ISSN 0378-4347

VOL. 163 NO. 1 MAY 1, 1979
(Biomedical Applications, Vol. 5, No. 1)

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1979

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

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	The publication schedule for the volumes 174–180 and for further *Chromatographic Reviews* issues (vol. 165) will be published later.						
Chromatographic Reviews				165/1									
Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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JOURNAL OF CHROMATOGRAPHY

VOL. 163 (1979)

(Biomedical Applications, Vol. 5)

JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

VOL. 5 1979

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands

Journal of Chromatography, 163 (1979) 1—8
Biomedical Applications

CHROMBIO. 313

MASS FRAGMENTOGRAPHIC DETERMINATION OF PROSTAGLANDIN $F_{2\alpha}$ IN HUMAN AND RABBIT URINE

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(Received November 3rd, 1978)

SUMMARY

Analysis of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in urine is a useful indicator of renal prostaglandin synthesis. A mass fragmentographic method for $PGF_{2\alpha}$ analysis in human urine was developed using [3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ as an internal standard and carrier. $PGF_{2\alpha}$ was extracted from urine (20 ml) with chloroform, purified by preparative thin-layer chromatography and converted to the methyl ester trimethylsilyl ether before analysis by gas chromatography—mass spectrometry. The specificity of the urine analysis was demonstrated by retention time and the use of two pairs of fragments *m/e* 494/498 and 513/517 with the same results. The coefficient of variation for duplicate analysis averaged 12.6%, n = 17. Urine from recumbent women contained 4.9 ± 2.6 (S.D.) ng/ml or 4.1 ± 1.0 ng $PGF_{2\alpha}$ per mg creatinine (n = 10) with little diurnal variation. Male urine contained 5.0 ± 2.7 (S.D.) ng/ml or 3.7 ± 2.1 ng/mg creatinine (n = 10). Similar concentrations were found in boys and in girls. These observations indicate that urinary $PGF_{2\alpha}$ originates from the kidneys with little contribution from the male accessory sexual glands. This method can also be applied to analysis of $PGF_{2\alpha}$ in rabbit urine.

INTRODUCTION

The kidneys have a large capacity to synthesize prostaglandins (PGs) [1, 2]. The renal PGs may be of physiological importance in the regulation of renal blood flow, renin release and sodium excretion [3,4]. Recently PGE_2 and $PGF_{2\alpha}$ were conclusively identified in human urine [5]. Stop-flow experiments indicate that PGs formed in the kidney enter the urine via the loop of Henle [6], while PGs infused into the renal artery are mainly recovered in urine as metabolites [7, 8]. Urinary excretion of primary PGs might therefore be used as a valuable indicator of renal PG synthesis [4, 5]. In this paper we describe a convenient and rapid mass fragmentographic method for analysis of $PGF_{2\alpha}$ in human and rabbit urine.

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METHODS

Materials

All reagents were of analytical grade. $PGF_{1\alpha}$, $PGF_{2\alpha}$, and [3,3,4,4-$^2H_4$]-$PGF_{2\alpha}$ were obtained from Upjohn (Kalamazoo, Mich., U.S.A.) and [^{3}H]-$PGF_{2\alpha}$ (150 Ci/mmol) from New England Nuclear (Darmstadt, G.F.R.). The purity of the compounds was checked by thin-layer chromatography (TLC) or by gas chromatography—mass spectrometry (GC—MS) (cf. Fig. 1). Thin-layer silicic acid plates (0.25 mm DC-Fertigplatten Kieselgel 60, Art. 5721) were obtained from Merck (Darmstadt, G.F.R.). The silylation reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), was obtained from Pierce (Rockford, Ill., U.S.A.).

Sample purification

Human urine. Fresh urine samples were obtained from healthy men, women and children before lunch. Urine was not collected from women during menstruation. The first few millilitres of urine were discarded. For diurnal analysis, urine was collected every 4 h during the day and overnight after 8 h and stored frozen.

All analyses were run in duplicate. To 20-ml portions of urine was added 0.50 μg of [3,3,4,4-$^2H_4$]$PGF_{2\alpha}$. The urine was acidified to pH 3 with 5—10 drops of 50% formic acid and extracted with chloroform (2 × 20 ml). To obtain phase separation, centrifugation at 1500 *g* for 15 min was often necessary. The combined chloroform extracts were dried by filtering through 4 g sodium sulphate on a filter paper and then evaporated. The sample was dissolved in 0.5 ml methanol and methylated with a fresh ethereal solution of diazomethane and evaporated to dryness under a stream of nitrogen. The samples were purified by TLC using ethyl acetate—methanol—water (80:13:50). In this system $PGF_{1\alpha}$ and $PGF_{2\alpha}$ methyl esters gave spots of the same R_F (R_F 0.67). Reference $PGF_{1\alpha}$ methyl ester was therefore put onto both sides of the TLC plate. The part of the plate containing the urine sample was covered and the references developed by spraying with phosphomolybdic acid and gentle heating of the sides of the plate. The zone corresponding to the $PGF_{1\alpha}$ and $PGF_{2\alpha}$ methyl ester was then scraped off and eluted twice with 2 ml methanol.

The sample was evaporated to dryness and the trimethylsilyl ethers were prepared by treatment with 50 μl BSTFA for 1 h at room temperature. After evaporation the residue was dissolved in 30 μl *n*-hexane and 5 μl was injected into an LKB 2091 combined gas chromatograph—mass spectrometer.

In some experiments [^{3}H] $PGF_{2\alpha}$ (2 × 10^5 dpm) was also added to the urine prior to extraction to obtain data on recovery. The recovery of [^{3}H]$PGF_{2\alpha}$ throughout the procedure averaged 35% with about 30% of the total radioactivity lost in the chloroform extractions and about 40% of the remaining activity lost during TLC.

Rabbit urine. Female non-pregnant white New Zealand rabbits (2—3 kg), were housed in metabolic cages. The cages were cleaned mechanically daily and rinsed with water. Urine was collected daily in traps on dry ice and kept frozen until analysis [9]. All samples were run in duplicate. To 5 ml of rabbit urine

were added 15 ml of water and 1.0 μg [2,3,4,4-$^2H_4$]$PGF_{2\alpha}$ but the analysis was otherwise performed exactly as described above for human urine.

