so than those incubated at 37°. The half-lives of AZA decline at 37° were 28 min and 46 min for blood drawn from two monkeys. This compares well with the half-life of 47 min determined for the conversion of AZA to 6MP by the addition of glutathione [17].

Plasma levels of 6MP following a 1.47 mg/kg dose of 6MP to a 6.8-kg rhesus monkey are depicted in the upper curve in Fig. 3. The concentration time curve appears to follow a 2-compartment body model. This same monkey also received a 3 mg/kg intravenous dose of AZA (equivalent to 1.65 mg/kg 6MP if AZA is quantitatively converted to 6MP). AZA concentrations fell

TABLE I

STABILITY STUDY OF 6MP AND AZA IN PLASMA

Day	Concentration	
	6MP (ng/ml)	AZA (μg/ml)
0	44	0.37
	47	
2	41	0.40
	41	0.41
8	50	0.35
	46	0.41
52	44	0.38
	39	0.39
Mean ± S.D.	44 ± 4	0.39 ± 0.02

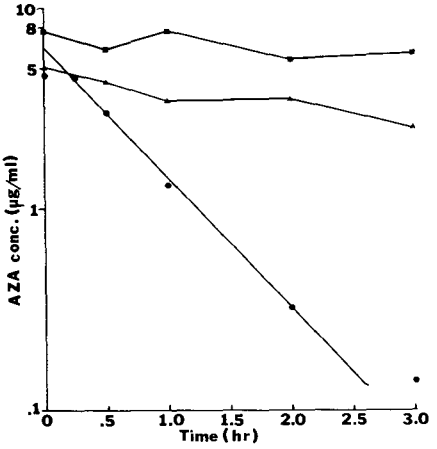

Fig. 2. Stability study of AZA in blood from a rhesus monkey, □, In ice; 7, at room temperature; •, at 37°.

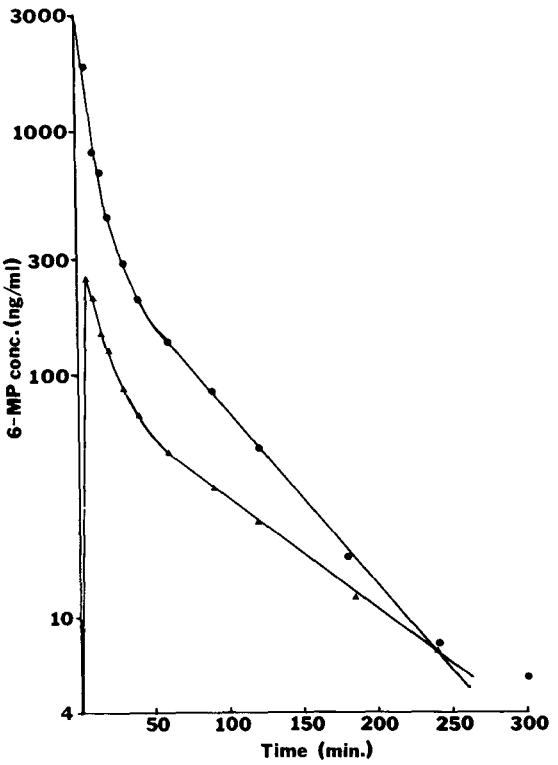

Fig. 3. Plasma levels of 6MP in a 6.8-kg rhesus monkey after an intravenous bolus dose of 6MP (•) at 1.47 mg/kg and AZA (▲) at 3 mg/kg.

rapidly to levels below assay sensitivity. However, 6MP concentrations following this AZA dose are depicted in the bottom curve of Fig. 3. Note that peak 6MP levels are achieved at the first 5 min sampling point after AZA dosing. Plasma levels of 8-OHMP were found following a 9 mg/kg oral dose of AZA to one rhesus monkey. 8-OHMP is derived from the oxidation of AZA to 8-OHAZA in the animal by the enzyme aldehyde oxidase [18]. 8-OHAZA is subsequently cleaved to 8-OHMP and the imidazole moiety.

Randomized blood samples were collected from three renal transplant patients on daily oral doses of AZA. Plasma was analyzed for 6MP as shown in the semilogarithmic plot of the data in Fig. 4. Absorption of the drug appears to be rapid and levels of 6MP can be detected for up to 7 h. Further studies to define the kinetics of AZA and 6MP in rhesus monkeys and renal transplant patients are presently ongoing in our laboratory using the assay procedures described in this paper.

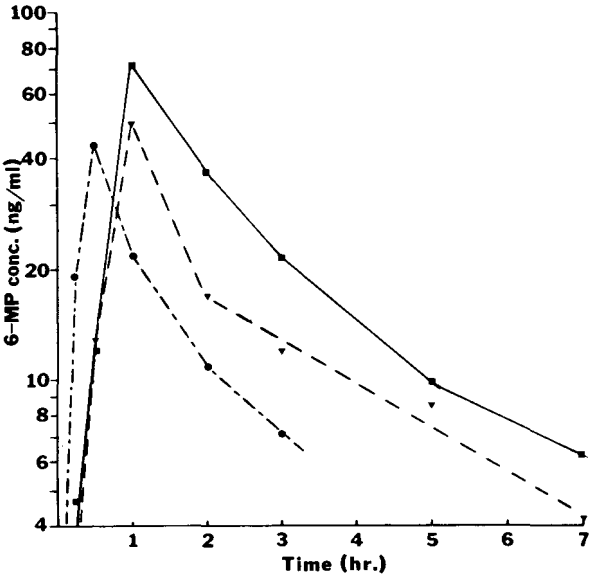

Fig. 4. Plasma levels of 6MP in 3 renal transplant patients, each receiving an oral dose of AZA daily. Key: •, D.H., 74.3 kg, 100 mg AZA; ▲, A.R., 65.3 kg, 50 mg AZA; ■, A.V., 54.4 kg, 50 mg AZA.

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Note

Combined capillary column gas chromatography—mass spectrometric method for the quantitative analysis of urinary prostaglandins

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The development of accurate, sensitive, and highly specific methods for the quantitative analysis of prostaglandins in biological materials has received considerable attention recently [1—5]. Of particular interest is the determination of urinary prostaglandins [6]. It has been postulated that urinary prostaglandin excretion is a reflection of renal prostaglandin production and it has been used as a tool to study renal prostaglandin physiology and pathology.

In 1970 Samuelsson and coworkers employed for the first time the stable-isotope dilution technique of selected ion monitoring for prostaglandin analysis [1]. Since then gas chromatography—mass spectrometry (GC—MS) has been successfully applied to the quantitative analysis of prostaglandins, prostaglandin metabolites, and thromboxanes [3, 5], and is considered to be the most accurate method for prostaglandin determination. However, one of the main difficulties in the use of this methodology is caused by the very low concentration of prostaglandins in complex biological samples such as urine. This analytical problem may be improved by increasing the poor chromatographic resolution usually achieved with conventional packed columns.

This paper describes the quantitative analysis of PGE_2, $PGF_{2\alpha}$ and 7α-hydroxy-5,11-diketotetranor-prostane-1,16-dioic acid (PGE-M), the major urinary metabolite of the E-prostaglandins, using a glass capillary column combined with a mass spectrometer, which was operating in the selective ion monitoring mode. Compared to packed columns, resolution, sensitivity, and specificity were greatly improved by the capillary column. Similar results have been reported by Maclouf et al. [7] and by Fitzpatrick [8], although they did not consider quantitative aspects in their prostaglandin analysis.

EXPERIMENTAL

Materials

All solvents were purchased from the Prochem Company (Wesel, G.F.R.). The tetradeutero (D_4) analogs of PGE_2 and $PGF_{2\alpha}$ were a generous gift from Dr. U. Axen, The Upjohn Company. Tritium-multilabelled PGE_2 and $PGF_{2\alpha}$ (specific activity 120—170 Ci/mol) were purchased from Amersham/Buchler Cooperation (Braunschweig, G.F.R.). Heptatritio and heptadeutero PGE-M were provided by Dr. W.J.A. VandenHeuvel, The Merck, Sharp and Dohme Research Laboratories. Methoxyamine·HCL and N,O-bis(trimethylsilyl)trifluoroacetamide were purchased from Serva (Heidelberg, G.F.R.) and Fluka AG (Buchs, Switzerland), respectively. Diazomethane was prepared as described previously [4].

Extraction of prostaglandins from urine

Tritium- and deuterium-labelled PGE_2, $PGF_{2\alpha}$ and PGE-M were added to urine samples of different patients as internal standards and tracers. Extraction and separation were carried out essentially as described previously [9] by high-performance liquid chromatography (HPLC) on a microparticulate silicic acid column [10].

Gas chromatography—mass spectrometry

A Hewlett-Packard HP 5992A microprocessor-controlled GC—MS system was used. The system was alternatively equipped with a 1% Dexsil 300 packed column (1.8 m × 2 mm I.D.), and an SE-30 glass capillary column (30 m × 0.3 mm I.D.) with a Grob-type splitless injector [11]. The GC—MS system included an HP 5990A quadrupole mass spectrometer coupled to a 9825 HP computer and a 9885 flexible disk. The interface for the packed column was a one-stage jet separator. The capillary column was coupled directly via an open split connection with glass capillary restriction basically designed as the interface described by Henneberg et al. [12]. Helium flow-rates were 20 and 2 ml/min, respectively. Operation conditions were: injection port, 260°; interface, 240°; ion source, 170°; electron impact energy, 70eV. The electron multiplier voltage was 2.4 kV.

RESULTS AND DISCUSSION

Sensitivity

Selected ion chromatograms of *m/e* 512 from the tetradeutero PGE_2-methyl ester-methyloxime-bis-trimethylsilyl ether (Me-Mo-bis-TMS) and *m/e* 508 from the protium form of the PGE_2 derivative are shown in Fig. 1. Unlabelled PGE_2 added to a constant amount of tetradeutero PGE_2 ranged from 0 to 1% of the tetradeutero PGE_2 derivative. Approximately 50 ng of tetradeutero PGE_2 were injected either into the packed column (upper tracings of Fig. 1) or into the glass capillary column (lower tracings of Fig. 1). Using the selected ion chromatograms obtained with the capillary column, the lower detection limit was 50 pg unlabelled PGE_2 with a signal-to-noise ratio of 2:1. After subtraction of the blank value the constructed standard line of ten different unlabelled standards

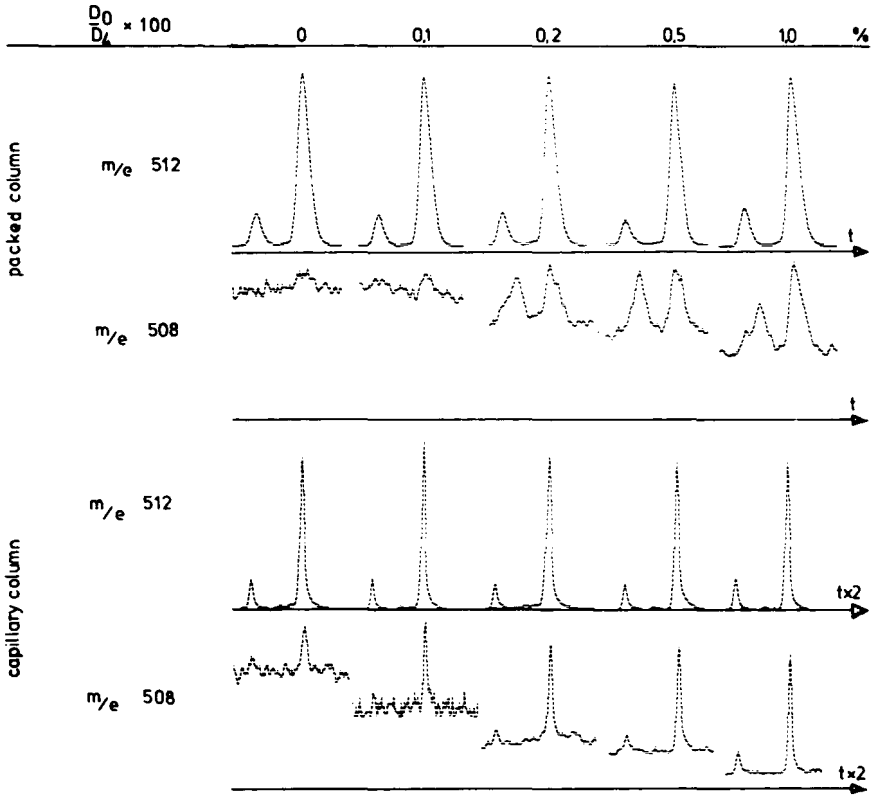

Fig. 1. Comparison of selected ion chromatograms obtained with a 1% Dexsil 300 packed column (upper tracings) and an SE-30 glass capillary column (lower tracings). The vertical axis represents abundance, the horizontal axis time (t). The recording speed was doubled by means of dwell time for the tracings obtained with the capillary column. Selected ions of the Me-Mo-bis-TMS derivatives are *m/e* 512 for tetradeutero PGE_2 (D_4) (full-scale 180 for the packed column and 1000 for the capillary column) and *m/e* 508 for unlabelled PGE_2 (D_0) (full-scale from 2 to 4 for the packed column and from 4 to 28 for the capillary column). The amount of D_4-PGE_2 injected was approximately 50 ng.