GC—MS analysis

The samples were run on an LKB 2091 gas chromatograph—mass spectrometer equipped with a multiple ion detector. A 1% OV-17 column, 80 cm × 3 mm, operated at 240°, was used. The energy of the electrons was 22.5 eV and the trap current was 100 μA. The instrument was initially focused both on *m/e* 494/498 (M^+ —90) and 513/517 (M^+ —71), but for routine analysis only on *m/e* 494/498. A mass spectrum of the [3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ methyl ester trimethylsilyl ether, demonstrating the main ion intensities, is shown in Fig. 1. The deuterated $PGF_{2\alpha}$ standard contained 1% of the protium form.

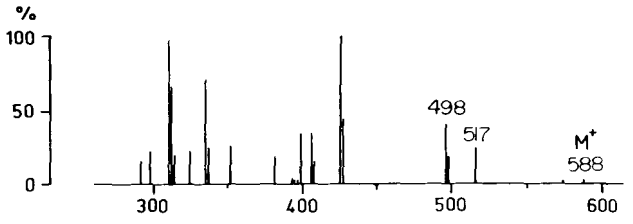

Fig. 1. Mass spectrum of [3,3,4,4-$^2H_4$]$PGF_{2\alpha}$ methyl ester trimethylsilyl ether. The deuterated $PGF_{2\alpha}$ contained 1% of the protium form. For mass fragmentographic analysis *m/e* 494/498 (M^+ —90) were used.

Standard curves were prepared by plotting the ratio between the maximum ion intensities of the ions in focus on the ordinate and the known ratio between unlabelled and deuterium-labelled $PGF_{2\alpha}$ in the standard solutions on the abscissa. Linearization by the least squares method gave lines with slope 1.05 ± 0.09 (S.D.) and intercept of ordinate 0.023 ± 0.012. The regression coefficient was 0.996 ± 0.006.

Other analyses

Radioimmunoassay of $PGF_{2\alpha}$ in rabbit urine was performed as previously described [10]. The antibodies to $PGF_{2\alpha}$ were raised and characterized in our laboratory [10]. To reduce the error due to interradioimmunoassay variation, the urine samples were analyzed by four assays and the mean values compared with mass fragmentography (Table I).

Creatinine in urine was measured spectrophotometrically by the Jaffé colour reaction according to Poulsen [11]. Data are expressed as mean ± S.D.

RESULTS

Evaluation of the method

The typical mass fragmentogram in human urine gave $PGF_{2\alpha}$ as the main peak with little background interference. This is illustrated in Fig. 2a using *m/e* 494/498. The specificity of the urine analysis was demonstrated by using two different ion pairs (*m/e* 513/517, M^+ —71 and 494/498, M^+ —90) as shown in Fig. 2b.

TABLE I

URINE CONCENTRATION OF $PGF_{2\alpha}$ IN URINE SAMPLES OF SEVEN RABBITS MEASURED BY GC–MS AND RADIOIMMUNOASSAY

Sample	GC–MS (ng/ml)	RIA (ng/ml)	Difference* (%)
1	36	36	0
2	34	21	−38
3	37	37	0
4	23	20	−13
5	28	27	− 4
6	39	32	−18
7	37	26	−30
7 + 588 ng/ml	631	690	9
Recovery	101%	113%	

*$\bar{x}$ = −12%.

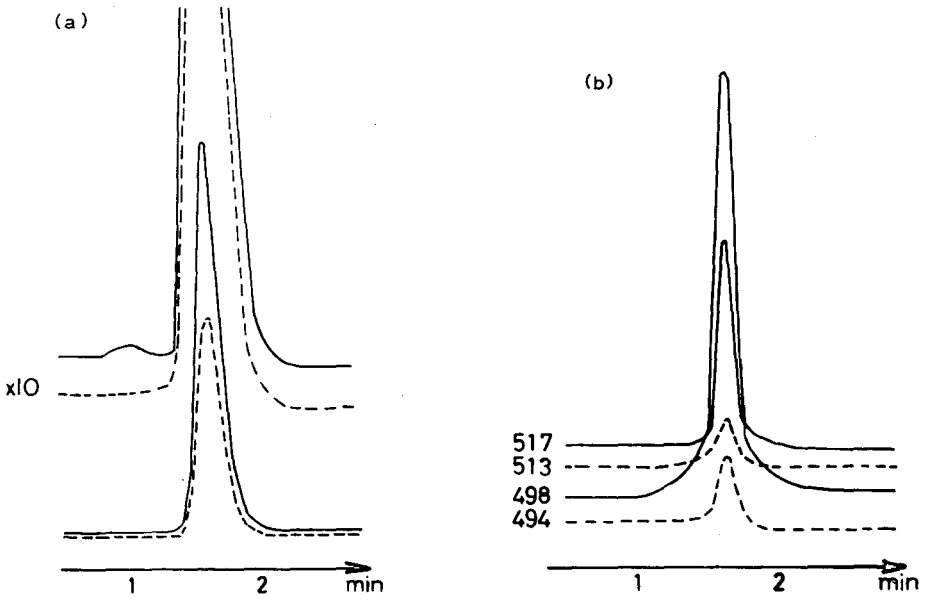

Fig. 2. (a) Mass fragmentogram from human urine using *m/e* 494/498. The main peak corresponds to $PGF_{2\alpha}$ derivatives. (b) Mass fragmentogram from human urine demonstrating the ratio between two different ion pairs *m/e* 513/517 (M^+ −71) and *m/e* 494/498 (M^+ −90).

The precision of the analysis was evaluated by replicate (n = 8) analyses of the same urine sample which gave 1.57 ± 0.31 (S.D.) ng/ml. In seventeen duplicate urine analyses the coefficient of variation was found to be 12.6%.

Unlabelled $PGF_{2\alpha}$ added to the urine was quantitatively recovered. The sensitivity of the analysis was usually adequate to measure 0.5 ng $PGF_{2\alpha}$ per ml corresponding to double background. Most of the background signal originated from the protium in the deuterium-labelled internal standard.

The stability of $PGF_{2\alpha}$ derivatives for GC—MS analysis was not particularly studied. However, the derivatives could be kept in a desiccator at 4° for at least one week, without decaying.

Analysis in human urine

The method was used to analyse the excretion of $PGF_{2\alpha}$ in urine from men, women and children. The results are shown in Table II and III. In the group of women aged 20—50 years the urinary concentration was 4.1 ± 1.0 mg $PGF_{2\alpha}$ per mg of creatinine. Young girls between the ages of 3 and 11.5 years had levels of the same order of magnitude, 4.6 ± 1.3 ng/mg of creatinine. The men (Table III) had slightly lower values than the women 3.7 ± 2.1 ng/mg of creatinine. It should be noted that there was greater tendency towards inter-individual variation among the men. Three boys between 3.5 and 7 years had $PGF_{2\alpha}$ levels between 4.7 and 5.6 ng/mg creatinine.

The possibility of a diurnal variation was explored in four healthy young women. The hourly $PGF_{2\alpha}$ output is shown in Fig. 3. No marked variation was seen. Two of the women had higher levels in the evening but the other two had not. One woman had an excretion rate of $PGF_{2\alpha}$ which was about 5 times higher than that of the other subjects. The reason for this was not apparent but it could be noted that she had had recent history of acute pyelonephritis.

Analysis in rabbit urine. Rabbit urine was generally found to contain higher concentrations of $PGF_{2\alpha}$ than human urine. A comparison between GC—MS analysis and radioimmunoassay of $PGF_{2\alpha}$ in rabbit urine (Table I) demon-

TABLE II

URINARY CONCENTRATION OF $PGF_{2\alpha}$ MEASURED BY MASS FRAGMENTOGRAPHY IN FEMALES

Females	Age	ng/ml	ng/mg creatinine
Women	20	7.9	3.2
	23	4.0	5.7
	26	8.5	3.1
	28	3.5	4.5
	31	4.9	3.2
	32	4.1	3.3
	33	8.3	4.1
	34	4.8	5.6
	38	0.9	3.8
	50	2.2	4.3
Mean ± S.D.		4.9 ± 2.6	4.1 ± 1.0
Girls	3	6.8	5.2
	5.5	1.6	2.3
	6	6.7	5.0
	10	6.8	5.5
	11.5	6.7	5.0
Mean ± S.D.		5.7 ± 2.3	4.6 ± 1.3

TABLE III

URINARY CONCENTRATIONS OF $PGF_{2\alpha}$ MEASURED BY MASS FRAGMENTOGRAPHY IN MALES

Males	Age	ng/ml	ng/mg creatinine
Men	23	8.8	3.9
	27	4.1	2.6
	29	1.5	3.2
	29	7.4	5.7
	30	5.1	1.7
	31	1.8	1.3
	31	2.9	4.1
	33	3.2	1.9
	38	8.4	8.2
	42	6.4	4.2
Mean ± S.D.		5.0 ± 2.7	3.7 ± 2.1
Boys	3.5	4.9	5.6
	5	3.0	4.7
	7	4.8	4.8
Mean ± S.D.		4.2 ± 1.1	5.0 ± 0.5

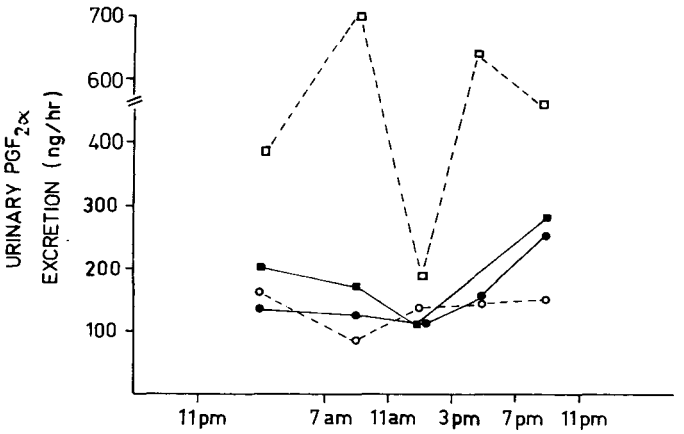

Fig. 3. Diurnal variation of urinary $PGF_{2\alpha}$ excretion in four women, measured by mass fragmentography.

strates little systematic difference between the two methods. Recovery of large amounts of $PGF_{2\alpha}$ (588 ng/ml) added to urine was quantitative with GC—MS but slightly overestimated by radioimmunoassay (Table I).

DISCUSSION

The present paper describes a mass fragmentographic method for analysis of $PGF_{2\alpha}$ in human and rabbit urine. $PGF_{2\alpha}$ has previously been identified in human urine [5] and analysed with GC—MS methods after different purifica-

tion procedures [12, 13]. The present method demonstrates that mass fragmentographic analysis of $PGF_{2\alpha}$ in urine is possible after solvent extraction, TLC and derivatization.

The concentration of $PGF_{2\alpha}$ in urine using this method is slightly higher than has previously been reported by GC—MS analysis [5]. The reason for this discrepancy is unknown. Dietary factors may be of importance since urinary $PGF_{2\alpha}$ excretion seems be inversely related to sodium excretion [9]. However, the difference is not likely to depend on diurnal changes in $PGF_{2\alpha}$ excretion.

In a recent publication [14] urinary PGE_2 excretion was found to change parallel to the diurnal changes in water and sodium excretion. Urine flow and sodium excretion were low during the night and increased after noon [15]. In this work $PGF_{2\alpha}$ excretion did not exhibit diurnal rhythm but remainded rather stable throughout the day.

The human semen and menstrual fluid contain large quantities of $PGF_{2\alpha}$. Urine was therefore not collected during menstruation and the first millilitres of voided urine were also discarded to avoid contamination with genital secretion. This is also important in males, where $PGF_{2\alpha}$ from the seminal fluid and the accessory sexual glands could give spuriously high values in urine. For urine collected in this way between 8 and 12 a.m. similar concentrations of $PGF_{2\alpha}$ were found in men, women and children. This indicates that the contribution of $PGF_{2\alpha}$ from the seminal fluid to the urine could be of little importance under these conditions.