(from 50 pg to 50 ng unlabelled PGE_2) had a slope of 0.996 with an intercept of −0.249 and a correlation coefficient of 0.9997. Using the packed column 1 ng of unlabelled PGE_2 was the minimum amount which could be detected quantitatively. This also applies to $PGF_{2\alpha}$ and PGE-M.

Detection of urinary prostaglandins

Some examples of selected ion chromatograms of PGE_2-Me-Mo-bis-TMS, $PGF_{2\alpha}$-methyl ester-tris-trimethylsilyl ether (Me-tris-TMS) and PGE-M-bis-methyl ester-bis-methyloxime-trimethylsilyl ether (bis-Me-bis-Mo-TMS) after extraction and separation from human urine are shown in Fig. 2. Despite intensive purification and isolation procedures using organic solvent extraction, open column chromatography, HPLC and GC, the selected ion chromatograms of the protium forms show a remarkably high background and peaks with different retention times from those of the corresponding deuterated prosta-

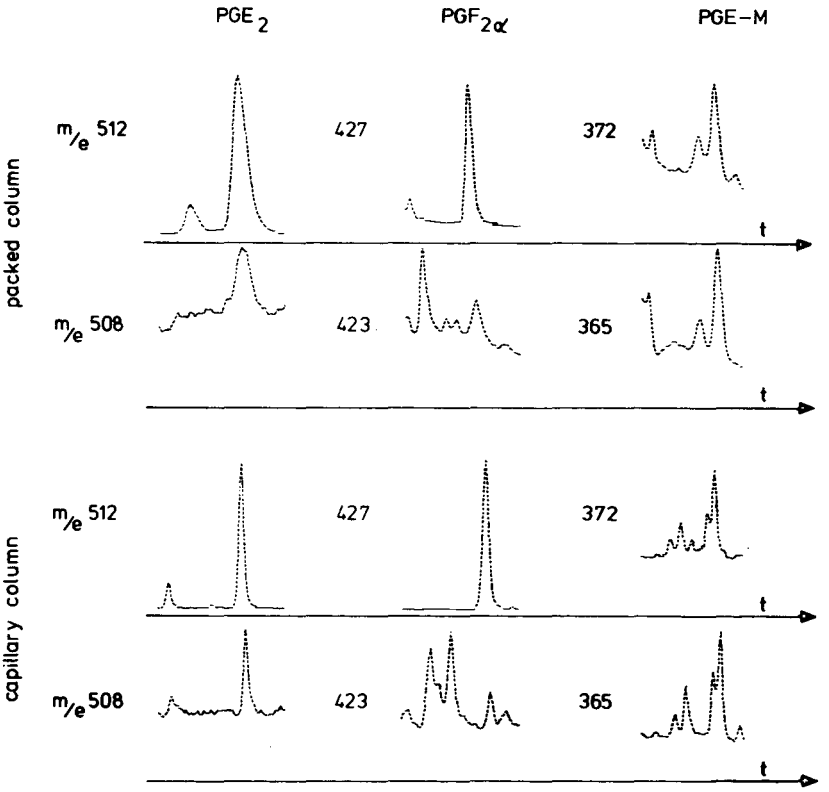

Fig. 2. PGE_2, $PGF_{2\alpha}$ and PGE-M from human urine. The vertical axis represents abundance, the horizontal axis time (t). The recording speeds for the capillary chromatograms (lower tracings) were increased two- to five-fold compared to the chromatograms of the packed column (upper tracings). The recorded ion pairs for Me-Mo-bis-TMS of unlabelled and tetradeutero PGE_2 were *m/e* 508 (full-scale 8 for the packed column and 6 for the capillary column) and *m/e* 512 (full-scale 85 for the packed column and 58 for the capillary column); the ion pairs for Me-tris-TMS of unlabelled and tetradeutero $PGF_{2\alpha}$ were *m/e* 423 (full-scale 35 for the packed column and 13 for the capillary column) and *m/e* 427 (full-scale 86 for the packed column and 109 for the capillary column); the ion pairs for bis-Me-bis-Mo-TMS of unlabelled and heptadeutero PGE-M were *m/e* 365 (full-scale 57 for the packed column and 33 for the capillary column) and *m/e* 372 (full-scale 62 for the packed column and 24 for the capillary column). The amounts of deuterated prostaglandin analogs injected as internal standard were approximately 50 ng for PGE_2 and $PGF_{2\alpha}$ but only 20 ng for PGE-M. The columns used were the same as described in the legend of Fig. 1.

glandin analogs. The poor resolution of packed columns may then lead to a low signal-to-noise ratio as shown by the SIM tracings of the unlabelled PGE_2 derivative. Using the packed column, the peak area of *m/e* 508 was only about half of that obtained with the capillary column. The measured values were 3.1 ± 1.5 versus 5.3 ± 0.5% unlabelled PGE_2 ($\bar{x}$ ± S.D. of four injections), which corresponds to an excretion rate of 718.6 ± 348.8 versus 1234.4 ± 111.6 ng/day. This discrepancy is explained in part by an automatic integration device triggered by slope sensitivity which results in distorted values when integrating only partially resolved peaks. The opposite was the case in the example of

$PGF_{2\alpha}$. Contaminating peaks at the nominal mass 423, which were unresolved from the signal of the unlabelled $PGF_{2\alpha}$ derivative, gave a false value of 12.1 ± 1.3% unlabelled $PGF_{2\alpha}$ with the packed column, while the percentage of unlabelled $PGF_{2\alpha}$ was only 1.7 ± 0.3 when the capillary column was used. The excretion of $PGF_{2\alpha}$ would be 499.0 ± 54.2 instead of 67.8 ± 10.7 ng/day. The four *syn* and *anti* isomers of the bis-methyloxime derivative of PGE-M did not show up in the mass chromatograms of the packed column. Quantitative analysis of PGE-M was distorted by unresolved isomers and a falling base-line. The PGE-M excretion rate would be either 2.7 or 3.8 μg/day depending which gas chromatographic system was used, either packed or capillary column.

These studies have demonstrated a successful attempt to introduce capillary columns into the quantitative analysis of prostaglandins. The determinations of urinary prostaglandins were more accurate when a glass capillary column was used instead of a conventional packed column because of the much higher resolution of the capillary column. This technique should be used for the quantitative analysis of other arachidonic acid metabolites in complex biological samples. Such work is currently under way in our laboratory.

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Note

Analysis of human axillary volatiles: compounds of exogenous origin

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Considerable speculation has appeared concerning the possibility of chemical communication among humans. Anecdotal information as well as scientific reports including those dealing with mammalian odor communication have fueled this speculation [1, 2]. The human axilla has been suggested as an area especially suited for the development of odors which may affect human reproductive biology. The following factors present in the axillae aid in odor production: (a) unique secretion from the apocrine gland which can serve as a bacterial substrate, (b) moisture from the eccrine glands, (c) a resident population of bacteria, (d) lipid secretions from the sebaceous gland to serve as an odor fixative, and (e) the presence of hair to assist in odor dispersal [3].

Despite the considerable resources expended on control and masking this odor, little is known concerning the nature of the odoriferous constituents being produced. Our efforts to isolate and identify these odorous compounds were begun by sampling the odor directly by sweeping the axillary headspace and also by collection of odors on cotton pads. These collections have led to the identification of a large number of volatile constituents many of which appear to be synthetic perfume components as well as pollutants in our atmosphere and drinking water. A number of these compounds were both odorous and physiologically active and may be contributing to our normal body odor.

METHODS AND MATERIALS

Subjects

A panel of ten subjects, four male and six female, for the pad study and three subjects (male) for the direct cup sampling (ages 18—23 years) followed a regimen involving use of only a non-bacteriostatic soap for two weeks and no soap 48 h prior to sampling. Previous investigations have shown this protocol to produce strong typical axillary odor in most individuals [4].

In this panel, the protocol gave axillary odors of weak to strong intensity. The odor was rated on a scale of 0—3 by several workers experienced in axillary odors, where the values represent none, discernable, moderate, and strong-unpleasant odors, respectively. Only individuals with strong odors were selected for direct sampling.

Odor collection

Direct sampling of the axillae employed a cup (plastic funnel with PTFE and metal fittings) which was held tightly over the axillae. A small pump was employed to draw room air through an activated charcoal filter and over the axillae. Attached to the exit of the cup was a 6 in. × 1/8 in. stainless-steel tube containing 70 mg of Tenax (Applied Science, State College, Pa., U.S.A.). Collections were 15 min for each axillae on the same tube at a flow-rate of 40—60 ml/min. Prior to collection, a fresh tube of Tenax was employed to sample the same amount of room air.

Collection of the odors used cotton pads which were previously extracted with chloroform—methanol (85:15, v/v), ether, and vacuum dried [5]. For each subject a pad was worn overnight in one axilla, while the other axilla was left uncovered and rated for odor upon the subjects's arrival in the laboratory. After removal, the pad was placed in a glass tube, warmed to 50° and swept with nitrogen (flow-rate 80 ml/min for 16 h) to transfer the volatiles to Tenax. Upon removal of the pads from the collection tube they were odorless, even those which initially had strong odors.

Gas chromatographic and gas chromatographic—mass spectrometric (GC—MS) analyses

The volatiles were thermally desorbed from the Tenax tube by heating in the helium stream at 220° for 10 min. Dry ice was placed around the front 15 cm of the chromatographic column to condense the desorbed volatiles. The chromatograph used was a Perkin-Elmer (PE) 990 equipped with flame ionization detector and a 10 ft × 2 mm I.D. Pyrex 10% Carbowax 20M column for separation. Analysis conditions were as follows: temperature 70° (4 min), 70°—220° (4°/min); helium flow-rate 40 ml/min; injector 250°; detector 250°. The GC—MS system consists of a PE 990 interfaced with a PE/Hitachi RMU-6L mass spectrometer via a Watson—Biemann separator [6]. Structural assignments were based upon mass spectral comparisons with either authentic samples or literature spectra. Relative retention times of unknown and authentic samples were obtained by comparing their elution times with a series of n-C_2 — C_{18} fatty acid ethyl esters. This yields a fatty acid ethyl ester (FAEE) index for each compound [7].

It was demonstrated that short-chain aliphatic acids were not transferable from a moist cotton pad (axillary pads absorb ca. 1 g moisture during sample collection). The aliphatic acids gave low to moderate efficiencies (10—50%) from aqueous solutions even when the solutions were acidified and salted. Esters, hydrocarbons, furfuryl alcohol, cresol, and indole were transferred efficiently (30—90%) from both pads and aqueous solution. In other studies, where other specific groups of compounds have been collected on Tenax, high efficiencies have been reported [8].

RESULTS

The volatiles found in the direct sampling of the axillae are listed in Table I. Many of these compounds have been identified in studies of urban air and are thought to arise from "man-made emissions" [9—12]. Room air controls showed the presence of alkylbenzenes and chlorinated ethylenes (Table I). These chemicals have also been identified in an analysis of volatiles from blood plasma [13—15].

TABLE I

CHEMICALS IDENTIFIED ON DIRECT SAMPLING OF AXILLAE

Benzene*, **, ***	Trimethylbenzenes*, **
Toluene*, **, ***	Methylene chloride*
Xylenes*, **, ***	Chloroform**
Ethylbenzene*, **, ***	Limonene*, **
2-Ethylhexanol	6-Methyl-hept-5-en-2-one
Trichloroethylene*, **	Acetone***
Tetrachloroethylene*, ***	

*Found in room air blanks.

**Found in rural and urban air samples (ref. 10).

***Found in normal blood plasma (ref. 11).

Fig. 1 shows a typical GC trace of volatiles collected from the cotton pads. The major identified components are summarized and include a series of isopropyl esters of fatty acids, principally isopropyl myristate and isopropyl palmitate. These esters are known components of deodorant and cosmetic preparations [16]. In addition, commercial samples of palmitic and myristic acids were shown to contain small amounts of other C-12 to C-18 fatty acids as impurities and this may account for their presence in the axillary samples [17]. A number of compounds in Fig. 1 are known fragrance chemicals which are added to soaps and cosmetics. Two compounds, 2-ethylhexanol and diethylphthalate, are commonly found in biological samples [18].