For duplicate analysis the method had a coefficient of variation of about 12%. This variation is comparatively high for a mass fragmentographic analysis. There are several reasons for the low precision: the levels of $PGF_{2\alpha}$ in urine are relatively low, only a few ng/ml and the recovery after extraction and purification on TLC was only 35%. Although the mass fragmentograms showed stable baselines with little background interference, the high coefficient of variation indicates the appearance of background noise from other compounds in the $PGF_{2\alpha}$ peaks. The background contribution from the protium form was of minor importance (1%), i.e. 0.5 μg $[^2H_4]PGF_{2\alpha}$ added to 20 ml urine contributed with 0.25 ng/ml. For a rapid and convenient method, a variation of 12% can often be accepted.

Many radioimmunoassays for primary PGs or PG metabolites in urine have been reported [16, 17]. Data on the concentration of PGs in urine or in other body fluids obtained by radioimmunoassays differ [16]; it is therefore important to validate radioimmunoassays by other methods. This is emphasised by Gill et al. [18], who found PGE_2 in human urine to be considerably higher by radioimmunoassay than by GC—MS. One application of the present method could therefore be to validate radioimmunoassays of $PGF_{2\alpha}$ in urine.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council (Nr. 2211), from Karolinska Institutets Fonder and from Svenska Läkaresällskapet. Dr Udo Axen at the Upjohn Company kindly supplied the prostaglandins and the deuterated $PGF_{2\alpha}$, which made this study possible.

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CHROMBIO. 309

ESTIMATION OF DISACCHARIDES IN PLASMA AND URINE BY GAS—LIQUID CHROMATOGRAPHY

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(Received November 15th, 1978)

SUMMARY

A technique for the estimation of disaccharides in plasma and urine using gas—liquid chromatography is described. The procedure involves the formation of trimethylsilyl derivatives followed by injection of the reaction mixture directly onto the column. The method is precise, linear over a wide range and gives recoveries of 93—99%. The limit of sensitivity is 80 μg per 100 ml, but with modification of the volumes used, levels of 40 μg per 100 ml may be quantitated.

INTRODUCTION

It is well known that small amounts of dietary disaccharide cross the intestinal wall intact and are excreted in the urine [1—3]. Abnormally high urinary excretion of disaccharide has been reported in patients suffering from various diseases of the gastrointestinal tract [4, 5], the explanation for which must be increased absorption. This has been postulated to be due to impaired disaccharide hydrolysis, increased permeability of the damaged intestinal wall, or a combination of both mechanisms [5].

Disaccharides which resist hydrolysis in the normal human small intestine have been used as probe markers of permeability in several studies [6—9], quantitative paper chromatography [10] being used to estimate the urinary excretion of sugars following the ingestion of oral loads.

Since the first report of gas—liquid chromatography of carbohydrates [11], numerous reports have appeared describing applications in the field of carbohydrate chemistry, but comparatively few on the adaptation of the technique for biomedical purposes. The present method was developed to estimate the concentrations of disaccharide appearing in plasma following oral loading, in addition to measuring levels in urine.

MATERIALS

Sugars

Cellobiose (4-*o*-β-D-glucopyranosyl-D-glucopyranose), lactose (4-*o*-β-D-galactopyranosyl-D-glucopyranose), maltose (4-*o*-α-D-glucopyranosyl-D-glucopyranose), melibiose (6-*o*-α-D-galactopyranosyl-D-glucopyranose) and sucrose (α-D-glucopyranosyl-β-D-fructofuranose) were supplied by BDH (Poole, Great Britain). Gentiobiose (6-*o*-β-D-glucopyranosyl-D-glucopyranose), palatinose (6-*o*-α-D-glucopyranosyl-D-fructofuranose), trehalose (1-*o*-α-D-glucopyranosyl-α-D-glucopyranose) and turanose (3-α-D-glucopyranosyl-D-fructofuranose) were obtained from Sigma (London) (Poole, Great Britain). Lactulose (4-*o*-β-D-galactopyranosyl-D-fructofuranose) was obtained from Philips-Duphar (Weesp, The Netherlands).

Chemicals

Zerolit DM-F and hydroxylamine chloride (AnalaR) were obtained from BDH. Hexamethyldisilizane, trimethylchlorosilane, dimethyldichlorosilane, N,O-bis(trimethylsilyl)acetamide, bis(trimethylsilyl)trifluoroacetamide and methoxyamine hydrochloride were obtained from Applied Science Labs. (Twickenham, Great Britain). Analytical grade solvents were used apart from pyridine which was refluxed over anhydrous potassium hydroxide and redistilled before use.

Stationary phases

Pre-coated stationary phases on Gas-Chrom Q (80—100 mesh) or Chromosorb W HP were purchased. OV-1, OV-17, SE-30 and SE-52 were obtained from Phase Separations (Queensferry, Great Britain). QF-1, XE-60, Poly 101A and ASI 50 phenyl 50 cyanopropyl were supplied by Applied Science Labs.

Gas chromatograph

A Pye 104 dual column gas chromatograph with flame ionisation detectors and heated injection ports and detectors was obtained from Pye Unicam (Cambridge, Great Britain). The amplifier attenuation used was 10×10^2, the backing-off range $0—10^{-8}$. Argon was used as the carrier gas with a flow-rate of 55 ml/min. The flow-rate for hydrogen was 60 ml/min and for hydrocarbon-free air, 500 ml/min. The detector temperature was 350°. The injector temperature was 20° above that of the column.

Integrator

A Hewlett-Packard 3380A integrator was obtained from Hewlett-Packard (Wokingham, Great Britain). The slope sensitivity used was 0.1 mV/min.

Columns

Empty coiled glass columns (1.5 m and 2.7 m × 6 mm O.D.) were purchased from Pye Unicam, and silylated before use with 5% dimethyldichlorosilane in toluene. After filling with coated support to a level above the injector site, a silylated glass yarn plug was placed on top of the packing. The columns were conditioned by passing carrier gas at a flow-rate of 30 ml/min and increasing

the temperature by 50° every 2 h until the final temperature was reached. This was 15° above the maximum temperature to be used during analysis and was maintained for 24—48 h, or until a stable baseline was achieved. During conditioning the columns were not connected to the detector.