Antioxidants, such as di-*tert.*-butyl-hydroxytoluene (BHT), are found in approximately half of the samples including one subject from direct sampling. In addition, the mass spectral data also suggest the presence of di-*tert.*-butyl-hydroxyanisole.

The aldehydes and isopropyl esters were found in most subjects. Compounds identified from only a few subjects include nonenyl salicylate (T), methyl and

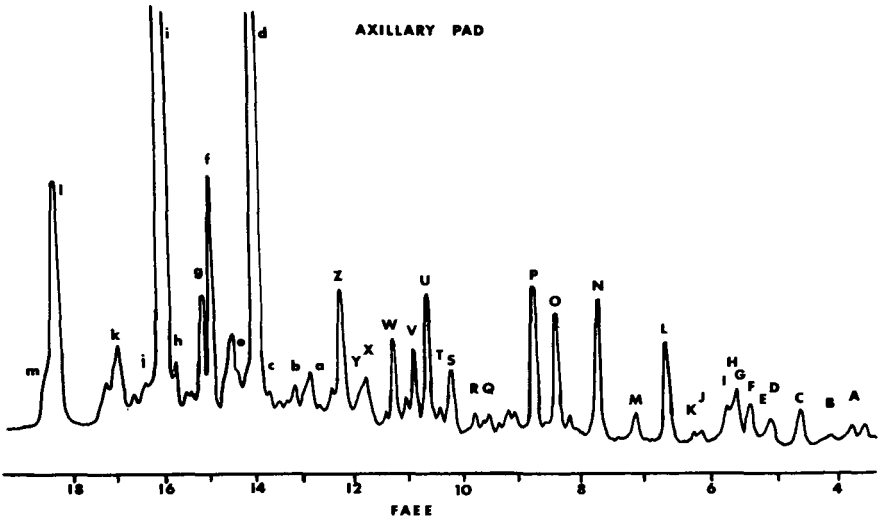

Fig. 1. GC analysis of volatiles from axillary pad concentrated on Tenax and separated on 10 ft. × 2 mm I.D. 10% Carbowax 20M column programmed at 70° (4 min), 70°—220° (4°/min). Ordinate values refer to FAEE Indices. Numbers refer to *m/z* values for assumed molecular ion (*) and significant fragment ions (underlined). T = Tentative. Peaks: A = propylfuran (T), B = tetrachloroethylene, C = hexanal, D = butylfuran, E = xylene, F = C_9-ketone (142*), G = heptanal, H = cyclopentanone (T), I = limonene, K = unknown (122*), L = octanal, mesitylene C_3-alkylbenzene, M = 6-methyl-hept-5-en-2-one, N = nonanal, O = unknown (ketone, T), P = decanal, Q = unknown (204*, 142) R = undecanal, S = furfuryl alcohol, T = unknown (126*, 111), U = naphthalene, unknown (210*, 192), V = heptadecane, W = unknown, X = octadecane, Y = isopropyl laurate, Z = geranyl acetone, unknown (243, 71, 43), a = butylated hydroxytoluene, b = isopropyl tridecanoate, lauryl alcohol (C-12), c = methyl myristate, d = isopropyl myristate, e = di-*tert.*-butyl-hydroxyanisole (T), f = isopropyl pentadecanoate, g = myristyl alcohol (C-14), h = methyl palmitate, i = isopropyl palmitate, j = ethyl palmitate, k = isopropyl heptadecanoate, diethyl phthalate, l = isopropyl stearate, m = nonenyl salicylate (262*, T).

ethyl esters of myristic and palmitic acids, diphenylamine, myristyl propionate (major product in one subject), and C-17, 18, 21, 22, 23, 24 hydrocarbons [19].

In both direct sampling and the pad users, the amounts and type of constituents present did not appear to correlate with the perceived odor intensity of the axillae or the pad. In addition, sampling the odors eluting from various positions of the chromatograph did not reveal any individual constituents which smelled like apocrine odor.

DISCUSSION

Our study to characterize the volatiles associated with axillary odor have shown that the major volatile constituents identified from the skin surface of the axillae are of exogenous origin. It is believed that many of the observed compounds are emissions from man-made sources in and around Philadelphia. These pollutants undoubtedly adhere to and perhaps are concentrated in the unwashed axillary region (particularly hair) of our subjects who spend a major-

ity of their days in an urban atmosphere. Both the atmosphere pollutants and the soap/cosmetic constituents may contribute to the observed skin odors but do not form part of the "natural" odor.

A number of these materials, such as limonene, toluene, benzene and chlorinated hydrocarbons, have been detected in serum and urine samples and may also be excreted to the skin surface through the sweat glands [13—15, 20]. These materials are not observed in our GC investigations of freshly collected sebaceous and apocrine secretions and are most likely of exogenous origin.

Preliminary analyses in our laboratory of sebum from the scalp extracts and "pure" apocrine secretion have shown antioxidants to be present in these samples also. This suggests that compounds such as these arise from food sources and are excreted through the apocrine and sebaceous glands to the skin surface [21]. Man reportedly consumes about 0.1 mg per kg body weight daily of phenolic antioxidants [22]. BHT can be metabolized by the body and has been shown to inhibit the activation of certain carcinogens [22, 23]. However, other materials we have identified, such as the chlorinated ethylenes and benzene (Table I), are suspected carcinogens [24].

A previous investigation of axillary odors employed GC profiles of collected volatiles with subsequent evaluation of individual peaks for malodors. No structure elucidation was performed in this study [25]. Consequently, many of the major constituents could well have been exogenous materials co-eluting with odorous substances.

Other studies have examined the chemical composition of total body volatiles. In one such report, room air was sampled in the presence and absence of individual subjects. The volatile compounds which were identified and thought to arise from sweat included C-1 to C-3 alcohols, acetone, and acetic acid [26]. Another study which examined whole body volatile effluvia identified 135 volatile compounds from a complex GC trace containing about 300—400 constituents [27]. Alkylbenzenes, acetone, heptanal, and 2-ethylhexanol were identified by Ellin et al. [23] and are also reported here. Odorants produced in the axillae are undoubtably major contributors to human whole body odors however, neither our results nor those of Ellin et al. have identified constituents such as androgen steroids and/or C_2—C_5 aliphatic acids which have often been suggested as comprising whole body/axillary odors [2, 28].

Synthetic body odors fashioned by perfumers will often contain short-chain aliphatic acids to give them the "sweaty" note [29]. In our experience, the odor of these compounds alone differs markedly from that of axillary odor; however, they may constitute some part of the "odor bouquet" originating in the axillae. As noted above (Methods and materials) our experiments with short-chain acids showed that they were not transferable from moist cotton pads. Also, other studies suggest free androgen steroids are present in axillary sweat but at such low concentrations that our present collection and analysis procedures would not detect them [28, 29].

The presence of large amounts of exogenous compounds in the axillae have led us to examine alternate schemes for identification of the volatiles responsible for axillary odor. The most promising approach appears to be the generation of the characteristic odor by in vitro manipulation of pure apocrine secretion by heating and bacterial action. Odorants produced by these techniques are currently being investigated.

ACKNOWLEDGEMENTS

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Note

Isotachophoretic analysis of isovalerylglycine in urine of a patient with isovaleric acidemia

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Isovaleric acidemia is an inborn error of leucine metabolism; large amounts of isovalerylglycine (300—1200 mg/day) are known to be excreted in the urine as a detoxication product [11]. The determination of this compound has been achieved by the use of gas chromatography [1] or thin-layer chromatography [2]. However, these methods are time-consuming for the pretreatment of the sample.

Recently, we had an opportunity of examining a patient with isovaleric acidemia who was found in Okayama, and a new simple and rapid method for detecting urinary isovalerylglycine was devised. The isotachophoretic method [3—10] presented here has several advantages over previously described techniques.

EXPERIMENTAL

The capillary apparatus used was a Shimadzu IP-IB isotachophoretic analyser [12] (Shimadzu Seisakusho, Kyoto, Japan). The separations were carried out in a capillary tube 20 cm long with an I.D. of 0.5 mm, which was maintained at a constant temperature of 20°. The detector cell had an I.D. of 0.5 mm and length 0.05 mm. The migration currents were 50, 100 and 150 μA. The leading electrolyte consisted of 0.01 *M* hydrochloric acid and β-alanine (pH 3.1 and pH

4.5) and 0.01 *M* hydrochloric acid, β-alanine and 0.001 *M* cupric chloride (pH 3.1). The terminating electrolyte was 0.01 *M* caproic acid.

The chemicals used were analytical grade. Authentic isovalerylglycine was synthesized from the corresponding isovalerylchlorides and glycine.

The normal urine samples were from laboratory personnel. The samples from the patient with isovaleric acidemia were obtained from Okayama National Hospital. The samples were kept frozen if not analysed immediately.

RESULTS AND DISCUSSION

The patient with isovaleric acidemia was found in Okayama using gas chromatography, but the method is time-consuming on account of the pretreatment of the samples. Therefore, the purpose of our investigation was to develop a simple and rapid method for the determination of urinary isovalerylglycine in cases of isovaleric acidemia. Isotachophoresis is a method of high resolution for the separation of compounds according to their net mobility in a given electrolyte system.

We tried to determine isovalerylglycine in the urine of the isovaleric acidemic patient by isotachophoresis. Aliquots (0.1 μl) of normal human urine and of the urine of the isovaleric acidemic patient were subjected to isotachophoresis. No zone was detected in normal human urine, but a large zone was detected in the urine of the isovaleric acidemic patient. It was necessary to identify this

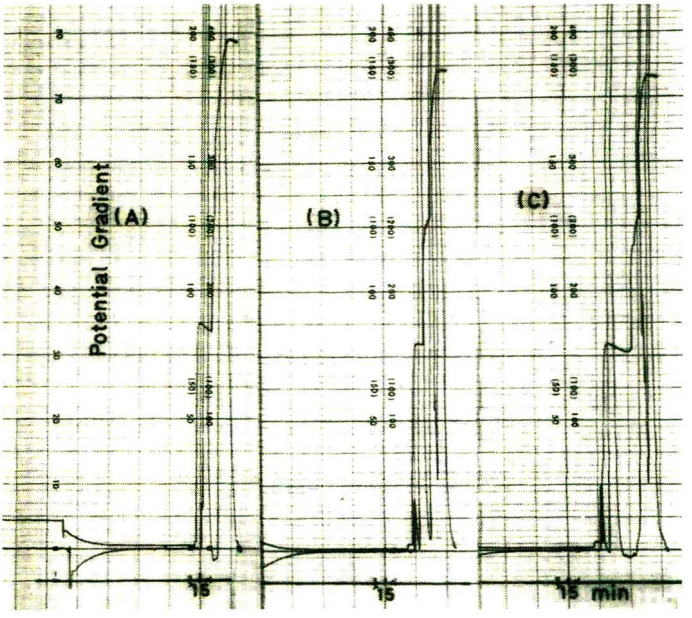

Fig. 1. Isotachophoretic runs of urine from a patient with isovaleric acidemia and of authentic isovalerylglycine. (A) Synthetic isovalerylglycine; (B) urine sample of patient with isovaleric acidemia; (C) mixture of synthetic isovalerylglycine and urine sample of patient. The leading electrolyte was 0.01 *M* HCl and β-alanine (pH 3.1) and the terminator was 0.01 *M* caproic acid. Migration current, 150—100 A; chart speed, 10 mm/min; temperature of electrolyte, 20°.

large zone as isovalerylglycine by the addition of authentic isovalerylglycine. Authentic isovalerylglycine was added to the urine of the isovaleric acidemic patient and the mixture was subjected to isotachophoresis. The zones were coincident (Fig. 1). However, when members of the tricarboxylic acid cycle and acidic amino acids were subjected to isotachophoresis, it was found impossible to distinguish the zones of aspartic acid and isovalerylglycine in the same electrolyte (pH 3.1). Isovalerylglycine and aspartic acid could be separated by ion exchange, but this method was as time-consuming as the gas chromatographic method for determining isovalerylglycine in the urine of the isovaleric acidemic patient. We then studied high-resolution methods for the separation of these compounds by changing the electrolyte system. When aspartic acid and isovalerylglycine were run separately (pH 4.5) good resolution was obtained, but their mixture did not give good separation in the same leading electrolyte.

Eventually, we obtained good results, as shown in Fig. 2, by adding 0.001 *M* cupric chloride to the leading electrolyte (pH 3.1).

The standard curves for aspartic acid and isovalerylglycine were linear. In a given electrolyte system, the separation pattern of isovalerylglycine was reproducible and its identification generally did not cause problems. The coefficient of variation for our technique was 2.15% ($n = 7$). riation of 2.15%

When there is any ambiguity, it is possible to add an authentic sample to an actual sample or change the leading electrolyte. The patient with isovaleric acidemia excreted 600–950 mg of isovalerylglycine per day.

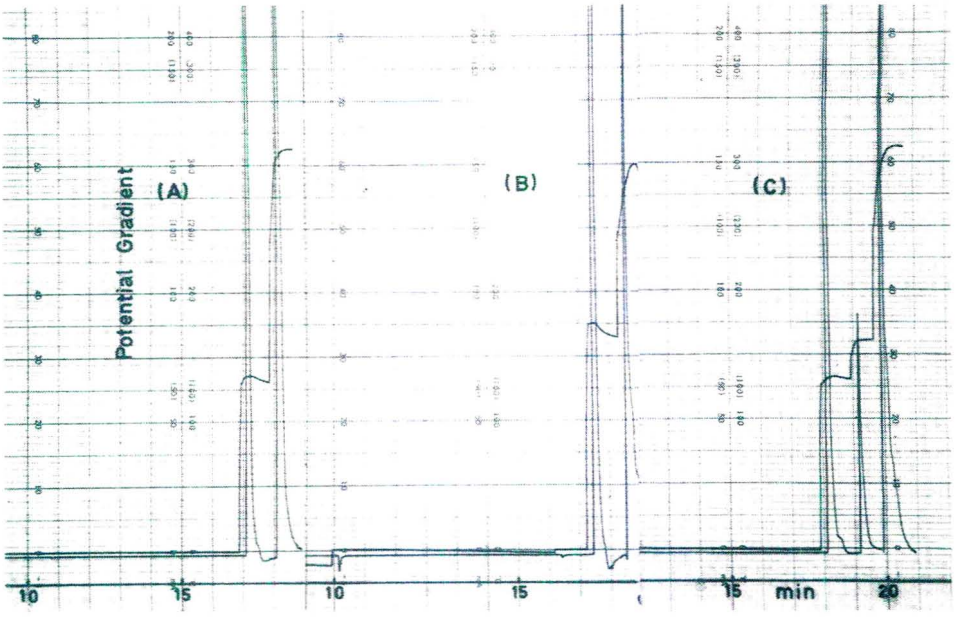

Fig. 2. Isotachophoretic runs of aspartic acid and isovalerylglycine. (A) Synthetic aspartic acid; (B) synthetic isovalerylglycine; (C) mixture of aspartic acid and isovalerylglycine. The leading electrolyte was 0.01 *M* HCl, β-alanine and 0.001 *M* $CuCl_2$ (pH 3.1) and the terminator was 0.01 *M* caproic acid. Conditions as in Fig. 1.

This method can measure isovalerylglycine by applying 0.1 μl of urine directly without any pretreatment. The method is very simple and rapid compared with gas chromatography or thin-layer chromatography and is very useful for screening inborn errors of metabolism such as isovaleric acidemia.

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Note

Sensitive gas chromatographic method for the determination of diazepam and N-desmethyldiazepam in plasma

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Diazepam is a tranquillizer of the benzodiazepine type and is widely used for the symptomatic relief of anxiety, insomnia, psychiatric disturbances, seizures, and as preoperative medication [1].

Diazepam and its metabolites have been studied more intensively than the other benzodiazepines [2, 3].

Various papers have been published on the determination of diazepam and its metabolites by methods including spectrophotometry, gas chromatography with flame ionization or electron-capture detection, high-performance liquid chromatography, thin-layer chromatography, polarography and radioimmunoassay [2—4]. Gas—liquid chromatography (GLC) has been used extensively in the analysis of the benzodiazepines.

Chromatography at low concentrations, such as those found in blood and saliva following a single therapeutic dose, requires the use of an electron-capture detector to obtain good sensitivity. At the nanogram level, column adsorption processes, especially with the N-desalkyl compounds, become a problem. Such adsorbed compounds exhibit long retention times or do not elute from the column at all [5]. For these reasons, some GLC methods involve chromatography of the benzophenone hydrolysis products rather than of the benzodiazepines themselves [6—9]. These methods are time-consuming because of the clean-up procedure involved. Another disadvantage is their lack of specificity, due to the fact that metabolites of the parent drug if present in sufficient amounts would also yield the same benzophenone derivative [3]. By derivatizing the functional group of the intact compound, polarity is

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decreased and volatility is increased, thus providing increased sensitivity together with a shorter analysis time [10].

In this paper we describe a sensitive and specific procedure for the analysis of diazepam and N-desmethyldiazepam by electron-capture gas chromatography (EC-GC) after N-butylation of N-desmethyldiazepam based on a method reported by Greeley [11] for barbiturates. The method described below was developed to obtain a reliable analysis of diazepam and N-desmethyldiazepam at the nanogram level in dose–response studies. Our procedure is based on an EC-GC procedure which employs a liquid phase of 3% OV-17 and prazepam as internal standard, and requires a minimal amount of sample clean-up prior to EC-GC analysis. The method described provides adequate sensitivity for therapeutic monitoring of diazepam and N-desmethyldiazepam.

EXPERIMENTAL

Materials

All solvents were analytical-reagent grade and all inorganic reagents were prepared in distilled water. Buffer solution of pH 9 was prepared by mixing 37.5 ml of 0.1 *M* HCl with 0.05 *M* sodium borate to 250 ml final volume. Diazepam and N-desmethyldiazepam were kindly supplied by Hoffmann-La Roche Nederland, Mijdrecht, The Netherlands). Prazepam was supplied as a gift from Warner-Lambert Nederland, Mijdrecht, The Netherlands). Stock solutions of diazepam, N-desmethyldiazepam and prazepam were prepared by dissolving 10 mg of each compound in 100 ml of methanol; standard solutions were obtained by diluting these stock solutions to a concentration of 10 μg/ml. These standard solutions were diluted with plasma to concentrations covering the range 50–600 ng/ml in order to obtain calibration graphs.

Tetrabutylammonium hydroxide (TBAH) 0.2 *M* was prepared as follows. To a solution of 0.6 g tetrabutylammonium iodide in 10 ml of methanol, 0.5 g of silver oxide was added and shaken gently for 2 h at room temperature. After centrifugation the liquid phase was stored in a dark container at ca. 4°.

Samples

Blood samples drawn from patients (some on chronic administration of three times a day 2–5 mg diazepam) 2–3 h after an oral dose of 5 or 10 mg diazepam were taken with lithium heparin as anticoagulant and immediately stored at 4°. Separated plasma was frozen at −20° until analyzed.

Extraction

Plasma samples (0.5 ml) were spiked with 40.0 μl internal standard solution (400 ng), mixed with buffer (1.0 ml) and adjusted to pH 9 if necessary. This mixture was extracted by shaking it on a Vortex Genie mixer for 10 min with 5.0 ml of toluene–heptane (90:10). After centrifugation (2500 *g*) for 10 min, the organic phase (4.0 ml) was separated and transferred to a 10-ml conical-bottomed flask and evaporated under reduced pressure in a Büchi Rotavapor at 60°. Next, the residue was evaporated to complete dryness under a stream of nitrogen for 10 min at 50°. The residue was then dissolved in

50 μl of methanol and the butylation of N-desmethyldiazepam was performed as follows. To the 50 μl methanolic solution of the residue 4.0 μl of N,N-dimethylacetamide, 5.0 μl of TBAH (0.2 *M*) diluted (1:1) with methanol, and 100 μl of 1-iodobutane were added. Then the solution was mixed thoroughly on a Vortex Genie mixer for 10 sec. This mixture was allowed to react completely at room temperature for 10 min. After evaporation under a stream of nitrogen at 75°, the residue was dissolved in 500 μl of toluene. Volumes of 1 μl were injected into the gas chromatograph. The solution was stable for at least one week when stored in a refrigerator.

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with an Aerograph® ^{63}Ni pulsed electron-capture detector and a Varian Model A 25 1-mV recorder was used. A Varian CDS 111 Chromatography Integrator was employed for measurement of peak retention times and peak areas. A coiled glass column (1.8 m × 3 mm I.D.) packed with 3% OV-17 on Chromosorb W HP (80—100 mesh) was used. The injection port was maintained at 290°, the column oven at 265°, and the detector at 300°. Pre-purified nitrogen was used as the carrier gas, at a flow-rate of 40 ml/min.

RESULTS

Recovery and precision

The recovery of internal standard from plasma (400 ng/ml) was 96 ± 7% (mean ± standard deviation; $n = 12$).

The over-all recoveries of diazepam (97 ± 8%) and N-desmethyldiazepam (as N-butyl derivative) (100 ± 12%) were calculated over the concentration range 50—600 ng/ml at 50, 250 and 600 ng/ml. The calibration graphs were constructed from three replicate measurements of five concentrations over that range (calibration graphs for diazepam, $y = 1.0869 \cdot 10^{-3}\ x - 2.2963 \cdot 10^{-2}$, $r = 0.996$; N-desmethyldiazepam as N-butyl derivative, $y = 1.7023 \cdot 10^{-3}\ x - 2.6617 \cdot 10^{-2}$, $r = 0.997$; concentration of internal standard; 400 ng/ml).

Within-run and between-run precision (four intervals of three days) were established. Diazepam and N-desmethyldiazepam were added to plasma at three different concentrations: 50, 250, and 600 ng/ml. The within-run precision of diazepam and N-desmethyldiazepam showed coefficients of variation varying from 5.4 to 1.6% and 10.5 to 5.9%, respectively, for this range. The between-run precision of diazepam and N-desmethyldiazepam showed coefficients of variation varying from 7.4 to 5.2% and 9.9 to 3.7%, respectively, for this range. The data on the precision are summarized in Table I.

Determination in plasma

Under the gas chromatographic conditions used, diazepam, N-desmethyldiazepam and the internal standard, prazepam, were eluted with retention times relative to prazepam of 0.63, 0.79, and 1.0, respectively (Fig. 1). A chromatogram obtained after analysis of a spiked plasma sample containing 400 ng/ml of each compound is given in Fig. 1b. Fig. 1a and c show chromatograms of blank and sample extracts of plasma. The limit of detection for

TABLE I

PRECISION DATA FOR DIAZEPAM AND N-DESMETHYLDIAZEPAM IN PLASMA

Drug	Within-run (n = 4)		Between-run (n = 4)	
	ng/ml (± S.D.)	C.V. (%)	ng/ml (± S.D.)	C.V. (%)
Diazepam	50 ± 3	5.4	51 ± 4	7.4
	234 ± 6	2.6	223 ± 14	6.3
	658 ± 11	1.6	637 ± 33	5.2
N-Desmethyldiazepam	49 ± 5	10.5	47 ± 5	9.9
	225 ± 7	3.2	220 ± 8	3.8
	611 ± 36	5.9	625 ± 23	3.7

diazepam and N-desmethyldiazepam (as N-butyl derivative) was 10 and 5 ng/ml, respectively, for 1-ml plasma samples.

DISCUSSION

The method reported here is based on the optimal extractability of diazepam and N-desmethyldiazepam into toluene—heptane at pH 9 [12]. The choice of an OV-17 liquid phase is based on its well-documented use in the quantitation of benzodiazepines utilizing gas chromatography. In a recent paper McAllister [13] showed the advantage of using prazepam as internal standard in the estimation of diazepam in plasma. It is assumed that high recoveries of both compounds, which are of comparable magnitude, indicate the reliability of the internal standard as such.

Rutherford [14] also used prazepam as the internal standard on the liquid phase OV-17. This column was deactivated every 4 h by means of 3-μl injections of a 5 g/l solution of dipalmitoyl phosphatidylcholine in ethanol. This treatment decreased the retention time of N-desmethyldiazepam by approximately 10% and the broad, tailing peak initially obtained became sharp and almost symmetrical. It is known that the tailing effect of standard solutions is diminished in the presence of a blood extract, probably due to the formation of an adsorption complex of the extracted blood lipids with active sites on the column [5]. A solution of cholesterol in acetone is also used as a column conditioner. In our experience these methods for deactivating adsorption sites on the column did not provide us with the required reproducibility for the chromatography of N-desmethyldiazepam.

The analytical method described above has an advantage over previous reported methods because of its great sensitivity for N-desmethyldiazepam.