EXPERIMENTAL

Selection of stationary phases

For most stationary phases Gas-Chrom Q (80—100 mesh) was used as the support although Chromosorb W HP was satisfactory. In general, 3% phases were used but this was increased to 10% for OV-17. The length of columns used was 1.5 m for OV-1 and SE-30; 2.7 m for the other stationary phases. SE-30 and SE-52 gave similar qualitative separations to OV-1 and OV-17 respectively. As the latter phases were more thermostable they were used in preference. OV-1 and OV-17 were stable to injected reagents, symmetrical peaks with minimal tailing being obtained. Poly 101A and ASI 50 phenyl 50 cyanopropyl gave satisfactorily shaped peaks but were not as stable as OV-1 and OV-17. There was some tailing of peaks with QF-1 and XE-60. Of the phases tested OV-1 and OV-17 appeared to be the most suitable for the measurement of disaccharides. The operating temperature for each phase is given in Table I.

Derivatisation procedures

All derivatisations were performed in glass Excelo centrifuge tubes.

Trimethylsilylation. Three derivatising reagents were evaluated: (i) hexamethyldisilazane (HMDS) with trimethylchlorosilane (TMCS) as catalyst, (ii) bis(trimethylsilyl)acetamide (BSA) with TMCS as catalyst, and (iii) bis(trimethylsilyl)trifluoroacetamide (BSTFA).

All three reagents were used with redistilled pyridine as solvent. HMDS—TMCS—pyridine formed an insoluble precipitate, presumably ammonium chloride, which if injected directly onto the column led to the accumulation of deposits at the injection site. In addition, derivatives formed from plasma using this reagent were unstable because traces of sulphosalicylic acid, which was used to precipitate proteins, remained after desalting, and caused breakdown of products. BSA—TMCS—pyridine formed a clear reagent and derivatives formed from plasma were stable for several days if kept in airtight glass tubes. BSTFA, which incorporates halogen groups and should be self-catalysing was not as effective as BSA—TMCS unless TMCS was added, presumably to act as a more efficient catalyst. All three reagents produced derivatives with the same retention characteristics. BSA—TMCS—pyridine was used for further studies. Although BSTFA—TMCS—pyridine was equally effective, it was more expensive. If pyridine was omitted crystallisation of reaction products occurred, particularly when urine was being analysed.

Conditions of derivatisation were assessed by two methods:

(a) By comparing the peak area of derivatised test sugar to that of a non-derivatised hydrocarbon with a suitable retention time (octadecane, 100 mg/100 ml).

(b) By thin-layer chromatography (TLC). Reaction mixtures were spotted on

TABLE I

SEPARATION OF TMS–DISACCHARIDE DERIVATIVES USING VARIOUS STATIONARY PHASES

Retention times relative to that of turanose are given.

	OV-1 (220°)	OV-17 (255°)	Poly 101A (240°)	ASI 50 phenyl 50 cyanopropyl (230°)	QF-1 (230°)	XE-60 (230°)
Cellobiose	0.92, 1.32	0.94, 1.22	0.93, 1.42	1.04, 1.45	0.91, 1.27	0.91, 1.27
Gentiobiose	1.88	1.79	2.30	1.79	1.70	1.96
Lactose	0.76, 1.18	0.86, 1.13	0.86, 1.39	0.97, 1.39	0.83, 1.22	0.85, 1.27
Lactulose	0.76	0.73	0.76, 0.79	0.89, 0.97	0.82	0.83
Maltose	0.89, 1.03	0.94, 1.22	0.76, 0.86, 1.06	0.96, 1.06	0.01, 1.11	0.90, 1.00
Melibiose	1.58, 1.74	1.63*	1.77, 2.06	1.80, 1.96	1.47, 1.57	0.90, 1.00
Palatinose	1.07, 1.13	1.12	0.91, 1.09	1.16, 1.28	1.16	1.02, 1.09
Sucrose	0.79	0.81	0.70	0.74	0.80	0.77
Trehalose	1.04	1.10	1.11	1.02	1.00	1.00
Turanose	1.00 (16 min)	1.00 (21 min)	1.00 (8 min)	1.00 (11 min)	1.00 (9 min)	1.00 (9 min)

*Marked shoulder on the ascending limb of the peak.

silica gel plates (Merck, Darmstadt, G.F.R.) and developed in toluene. Chromatograms were located by spraying with 4-aminobenzoic acid reagent (0.7 g 4-aminobenzoic acid and 0.4 ml 88% orthophosphoric acid in 100 ml methanol).

The most effective derivatising reagent included BSA and TMCS in a ratio of 1:1, maximum derivatisation being obtained with wide variations in the relative volume of pyridine. The volume of reagent was minimised for plasma estimations to ensure maximum sensitivity, 50 μl pyridine—BSA—TMCS (2:1:1) being used. For urine 200 μl pyridine—BSA—TMCS (5:1:1) was used.

For lactulose, the peak area of derivatised sugar was maximal within 45 min of adding reagent if kept at room temperature, or 20 min if incubated at 60°. A single spot was obtained on TLC which was widely separated from native lactulose. Recovery experiments showed derivatisation was quantitative.

Oxime and methyl oxime formation combined with trimethylsilylation. For oxime formation, the method described by Sweeley et al. [12] was followed. A solution of hydroxylamine hydrochloride in pyridine (6 mg/ml) was prepared, 100 μl of which was added to 1 mg test sugar (lactose). After heating at 70° for 30 min, silylating reagent was added until a clear solution was obtained, 700 μl usually being required. TLC studies showed oxime formation to occur within 10 min and oxime—TMS formation to occur within 2 min.

For methyl oxime—TMS formation a modification of the method described by Horning et al. [13] was used. Methoxylamine hydrochloride (0.5 ml, concentration 20 mg/ml in pyridine) was added to 1 mg test sugar. After heating at 70° for 30 min silylating reagent was added until a clear solution was obtained, 500 μl usually being required. Assessed by TLC, methyl oxime formation occurred within 5 min and methyl oxime—TMS formation within 10 min.