Derivatization by means of alkylation at position N_1—H was employed in order to reduce adsorption and yield symmetrical peaks. The results obtained by conversion to the trimethylsilyl derivative at the N_1 position, as reported by Greaves [15], did not provide us with reliable results. Flash-heater alkylation of N-desmethyldiazepam to obtain a methyl, ethyl, propyl or butyl derivative, based on methods reported by Kowblansky et al. [16] and Pecci and Giovanniello [17], for the determination of xanthines and barbiturates, was not reproducible. During these experiments we found that the N-butyl

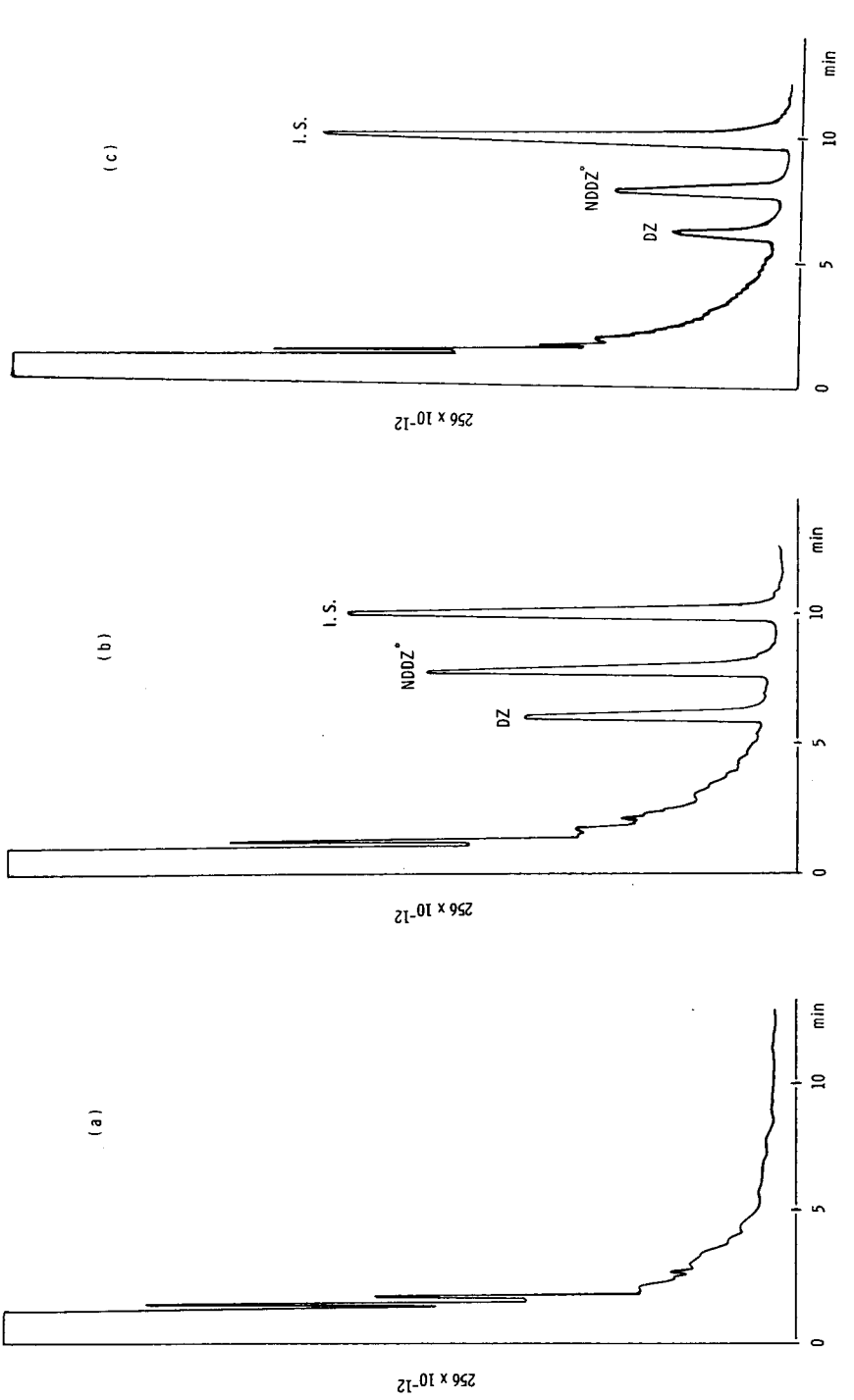

Fig. 1. Gas chromatograms of (a) plasma blank; (b) spiked plasma sample containing diazepam (DZ) 400 ng/ml, N-desmethyldiazepam (as N-butyl derivative, NDDZ*), and prazepam (as internal standard, I.S.); (c) plasma sample obtained from a patient (on chronic administration of three times a day 2 mg diazepam) 2.5 h after an oral dose of 5 mg diazepam, containing DZ 178 ng/ml and NDDZ* 192 ng/ml.

derivative yielded good separation. Finally, the procedure reported by Greeley [11] for alkylation of barbiturates has proved extremely successful for the preparation of a volatile N-butyl derivative of N-desmethyldiazepam for gas chromatographic analysis.

This method, with minor modifications, has also been used successfully to determine concentrations of diazepam and N-desmethyldiazepam in saliva [18].

Compared with the high-performance liquid chromatographic method recently reported by Brodie et al. [19] the procedure described here has comparable sensitivity and selectivity.

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Note

Quantification of imipramine and desipramine in plasma by high-performance liquid chromatography and fluorescence detection

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Although imipramine is a widely prescribed tricyclic antidepressant, little is known of the drug's pharmacokinetics in man. The lack of suitable methods for precisely quantitating plasma levels of imipramine and desipramine following administration of single doses is the probable explanation for this deficiency. Although many of the available assay methods [1—10] are adequate for the monitoring of plasma levels during chronic therapy they are either time consuming or lack the sensitivity required to measure the low plasma concentrations following single oral doses.

Gas chromatography—mass spectrometry [1—3] and gas chromatography with alkali-flame detection [4—7] have provided the most sensitive means of measuring plasma levels of imipramine and its major metabolite, desipramine although both procedures involve a time consuming preparation of samples before injection. Adsorption [9] and reversed-phase [10] high-performance liquid chromatographic methods employing ultra-violet detection have also been reported although this method of detection has limited the assay sensitivity to 5—10 ng/ml (15—30 nmole/l). This may be insufficient to measure plasma levels at times greater than 12 h after a single 50-mg oral dose of imipramine hydrochloride. In the present method, the inherent fluorescence of imipramine and desipramine allowed the use of the sensitive fluorescence detector with the high-performance liquid chromatograph to conveniently

measure plasma levels of these compounds and extend the range of their detection in plasma to 2—3 nmole/l.

MATERIALS AND METHODS

Reagents

All reagents were analytical-reagent grade and aqueous solutions were prepared using glass-distilled water. The extracting solvent was a hexane mixture (Nanograde Hexanes, Mallinckrodt, St. Louis, Mo., U.S.A.). Specially purified acetonitrile (Unichrom, Ajax Chemicals, Melbourne, Australia) was used for the high-performance liquid chromatography. Imipramine hydrochloride was obtained from Protea (Sydney, Australia), desipramine hydrochloride from Ciba-Geigy (Sydney, Australia) and trimipramine maleate from May and Baker (Melbourne, Australia).

Standards

A standard solution of imipramine and desipramine hydrochloride (10 μmole/l of each) was prepared in water and stored at 4° (stable for at least 2 months). This solution was then diluted as necessary and used to prepare the appropriate plasma standards for each assay run. The internal standard solution of trimipramine was also prepared in water (10 μmole/l) and stored at 4°. Peak height ratios of imipramine and desipramine to trimipramine were determined for plasma standards and unknowns and quantitation performed by reading unknown values from a plotted standard curve.

Extraction procedure

To 2 ml of plasma (either patient sample or standard) were added 200 μl of the internal standard solution followed by 2 ml of 1 *N* sodium hydroxide solution. The basified plasma was then extracted with 5 ml of hexane—isoamyl alcohol (99:1) by shaking at 200 rpm for 10 min. After separation of the phases by centrifugation at 20°, the organic layer was transferred to a 15-ml tapered centrifuge tube containing 0.2 ml of 0.05% orthophosphoric acid. The mixture was vortexed for 2 min (or shaken for 10 min at 200 rpm) and the phases again separated by centrifugation. A 100-μl aliquot of the aqueous layer was injected into the high-performance liquid chromatograph.

High-performance liquid chromatography

The chromatograph was a Spectra-Physics (Model SP 8000) instrument equipped with a 10-μm alkyl phenyl reversed-phase column (μBondapak/Phenyl from Waters Assoc., Milford, Mass., U.S.A.) and a 100-μl injector loop. The column oven temperature was 50° and the eluting solvent a helium-degassed mixture of acetonitrile and 0.015% aqueous phosphoric acid (71:29) at a flow-rate of 2 ml/min. The instrument was operated in the constant flow mode and all solvent lines from the column to the detector were carefully thermally insulated. The fluorescence of the eluent was monitored using a Schoeffel Model 970 fluorometer at an excitation wavelength of 252 nm with an emission cut-off filter allowing 90% transmission at 360 nm. The fluorometer sensitivity setting was 3.5, range 0.04 μA full scale and time constant 9.0 sec.

Recovery and reproducibility

Recovery of the extraction procedure was determined at concentrations of 10, 25, 100, 500 and 1000 nmole/l in plasma by comparison of the peak heights with those obtained for an aqueous solution containing known concentrations of imipramine and desipramine. Reproducibility was determined at concentrations of 10, 25, 100, 500 and 1000 nmole/l by assaying five plasma samples at each concentration.

RESULTS

High-performance liquid chromatography with fluorescence detection was found to be a convenient and sensitive means of quantitating plasma levels of imipramine and desipramine. The extraction time for five standards and twenty samples was approximately 1 h and the run time for each chromatogram was approximately 15 min. The minimum detectable level (determined at peak height twice noise) was 2 and 3 nmole/l for imipramine and desipramine, respectively. The reproducibility of the assay determined by replicate analyses of known concentrations of imipramine and desipramine over the range 10—1000 nmole/l is shown in Table I. Recovery of imipramine and desipramine was 90% for both and independent of concentration over the range 10—1000 nmole/l.

TABLE I

COEFFICIENTS OF VARIATION (C.V., %) FOR IMIPRAMINE AND DESIPRAMINE DETERMINATIONS

Five determinations at each concentration.

Concentration (nmole/l)	C.V. (%)	
	Imipramine	Desipramine
1000	2	3
500	2	3
100	2	3
25	5	5
10	10	15

No interference was observed from the tricyclic antidepressants amitriptyline, nortriptyline and protriptyline and the following fluorescent drugs and metabolites: propranolol, 4-hydroxypropranolol, propranolol glycol, N-desisopropylpropranolol, quinidine, dihydroquinidine and 3-hydroxyquinidine all of which eluted before imipramine and desipramine. The assay of plasma samples from a number of patients and volunteers not taking imipramine showed a plasma peak eluting at approximately 3 min with no potentially interfering peaks eluting after that time.

Chromatograms obtained by analysis of samples from a volunteer prior to taking an imipramine hydrochloride dose (50 mg), 4 h after the dose and from a patient on chronic oral medication are shown in Fig. 1a, b and c, respectively. The retention times for the eluted components were 3.0 min (plasma peak), 9.8 min (desipramine), 11.3 min (imipramine) and 14.1 min (trimipramine).

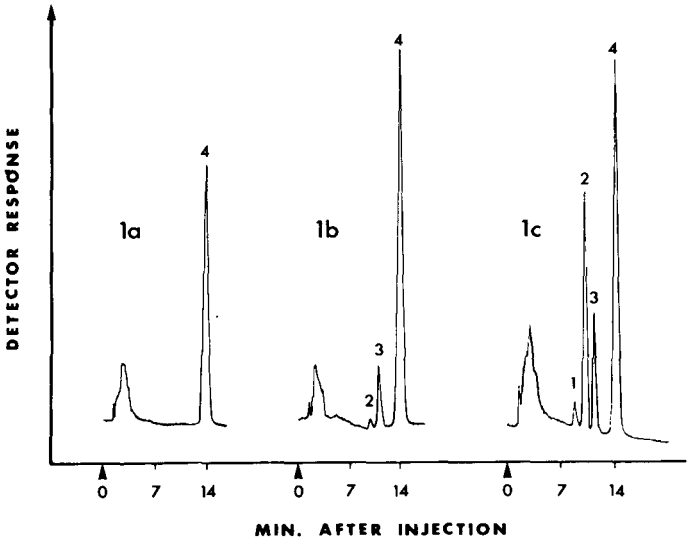

Fig. 1. Chromatograms obtained for the assay of plasma samples from a volunteer prior to taking an imipramine hydrochloride dose (50 mg) (1a), 4 h after the dose (1b) and from a patient on chronic oral therapy (1c). Peaks: 1 = unknown metabolite, 2 = desipramine, 3 = imipramine and 4 = trimipramine. In Fig. 1b, peak 2 = 20 nmole/l and 3 = 90 nmole/l; in Fig. 1c, peak 2 = 590 nmole/l and 3 = 190 nmole/l.

The small additional peak (peak 1) in Fig. 1c was only observed for patients taking imipramine and is probably attributable to an additional imipramine metabolite.

The plasma level time course following administration of a single 50-mg oral dose of imipramine hydrochloride to a healthy volunteer is shown in Fig. 2. The imipramine concentration peaked at 3.5 h (90 nmole/l) and then declined to a level of 8.0 nmole/l 28 h after the dose. The desipramine concentration also peaked at 3.5 h (30 nmole/l) but the levels at times 1—3 h and 12—28 h after the dose were too small to allow precise quantification (C.V.>15%).

DISCUSSION

The presently described method is applicable to the assay of plasma levels of at least two tricyclic antidepressants, imipramine and desipramine and possibly trimipramine, clomipramine and demethylclomipramine. Since trimipramine was used as the internal standard it is apparent that plasma levels of this drug could also be measured if another internal standard such as imipramine or desipramine was used.

The procedure involves a single hexane—isoamyl alcohol extraction of the drug and metabolite from basified plasma, back extraction into phosphoric acid and injection into the chromatograph. This allows approximately 20—30 plasma samples to be assayed per day. A combination of the separative power of reversed-phase high-performance liquid chromatography and the sensitivity of fluorescence detection has extended the separate detection of these compounds in plasma down to 2—3 nmole/l with a high degree of reproducibili-

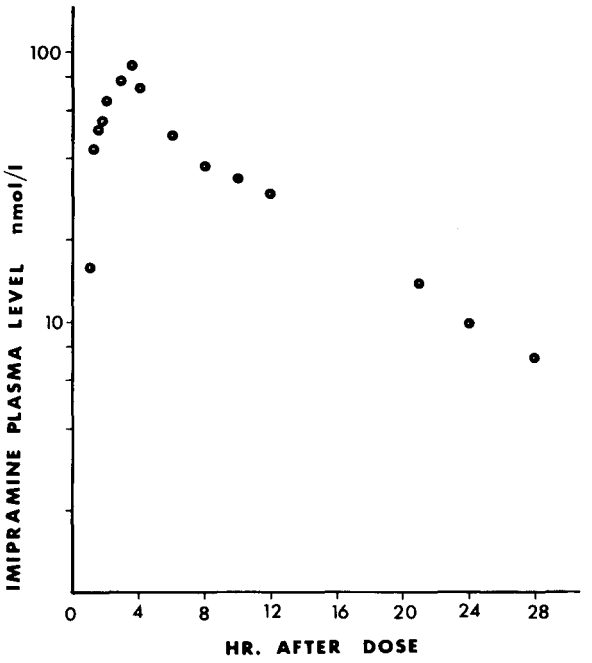

Fig. 2. Imipramine plasma level time course following administration of a single 50-mg oral dose of imipramine hydrochloride to a healthy volunteer.

ty. The sensitivity is sufficient to measure the very small levels of drug present in plasma more than 24 h after a single oral dose of imipramine. The convenience and speed of the procedure makes it suitable for the routine monitoring of steady-state plasma levels during chronic therapy.

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Note

High-performance liquid chromatographic assays for furosemide in plasma and urine

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Fluorometric [1, 2], gas chromatographic [3], thin-layer chromatographic [4, 5] and high-performance liquid chromatographic (HPLC) [6, 7] assays are available for the determination of the diuretic furosemide in biological fluids. All require prior derivatization and/or extraction. In addition, one HPLC assay [6] uses a mobile phase containing 0.02 *M* chloride which can damage the stainless-steel fittings on the instrument. A third HPLC assay has been described [8]. It has the advantage of not requiring any extraction or derivatization steps. The disadvantages of this method relate to the fact that no internal standard is used and to the necessity of bringing samples into an acid pH range (i.e., 2.5) for fluorometric measurement. As pointed out by the authors, low pH apparently leads to a degradative breakdown of furosemide. Moreover, the lower limit of detection for this assay is only 1 mg/l [8].

The purpose of the present investigation was to develop an assay for furosemide that could be used in our bioavailability/pharmacokinetic studies and that could also be used for routine monitoring of furosemide levels in adult, children and neonate patients. For these reasons it was imperative that we develop an assay capable of rapidly but reproducibly measuring low levels of furosemide in small volumes of plasma and urine. We have developed two different HPLC assays. The two methods differ in their mobile phase, internal standards, and methods of detection. Neither method requires prior extraction and/or derivatization of the plasma or urine samples. Both methods are rapid, sensitive and accurate.

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EXPERIMENTAL

Apparatus

We used a high-pressure liquid chromatograph (Model ALC/GPC 244; Waters Assoc., Milford, Mass., U.S.A.), characterized by a constant solvent flow at working pressures up to 420 kg/cm^2. This model includes a U6-K universal injector and a dual-channel fixed-wavelength, ultraviolet absorption detector. The instrument was fitted with a 30 cm × 3.9 mm I.D. μBondapak C_{18} reversed-phase column, particle size 10 μm (Waters Assoc.). The chromatograph was operated isocratically at a flow-rate of 2 ml/min, at ambient temperature. For Method 1 the wavelength of detection was fixed at 280 nm. For Method II the wavelengths of detection were fixed at 254 and 280 nm. A dual-pen recorder was used (Omniscribe Model A5211-1; Houston Instruments, Austin, Texas, U.S.A.). Chart speed was 2.5 cm/min and full-scale response was 1 mV.

Reagents

Chemicals. The furosemide, Lot RW 1793, was obtained from Hoechst-Roussel (Sommerville, N.J., U.S.A.), sodium cephalothin Lot 96123 was obtained from Eli Lilly (Indianapolis, Ind., U.S.A.), sodium phenobarbital, Lot 63453, was obtained from Merck (Rahway, N.J., U.S.A.).

The methanol (glass-distilled, Burdick and Jackson Labs., Muskegon, Mich., U.S.A.), acetonitrile, Analytical Reagent (Mallinckrodt, St. Louis, Mo., U.S.A.) and distilled water (glass-redistilled and stored in glass) were filtered through a 0.45-μm filter (HAWPO 4700 and FHLPO 4700; Millipore Corp., Bedford, Mass., U.S.A.) before use. All other chemicals were ACS reagent grade or better.

Mobile phase. Method I: The mobile phase consisted of methanol—0.01 *M* sodium acetate, pH 5.0 (35:65), prepared by mixing 350 ml of methanol with 650 ml of water, adding 0.6 ml of glacial acetic acid, and adjusting the pH of the solution to 5.0 with 4 *M* sodium hydroxide. Method II: The mobile phase consisted of acetonitrile—0.01 *M* sodium acetate, pH 5.0 (25:75), prepared by mixing 250 ml of acetonitrile with 750 ml of water, adding 0.6 ml glacial acetic acid, and adjusting the pH of the solution to 5.0 with 4 *M* sodium hydroxide. These mobile phases are degassed under vacuum before use.

Biological fluids. Human plasma that had been stored at −20° for four weeks was obtained from the blood bank of the University of California, San Francisco. The plasma was thawed to room temperature before use. Human urine from a male donor was collected daily.

Urine was collected from a normal male volunteer given 40 mg of furosemide by intravenous injection. Blood from the same patient was collected in a heparinized tube (Vacutainer; Becton-Dickinson, Rutherford, N.J., U.S.A.). It was centrifuged (3000 *g*, 4 min) and the plasma fraction was decanted. These plasma and urine samples were stored at −20° before analysis.

Procedure

Method I. Plasma: Add 20 μl of a 68 mg/l aqueous solution of sodium cephalothin, the internal standard, to 200 μl of plasma. Shake well, add 200 μl of methanol and shake to mix. Centrifuge for 15 min at 10,000 *g* to pre-

cipitate the proteins. Pour the supernatant into a 4-ml glass tube. Inject 25—30 μl of these solutions onto the chromatograph. Set the 280 nm detector at 0.01 a.u.f.s. Operate the chromatograph at a flow-rate of 2.0 ml/min at room temperature using methanol—sodium acetate (35:65) as the mobile phase. Retention times for sodium cephalothin and furosemide are 6 and 8 min, respectively.

Urine: Add 20 μl of the 68 mg/l aqueous sodium cephalothin solution to 200 μl of urine. Shake well to mix. Inject directly onto the chromatograph. The chromatographic conditions are identical to those described above for plasma.

Method II. Plasma: Add 20 μl of a 150 mg/l aqueous solution of sodium phenobarbital, the internal standard, to 200 μl of plasma. Shake well, add 400 μl of acetonitrile and shake to mix. Centrifuge at 3000 *g* for 10 min to precipitate the protein. Pour the supernatant into a 4-ml test tube. Evaporate under nitrogen at ambient temperature to a volume of about 150 μl. Add 30 μl of the mobile phase, mix and inject onto the chromatograph. Injection volumes of 15—20 μl were satisfactory at 0.01 a.u.f.s. at both 280 and 254 nm. Operate the chromatograph at a flow-rate of 2.0 ml/min at room temperature using the acetonitrile—sodium acetate (25:75) mobile phase. Retention times were 5 and 7 min, respectively, for furosemide and sodium phenobarbital. A dual-channel ultraviolet absorption detector must be used to monitor simultaneously the furosemide at 280 nm and the sodium phenobarbital at 254 nm.

Urine: Add 50 μl of the 150 mg/l aqueous sodium phenobarbital solution to 200 μl of urine. Inject directly onto the chromatograph. Volumes of 10 to 15 μl were satisfactory when the sensitivity of the detector was set at 0.01 a.u.f.s. at both 280 and 254 nm. All other chromatographic conditions were identical to those described above for plasma.

With both methods standard curves are prepared by adding furosemide and the appropriate internal standard to plasma or urine. The concentration of furosemide in samples is determined by comparing the furosemide/internal standard peak height ratios to standard curves of peak height ratios versus furosemide concentration. With all curves, we made a straight-line fit of the data by least squares linear regression analysis using the PROPHET system, a specialized computer resource developed by the Chemical/Biological Information Handling Program of the National Institutes of Health.

RESULTS AND DISCUSSION

We have developed two HPLC methods for quantitating furosemide in plasma and urine. Both are rapid, sensitive and accurate. Neither requires extraction and/or derivatization. Fig. 1 shows a chromatogram for the quantitation of furosemide in urine using Method I. The retention times for sodium cephalothin and furosemide are 6 and 8 min, respectively. The peak height ratio indicates the furosemide concentration is 3.6 mg/l. For Method I standard curves were constructed by adding known amounts of furosemide and sodium cephalothin, the internal standard, to urine and plasma and plotting the peak height ratio versus concentration of furosemide in mg/l. Over a

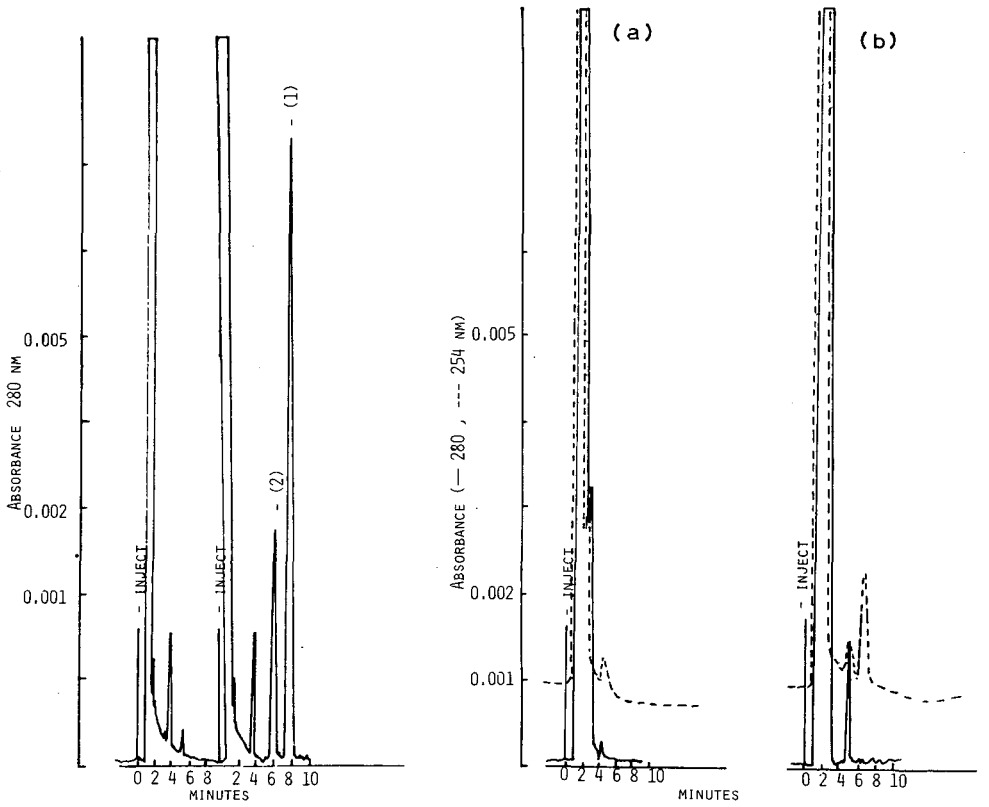


Fig. 1. Chromatograms developed using Method I of (a) blank urine and (b) urine with added furosemide (1) and sodium cephalothin (2). The peak height ratio indicates a furosemide concentration of 3.6 mg/l.