Unlike the formation of TMS—disaccharides, recovery experiments showed that oxime—TMS and methyl oxime—TMS derivatisations were not quantitative.

Internal standard

Turanose was used as an internal standard after it was demonstrated that this disaccharide was absent from the urine and plasma of subjects on a normal diet. TMS—turanose gave a single peak when chromatographed on all stationary phases tested.

Quantitative procedure

To 1 ml plasma was added 1 ml internal standard solution (10 mg/100 ml) and 1 ml sulphosalicylic acid (7 g/100 ml). After centrifugation the supernatant was desalted by adding Zerolit DM-F, which was in the H^+ $acetate^-$ state. Resin was added to occupy 50% of the volume of the mixture. The samples were shaken for 3 min and then centrifuged. One millilitre supernatant was transferred to a glass centrifuge tube in which the sample was dried at 50° under a stream of air. When dry the tubes were placed in an evacuated phosphorus pentoxide-containing desiccator for at least 30 min. After the addition of 50 μl derivatising reagent the tubes were closed with ground glass stoppers and placed in an incubating oven at 60° for 20 min. One microlitre of resulting solution was injected directly onto the chromatograph.

Urine was treated in a similar manner except that the protein precipitation stage was omitted and a derivatising reagent of differing proportions and amount (see above) was used. Aqueous standards of appropriate concentration were prepared for each batch of specimens. It was not necessary to add sulphosalicylic acid or desalting resin to standards.

RESULTS

Relative retention times of disaccharides

Relative retention times of disaccharides were calculated by analysing aqueous solutions of sugars (100 mg/100 ml) as urines. The results, given in Table I, are expressed as the retention time of test disaccharide relative to that of turanose, for which the retention time in minutes is given. Two peaks, presumably α- and β-anomers were obtained for reducing disaccharides. For lactulose and palatinose, the two peaks coincided on several of the stationary phases tested.

Quantitative studies

Quantitative estimations of lactulose, lactose and sucrose were performed using a 2.7 m 10% OV-17 column; for melibiose a 1.5 m 3% OV-1 column was used.

Precision: comparison of methods of standardisation

A known amount of lactulose was added to plasma which was shown to be free of disaccharide. A batch of 21 samples was prepared (lactulose concentration 17.3 mg/100 ml) and the results of analysis calculated by internal and external standardisation techniques. Peaks were quantitated by peak height, peak area and peak height × width at half height measurement. The results are shown in Table II. The coefficient of variation was less than 6% if internal

TABLE II

COMPARISON OF PRECISION OF METHODS OF QUANTITATION

Lactulose in plasma was measured. Twenty-one samples were analysed, mean value 17.28 mg/100 ml.

	Internal standardisation (coefficient of variation, %)	External standardisation (coefficient of variation, %)
Peak height	3.8	36.5
Peak area	2.7	28.0
Peak height × width at half height	5.5	32.0

standardisation was used, but greater than 28% with external standardisation. The most precise method was peak area measurement using internal standardisation. This method was used for quantitation unless partially overlapping peaks were obtained, when peak heights were measured (see below). Precision studies for lactulose in plasma at a level of 2 mg/100 ml (13 samples) gave a coefficient of variation of 1.8%.

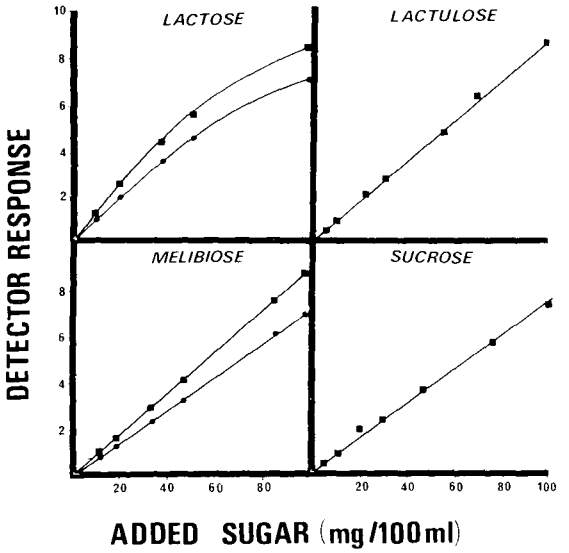

Fig. 1. Linearity of various disaccharides calculated by adding varying amounts of test sugar and constant amounts of internal standard to blank urine.

Linearity

The linearity of the method was tested for lactulose, lactose, melibiose and sucrose. Varying amounts of test sugars were added to urine, the concentration of internal standard being constant. For sugars forming two derivatives each peak was quantitated with reference to the equivalent anomer in the calibration standard, the concentration of which was in the range 15—30 mg/100 ml. The results obtained are shown in Fig. 1. All estimation were linear up to 100 mg/100 ml except lactose, which was linear up to 50 mg/100 ml.

Sensitivity

Sugar concentrations of 80 μg/100 ml were measured in plasma using the method described, with peak height measurement. By increasing the volume of plasma used to 2 ml, levels of 40 μg/100 ml were estimated.

TABLE III

RECOVERY OF DISACCHARIDES FROM PLASMA AND URINE

Sugar	Fluid	Weighed amount (mg/100 ml)	Calculated concentration (mg/100 ml)	Recovery (%)
Lactose				
2nd peak	urine	50.8	47.1	92.8
1st peak			44.0	86.6
Lactulose	urine	21.5	20.7	96.3
Lactulose	plasma	2.02	1.995	98.8
Melibiose				
2nd peak	urine	18.4	17.7	96.2
1st peak			14.0	76.0
Sucrose	urine	20.7	19.4	93.7

Recovery

Recovery of sugars relative to that of internal standard was determined by adding known amounts to urine or plasma, and comparing the concentration estimated with the amount added. The results are shown in Table III. For lactose and melibiose one of the derivatives formed gave a lower percentage recovery than the other. This was noted in analysis of plasma and urine but not of aqueous samples. Increasing the temperature or time of derivatisation did not increase the recovery of these anomers. If these derivatives were excluded, recoveries varied between 92.8 and 98.8%.