Fig. 2. Chromatograms developed using Method II of (a) blank plasma, (b) plasma with added furosemide, peak at 5 min on 280 nm; and sodium phenobarbital, peak at 7 min on 254 nm. The peak height ratio indicates a furosemide concentration of 0.82 mg/l.

period of 20 days we constructed eight plasma standard curves. With 37 points the regression line for plasma was $y = (0.093 \pm 0.002)\,x + (0.012 \pm 0.045)$ with a coefficient of variation of the slope of 2% and a correlation coefficient of 0.99. With urine, we constructed three standard curves over 20 days. With 15 points the regression line for urine was $y = (0.089 \pm 0.002)\,x + (0.044 \pm 0.039)$ with a coefficient of variation of the slope of 2% and a correlation coefficient of 0.99. For both plasma and urine the concentration of furosemide ranged from 1.8 to 36 mg/l.

Fig. 2 shows a chromatogram for the analysis of furosemide in plasma using Method II. The retention times of furosemide and sodium phenobarbital are 5 and 7 min, respectively. The peak height ratio indicates that the furosemide concentration is 0.82 mg/l. For Method II standard curves were constructed by adding known amounts of furosemide and sodium phenobarbital, the internal standard, to urine and plasma and plotting the peak height ratios of furosemide to sodium phenobarbital against the concentra-

tion of furosemide in mg/l. Over a period of twelve days we constructed six plasma standard curves. With 50 points the regression line for plasma was $y = (1.161 \pm 0.046)\ x + (0.016 \pm 0.052)$ with a coefficient of variation of the slope of 4% and a correlation coefficient of 0.99. With urine we constructed five standard curves over a period of two months. With 34 points the regression line for urine was $y = (0.456 \pm 0.008)\ x + (0.030 \pm 0.042)$, with a coefficient of variation of the slope of 2% and a correlation coefficient of 0.99. For plasma the concentration of furosemide ranged from 0.081 to 2.45 mg/l and for urine from 0.205 to 10.25 mg/l.

Preliminary stability studies with 10 mg/l and 2 mg/l of furosemide in plasma were performed over a period of 20 days using Method I. Concentrations of furosemide in plasma were obtained by comparing the furosemide/sodium cephalothin peak height ratios with those of a standard curve obtained the same day. The results (Table I) show that furosemide can be stored frozen in plasma for at least three weeks.

For both methods, furosemide was detected at 280 nm. This wavelength is quite close to the absorption maximum of furosemide which is 275 nm. The internal standard, sodium cephalothin, for Method I was also detected at 280 nm. However, for Method II the sodium phenobarbital was detected at 254 nm. We do not suspect any interference from endogenous substances using either method because under either set of conditions, all the extraneous peaks have retention times of less than 4 min (Figs. 1 and 2).

The two compounds chosen as internal standards are therapeutic agents which may be administered to patients receiving furosemide. If a patient is receiving one of these drugs, then the alternate method and internal standard may be used. Under the unlikely circumstances that a patient is receiving phenobarbital, cephalothin and furosemide, it is still possible to use sodium phenobarbital as the internal standard. Since the measurement of this compound is carried out at a wavelength different from that for furosemide, higher concentrations of sodium phenobarbital may be used while decreasing the sensitivity of the detector at 254 nm, thus swamping out the relatively low plasma levels of phenobarbital usually observed in patients.

With urine samples, where no precipitation is necessary, analyses can be performed in less than 10 min per sample. In the case of plasma, centrifugation after protein precipitation is necessary because direct injections of plasma onto the chromatograph result in increases in operating pressure caused by the build up of proteins at one end of the column. In Method I after 15 min of centrifugation, analyses can be performed in less than 10 min. In

TABLE I

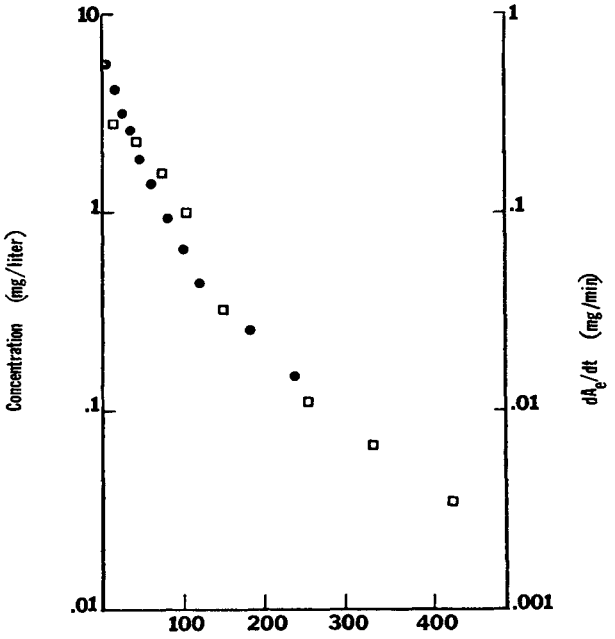
THE EFFECT OF STORAGE ON FUROSEMIDE CONCENTRATION

Furosemide (mg/l)	Time (days)					
	0	1	2	6	10	20
10	9.95	—	10.00	10.10	9.75	9.60
2	2.10	2.00	1.95	2.00	1.95	1.95

Method II 10 min of centrifugation and 10 min of evaporation are required after which analyses can be performed in less than 10 min.

Fig. 3 shows the plasma levels of furosemide (left axis) vs. time and the urinary excretion rate (right axis) vs. time curves obtained after intravenous administration of 40 mg of furosemide to a male volunteer. The terminal plasma elimination half-life of furosemide in this volunteer is 70 min similar to that seen in other volunteers [9].

Chronologically, we developed Method I first. It has the advantage of using a single fixed wavelength for detection of both the furosemide and the internal standard. It is also rapid, sensitive and accurate. However, we noticed some problems with the stability of sodium cephalothin. That is, the internal standard solutions had to be prepared fresh daily and could not be stored for even a few days. We had also noticed that precipitation of the plasma proteins by acetonitrile gave more complete precipitation than methanol. We discovered that with methanol precipitation column pressure built up fairly rapidly and peaks started spreading, thus, decreasing the column life-time. We also wanted to increase the sensitivity of the assay. Therefore, we developed Method II. The acetonitrile gives more complete protein precipitation. The acetonitrile—sodium acetate mobile phase also gives lower pressure at the same flow-rate than the methanol—sodium acetate mobile phase. Both of these factors contribute to longer column life-times. We could also increase the sensitivity by over twenty-fold since the furosemide peaks were sharper.



Thus, Method II is as rapid and accurate as Method I but is more sensitive. Method II has the disadvantage of requiring a dual-channel detector in order to measure furosemide at 280 nm and sodium phenobarbital at 254 nm. Because of its greater sensitivity we routinely use Method II. However, for investigators with only a single-channel detector, Method I is a rapid, accurate and sufficiently sensitive method for routine clinical monitoring of furosemide in biological fluids.

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Note

Determination of griseofulvin in rat plasma by high-performance liquid chromatography and high-performance thin-layer chromatography

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For the study of the resorption of different administration forms of griseofulvin (7-chloro-2',4,6-trimethoxy-6'-β-methylspiro-(benzofuran-2-(3H)-1'-(2)-cyclohexene)-3,4'-dione) a specific and sensitive method of determination for this antifungal antibiotic is required. Spectrofluorimetric methods are sensitive [1—4], but they also detect other metabolites, and they have been substituted by gas-chromatographic methods [5, 6] and a thin-layer chromatographic (TLC) process [7] (self-streaked Al_2O_3/SiO_2 plates with fluorescence detection). Bailey [8] has published a high-performance liquid chromatographic (HPLC) method with ultraviolet detection, but this is not sensitive enough. All these methods require the extraction of griseofulvin from plasma.

This paper describes a sensitive HPLC method using fluorescence detection, with a simplified extraction process, and a simple and effective high-performance TLC (HPTLC) method, also with fluorescence detection.

METHODS

HPLC method

Extraction of griseofulvin from plasma. One millilitre of plasma, diluted with 17 ml of distilled water, is extracted with 60 ml of peroxide-free diethyl ether (technical grade) through an Extrelut^R-Fertigsäule (Merck, No. 11737, Nachfüllpackung No. 11738). The eluate is evaporated to dryness at 40° and the residue dissolved in 1—5 ml methanol (p.a. grade) in water (50:50), according to the expected griseofulvin content. The performance of this extraction was tested with rat plasma spiked with different quantities of griseofulvin (0.5, 1.0, 5.0, 10.0 and 100.0 μg/ml).

Deproteinisation of plasma. One millilitre of plasma and 2 ml of ethanol (p.a. grade) are mixed, centrifuged and the supernatant is used for analysis.

Analytical procedure. The reagents used were p.a. grade from Merck (Darmstadt, G.F.R.). The column (10 cm × 3 mm I.D.) was filled with LiChrosorb RP-8 (Merck) or Nucleosil C_8 (Macherey, Nagel & Co., Düren, G.F.R.), particle size 5 μm, column temperature 20°. The fluorescence detectors were a Perkin-Elmer LC 1000 (λ_{exc} = 297 nm (filter), λ_{em} = 428 nm, scale expansion ×20); and a Perkin-Elmer 204, with an adjustable flow-through cell (Hellma, No. 176.70 QS), λ_{exc} = 295 nm, λ_{em} = 428 nm, cut-off filter FL 39 (Zeiss), respectively. Sensitivity control 12, selector ×10. The eluent was acetonitrile—water (40:60); the pump an Orlita DMPAE 10.4, providing a flow of 1.2 ml/min, at a pressure of 180 bar. The injector was a modified 7671 A-Automatic Sampler [9] (Hewlett-Packard) with a pneumatic sample injection valve 900048 L (Latek, Heidelberg, G.F.R.), and a 25-μl loop, filling with a peristaltic pump Mini S 820 (Ismatec).

The standard solution, according to the concentration expected, was 1.0 or 10.0 μg griseofulvin per ml methanol (50%). The standard was pure griseofulvin (Biochemie Ges.m.b.H., Kundl, Austria). Injection mode: after four samples one standard is injected. Recorder: Servogor S (Goerz), paper advance 0.5 cm/min. Calculations were made using the Laboratory Data System 3352 C from Hewlett-Packard.

Under these conditions the retention time of griseofulvin was 5 min.

HPTLC method

The reagents were p.a. grade from Merck. The plates were HPTLC-Fertigplatten Kieselgel 60 für die Nano-DC, 10 × 20 cm (Merck, No. 5641); the developing chamber was also from Merck (No. 11622). The solvent was butylacetate—acetone (4:1). Application: 500 nl microcaps (Drummond).

The standard solutions were: (1) 1 μg griseofulvin per ml methanol (50%); (2) 5 μg griseofulvin per ml methanol (50%); (3) 9 μg griseofulvin per ml methanol (50%). Samples A—J were different samples of rat plasma, diluted 1:1 or more with a 1% solution of 2,5-dimethylbenzosulphonic acid (ammoniumsalt, Merck, No. 3469). The solution to be applied should contain 1—9 μg griseofulvin per ml. The application scheme for 500 nl of each standard solution 1—3 and samples A—J was as follows: AB1CD2EFG3HIJAB1CD2EFG3HIJ. The migration distance was 3 cm (= 5 min), giving an R_F of 0.52 for griseofulvin. Drying time was 30 min at 120°.