Interferences

No non-sugar peaks were found in plasma or urine with the same retention time as disaccharides. When plasma was analysed, a peak was noted with a retention time of approximately 90 min using the OV-17 column, a similar retention time being obtained for authentic cholesterol. This interfered with quantitation of sugars but was overcome if the column was heated to 50° above the normal operating temperature for 10 min after two injections had been made. This cleared the column for the next two analyses.

Disaccharides of interest are shown in a chromatogram of urine in Fig. 2, the

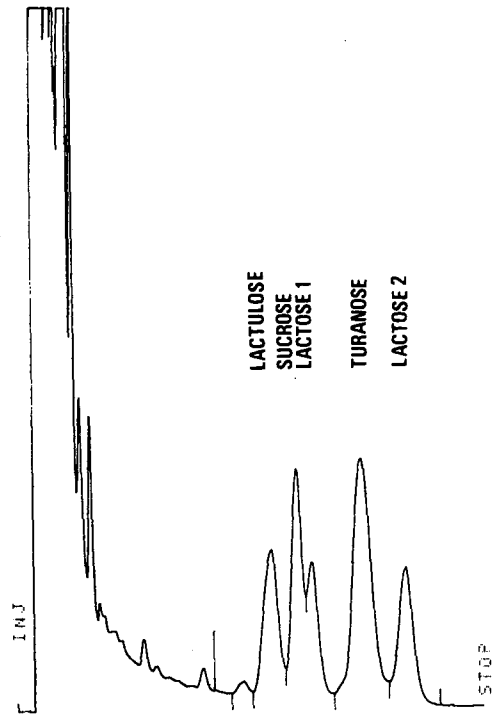

Fig. 2. Disaccharides of interest in a chromatogram of urine.

sugars having been added to blank urine. There was partial overlapping of several peaks. To test the effect of possible sucrose interference on lactulose quantitation, increasing amounts of sucrose were added to urine containing a

TABLE IV

EFFECT OF INCREASING AMOUNT OF SUCROSE ON LACTULOSE ESTIMATION IN URINE

Sucrose added (mg/100 ml)	Lactulose measured	
	by peak area (mg/100 ml)	by peak height (mg/100 ml)
0	11.7	11.7
10	9.6	11.2
30	9.1	11.2
50	10.3	11.3
75	11.3	11.3
100	10.2	11.4

constant amount of lactulose, the results being given in Table IV. Quantitation using peak area measurement was less accurate than if peak height was used.

DISCUSSION

Reports of GLC techniques for the determination of carbohydrates have mostly concentrated on three types of derivatisation: methylation, acetylation and trimethylsilylation. The first report of GLC of carbohydrates involved the formation of methyl derivatives of monosaccharides [11], the technique later being extended to the separation of di- and trisaccharides [14]. The procedures used are too cumbersome to cope with large numbers of specimens. Methods based on acetylation techniques have been used to measure monosaccharides [15] and sugar alcohols [16] in biological specimens. In this study, trimethylsilylation is preferred to acetylation for estimating disaccharides as the derivatives formed are more volatile, leading to shorter retention times, and columns can be operated at lower temperatures with less baseline noise. In addition, TMS—monosaccharide derivatives give sharper peaks than acetylated monosaccharides [17]. These factors result in improved sensitivity.

The formation of TMS derivatives of monosaccharides, disaccharides and sugar alcohols was described by Sweeley et al. [12] who used pyridine—HMDS—TMCS as the derivatising reagent. Methods based on this procedure have been described for the estimation of sugars in urine [18] and blood [19]. A disadvantage of these methods is that ammonium chloride is precipitated which will lead to column contamination if the reaction mixture is injected directly onto the chromatograph. This may be overcome by using an additional stage, the extraction of silyl derivatives from reaction mixture by hexane [19]. The present procedure, using pyridine—BSA—TMCS for derivatisation, has the advantage that no precipitate is formed and an extraction stage is unnecessary. In addition, derivatives formed from plasma are more stable than if pyridine—HMDS—TMCS is used.

A major potential disadvantage of the present technique is that reducing sugars give rise to two peaks, presumably due to α- and β-anomers. This might lead to lower precision because of variable anomerisation, reduced sensitivity and a greater chance of interferences because of an increased number of peaks.

However, for some disaccharides stationary phases may be selected on which anomers coincide, e.g., a single peak for lactulose is obtained on OV-1 and OV-17. For lactose and melibiose the recovery of disaccharide added to urine is less than 90% if quantitation is based on the peak with the shorter retention time. This is not due to variable anomerisation as the recovery of the equivalent later peak does not exceed 100%. Similar results are obtained with plasma but not with aqueous solutions. It is possible that an inhibitor is present in biological specimens that preferentially inhibits the derivatisation of one anomer type. If quantitation is based on the more completely recovered anomer, trimethylsilylation is satisfactory as a technique for the measurement of disaccharides.

In theory, the formation of oxime—TMS and methyl oxime—TMS derivatives would be an advantage as it should overcome the problem of double derivatives due to anomerisation. However, such derivatisations of disaccharides are not quantitative and multiple derivatives are formed.

The present technique improve on the sensitivity of some previous methods [15, 18] and uses smaller sample volumes than others [19]. Lactulose may be measured without the time consuming preliminary paper chromatographic stage used by Müller et al. [20]. It also has the advantage that plasma and urinary disaccharides may be measured using a single derivatising procedure, unlike the method described by Nakamura and Tamura [21].

ACKNOWLEDGEMENTS

Thanks are due to Professor R.V. Brooks for helpful advice. Equipment was purchased with money granted by the Research (Endowments) Committee of St. Thomas' Hospital. This work forms part of an M.D. thesis which has been submitted to the University of London.