Scanning conditions were: Zeiss-Chromatogrammspektralphotometer, excitation by Hg lamp St 41 at 295 nm, measuring through cut-off filter FL 39, F/II/10, measuring slit 3.5 mm, slit of monochromator, 1.3 mm, table advance 50 mm/min, paper advance on Servogor S 60 mm/min, scanning at right-angles to the direction of chromatography, to and fro. Fully automated computation [10] (calibration line: peak area against concentration).

RESULTS

The recoveries of griseofulvin added to plasma ranged between 94.0 and 100.0%. Results obtained from 40 samples, containing 6—100 μg of griseofulvin per ml and analysed by HPLC and HPTLC, showed the same results and a coefficient of correlation of 0.99868. For HPLC there was a close linearity

between peak area and concentration between 0.1 and 100 μg/ml, whilst for HPTLC this linearity was valid between 0.5 and 15 μg/ml (see calibration lines in Fig. 1). HPTLC is faster than HPLC, as can be seen in Table I; the peaks of samples, deproteinised by means of ethanol, were unsuitable for evaluation (Fig. 2). Both methods are of identical accuracy (see Table I); of course, when a sample is analysed once on ten different plates instead of ten times on one plate, the coefficient of variation increases to 2.0%.

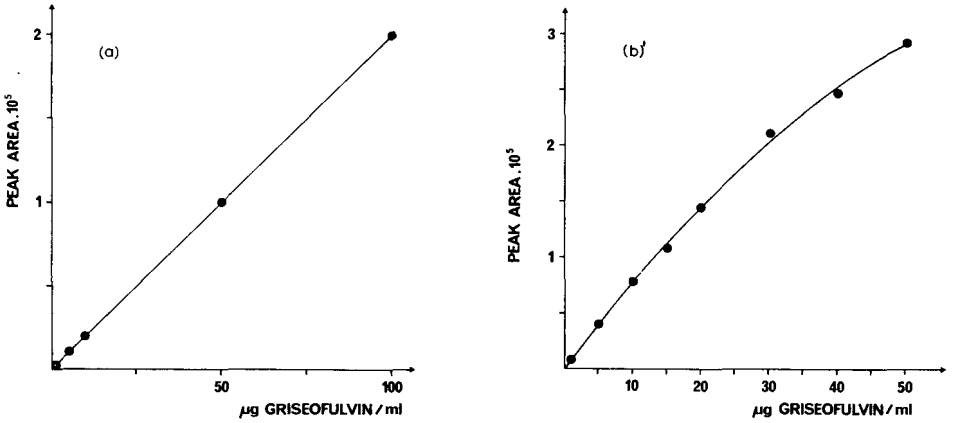

Fig. 1. Calibration curves for HPLC (a) and HPTLC (b).

TABLE I

COMPARISON OF HPLC WITH HPTLC

	HPLC		HPTLC	
Time (min) required for 20 samples	Extraction	270	Dilution	10
	Preparation*	115	Application	20
	Programming	5	Separation	5
	Separation, evaluation	220	Drying	30
			Programming	1
			Measurement adjusting	5
			Scanning, evaluation	25
	Total:	610		96
	Ratio of manipulation	68%		64%
Detection limits (μg/ml, signal-to-noise ratio = 3:1)	LC 1000, λ_{em} = 428 nm	0.1	Zeiss, FL 39	0.15
	204, λ_{em} = 428 nm	0.025		
	204, FL 39	0.06		
Accuracy (C.V. (%), n = 10)		0.8	Analysed on one plate	0.8
			Analysed on ten different plates	2.0

*The preparation time includes in proportion column filling, column testing, services to the apparatus and functional control of the analysis equipment.

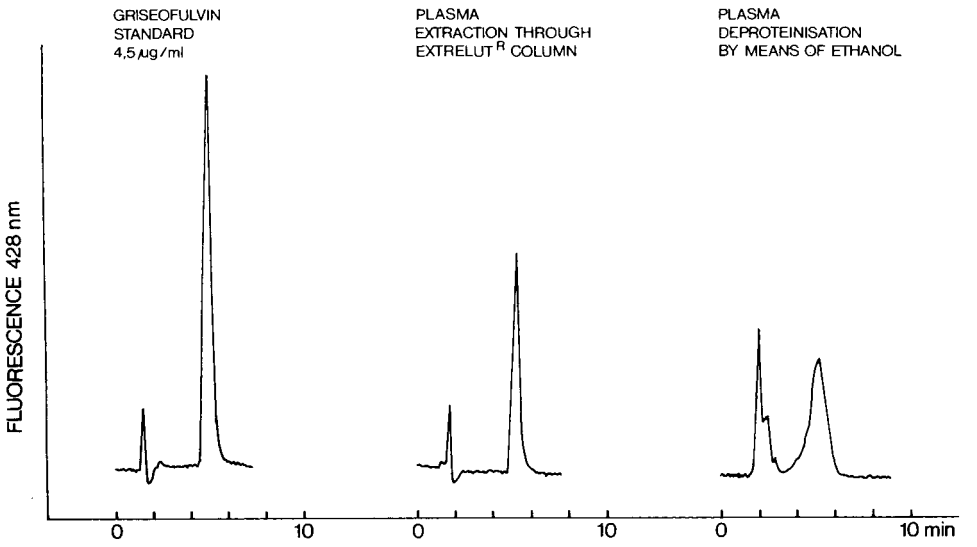

Fig. 2. HPLC chromatograms.

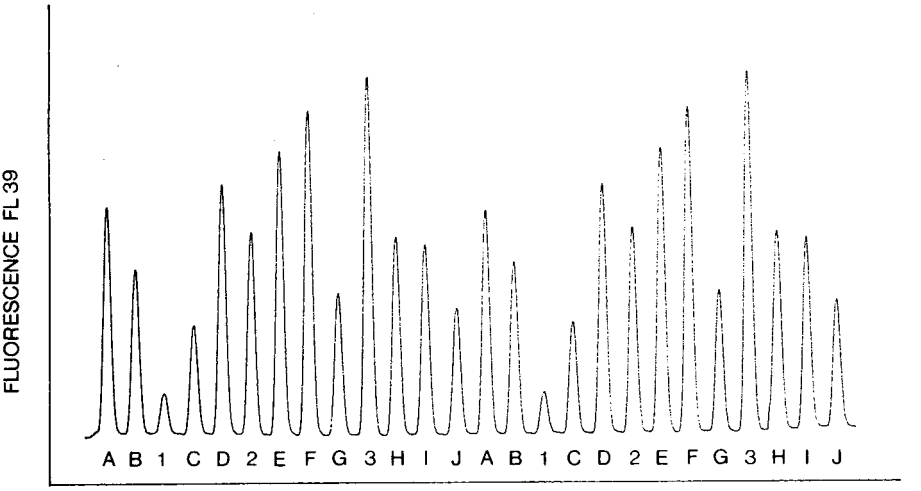

Fig. 3. HPTLC chromatogram.

DISCUSSION

The Extrelut^R extraction method for griseofulvin allows high recoveries over a wide range of concentrations, offers a sensible simplification in comparison to the conventional extraction, especially in serial analyses, and excludes faults to a large extent.

The advantage of HPLC is its high sensitivity, which could be enhanced by injecting larger volumes of the sample. The fact that the use of the cut-off filter with the Perkin-Elmer 204 yields a higher detection limit than with the monochromatic emission measurement, is due to the higher base-line noise in the cut-off filter measurement. The extraction of griseofulvin from plasma

is absolutely necessary and cannot be avoided by deproteinisation, as shown by the broader peak in Fig. 2.

The advantage of HPTLC is its simplicity, especially because no sample preparation is necessary. For this reason direct determination of griseofulvin in plasma samples is possible for the first time. The solvent causes deproteinisation at the site of application on the plate. As this precipitated protein causes enlarged spots after chromatography, dilution of the sample solution is necessary to reduce the amount of protein. It was of advantage to dilute with a solution of 2,5-dimethylbenzosulphonic acid, as in this way protein opacities of the plasma after a longer storage time are avoided. The speed of separation and measurement is remarkable; the measurement is executed at right-angles to the direction of chromatography (see Fig. 3). In contrast to the report of Fischer and Riegelman [7], the fluorescence signals are constant immediately. The only disadvantage of the HPTLC method is that with fluorescence excitation by an Hg lamp, the detection limit for griseofulvin is 0.15 μg/ml, and the useful measurement range begins at 0.5 μg/ml sample, that is at 1 μg griseofulvin per ml plasma. Using micro-optics for the Zeiss scanner this shortcoming can be overcome to a certain extent. There is the possibility to extract the sample in an Extrelut^{R} column, evaporate the ether and dissolve the residue in 0.5 ml of 50% methanol. This additional procedure augments the sensitivity four times.

As Table I shows, the HPTLC method requires only one sixth of the time necessary for the HPLC method, resulting in a cost ratio of 1:10. With an increase in sample numbers the preparation time decreases correspondingly. For both methods the ratio of manipulation is similar.

Consequently, for plasma containing more than 1 μg griseofulvin per ml, the HPTLC method should be preferred; for a lower griseofulvin content the HPLC method must be applied. This work shows how much the cost of analyses can be influenced when, according to the nature of the problem, one can choose among several chromatographic methods.

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I am indebted to Miss Anna Gapp for her assistance in developing the HPTLC method, to Ing. Johann Patka for the modification of the HP sampler and determinations performed with it, and to Mr. Josef Kleinlercher, Kufstein, for the construction of the Perkin-Elmer 204 fluorimeter cuvette-adjusting device.

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Book Review

Blood drugs and other analytical challenges (Methodological Surveys in Biochemistry, Vol. 7), edited by E. Reid, Ellis Horwood (Wiley), Chichester, 1978, X + 355 pp., price £ 19.50, ISBN 0-85312-124-9.

This book contains the proceedings of the second Bioanalytical Forum held in September 1977 at the University of Surrey, U.K. Like its predecessor in 1975 (Assay of drugs and other trace compounds in biological fluids, reviewed in J. Chromatogr., 150 (1978) 570—571) the meeting was held to discuss approaches and methods for the determination of trace organic compounds in biological fluids and to emphasize the rationale of developing suitable methods.

The first section, entitled The Framework, contains six excellent and authoritative general papers: Development of analytical methods (R.G. Cooper); The compromise between sensitivity and specificity in analyzing biological fluids for drugs (J.A.F. de Silva); Statistics of drug analysis and the role of internal standards (S.H. Curry and R. Whelpton): Quality control of results and sources of error (B. Scales); Approaches to the evaluation of analytical methods: an overview (J. Chamberlain); Recommended terminology and practices in chromatographic assays, especially GC-ECD, entailing sample work-up (E. Reid).

Then follow three technique-orientated sections namely on subtle gas chromatography, mass spectrometric approaches, and HPLC, TLC and non-chromatographic approaches (7, 6 and 9 papers respectively) and the final section deals with notes and comments related to the foregoing topics. These sections also offer very interesting reading, well-balanced papers and a treatment that will satisfy both the more experienced analyst as well as the novice. Without detracting the merits of the other papers here are just a few titles: Every day problems in glass-capillary GC (K. Grob); Applications of the alkali flame ionization detector in drug analysis (L.A. Gifford); Drug analysis by GC-ECD (J. Vessman); Derivatization for GC-ECD (H. Ehrsson); Assay of drugs and endogenous compounds in biological fluids by GC—MS (T.A. Baillie); Accuracy and precision in GC—MS quantitation (J.J. de Ridder); Possibilities of negative ion MS in forensic and toxicological analysis (H. Brandenberger and R. Ryhage); Ion-pair HPLC of acid and basic drugs, metabolites and endogenous compounds (G. Schill); Pre-concentration and chemical derivatization techniques in HPLC (R.W. Frei); New pre-chromatographic methods for biomedical trace analysis: problems and possibilities (W. Dünges).

The book has been produced from camera-ready type-set manuscripts, resulting in clear and well-readable text. Yet, the presentation of the figures is less satisfactory, whereas it would have been advisable for the captions to the figures to use a letter type different from that of the text. The subject index and a second index of types of compounds are well prepared and allow easy retrieval. A special word of appreciation should be given to the excellent cross-referencing between the individual papers.

This book can be recommended to all scientists engaged in the analysis of drugs in biological fluids.

Groningen (The Netherlands) ROKUS A. DE ZEEUW

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4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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