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Journal of Chromatography, 163 (1979)19—28
Biomedical Applications

CHROMBIO. 311

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ACID AND ALKALINE PHOSPHATASE IN SERUM

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(Received October 23rd, 1978)

SUMMARY

High-performance liquid chromatography was used to assay serum acid and alkaline phosphatase. Samples were incubated with adenosine-5'-monophosphoric acid (AMP) in a buffer of required pH, 5'-nucleotidase was inhibited with Ni^{2+} ions, and the phosphatase activity was determined by measuring the concentration of the reaction product, adenosine. The analysis time, after the incubation is terminated, is short (7 min), and the assay is quantitative and reproducible. Complete separation of the reaction product from the substrate and the naturally occurring serum constituents and the high sensitivity of the ultraviolet detection system eliminate some of the problems commonly encountered in spectrophotometric assays.

INTRODUCTION

Enzymatic assays play a prominent role in medicine and they have been recognized as routine tests to assist in interpreting pathological conditions [1, 2]. Serum enzymes have been increasingly used for these purposes in recent years and there is a great demand for simple assay for measuring enzyme activities accurately and rapidly in the clinical laboratory. This is particularly important since certain enzymes are used as early indicators of neoplasia and as management aids in following the progression of a disease.

Phosphatases are organ-specific enzymes which catalyze the hydrolytic cleavage to their pH optima. Alkaline phosphatase (EC, 3.1.3.1, orthophosphoric acid monoester phosphohydrolase, AP), with optimal activity at a pH of about 10, occurs in practically all tissues of the body, especially in the cell membranes, intestinal epithelium, kidney tubules, osteoblasts, liver and placenta [3].

Determination of serum alkaline phosphatase activity is of interest in several disease states. Elevated levels of alkaline phosphatase have been observed in patients with osteogenic sarcoma [4], parathyroid adenoma or carcinoma [5] and cancer metastatic to the bone [6]. Abnormal levels of alkaline phosphatase have also been found in serum of patients with viral hepatitis or cirrhosis [7] and in diseases which do not involve the liver or bone such as stage I or II Hodgkin's disease [8], myeloid metaplasia [9], congestive heart failure [9] and intra-abdominal infections [9].

Acid phosphatase (EC 3.1.3.2, orthophosphoric acid monoester phosphohydrolase, AP) includes a group of similar or related enzymes with optimal activity below a pH of 7.0.

Serum acid phosphatase elevations have been observed in a variety of diseases, such as carcinoma of the prostate [10], Gaucher's disease [11] and breast carcinoma [12]. Because of the great clinical significance and potential use of serum acid and alkaline phosphatase assays, chemists are constantly searching for better, more rapid methods for the determination of the activity of these enzymes.

Because of the presence of 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5., 5′-N) in serum, its activity must be inhibited prior to the assay for acid and alkaline phosphatase. This can be achieved by selective inhibition with Ni^{2+} ions [13]. Methods generally used for assaying phosphatases monitor as an index of activity the amount of phosphate liberated from a substrate or the absorbance of 4-nitrophenol liberated in alkaline solutions at a wavelength of 400—420 nm [14, 15]. We investigated the use of high-performance liquid chromatography (HPLC) for determining concentrations of alkaline and acid phosphatase in biological samples because the method is more selective than spectral techniques and quicker and easier to carry out than radiochemical assays.

EXPERIMENTAL

Reagents

Adenosine-5′-monophosphoric acid (AMP), adenosine, alkaline phosphatase (EC 3.1.3.1, Type I), acid phosphatase (EC 3.1.3.2, Type I), 5′-nucleotidase (EC 3.1.3.5, Grade V), and tris(hydroxymethyl)aminomethane (Trizma base) were purchased from Sigma (St. Louis, Mo., U.S.A.); reagent-grade sodium citrate and diethanolamine were from J.T. Baker (Phillipsburg, N.J., U.S.A.); methanol distilled in glass was from Burdick and Jackson (Muskegon, Mich., U.S.A.); and potassium dihydrogen phosphate was from Mallinckrodt (St. Louis, Mo., U.S.A.). Water used for the preparation of eluents and standard solutions was distilled—deionized and filtered through Millipore membrane filters, pore size 0.45 μm (Millipore, Bedford, Mass., U.S.A.).

Instrumentation

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 liquid chromatograph, equipped with solvent delivery system and a Model U6K universal injector were used in all determinations. An SF 770 Spectroflow monitor with a deuterium lamp, SF 339 wavelength drive and MM 700 memory module (Kratos,

Schoeffel Instrument Division, Westwood, N.J., U.S.A.) were used for the detection and stopped-flow ultraviolet (UV) scanning. A prepacked RP 8 Knauer column, particle size 7.0 μm (4.6 mm I.D. × 10.0 mm O.D., 250 mm long), was obtained from Unimetrics (Anaheim, Calif., U.S.A.).

Separation conditions

Samples were analyzed using an isocratic elution mode of reversed-phase partition HPLC. The eluent was anhydrous methanol—0.02 *M* KH_2PO_4 (pH 5.5) (1:9, v/v). Solvents were filtered through Millipore membrane filters, Type HA, pore size 0.45 μm, and degassed under vacuum. The flow-rate was 1.5 ml/min, and the temperature was ambient for all determinations.

Sample preparation

Serum samples were obtained from freshly drawn blood collected in tubes without anticoagulant. The blood was allowed to clot spontaneously for 10—15 min at room temperature. The supernatant fluid was then spun down at 630 *g* for 10 min, the liquid withdrawn and filtered through Millipore membrane filters (pore size 0.22 μm).

Enzyme assays

For the assay of serum acid and alkaline phosphatase, 0.1 ml of a 0.1 *M* solution of nickel chloride was added to 0.3 ml of serum to inhibit the activity of 5′-nucleotidase, and the mixture was incubated for 5 min at 25°. Then 0.8 ml of either 0.1 *M* diethanolamine buffer (pH 9.8), or sodium citrate buffer (pH 4.8) was added. The mixture was then incubated with 1.0 ml of 0.1 *M* AMP solution for 25 min at 25°. The reaction was stopped by heating the sample in boiling water until the protein coagulated. Samples were then spun down and the supernatant liquid was withdrawn and filtered through a Millipore filter, pore size 0.22 μm. The pH of the incubated samples was adjusted to 7.0 with solid Trizma base prior to chromatography.

Peak identification and confirmation of purity

In order to confirm the identity and the purity of the adenosine peak in incubated samples, UV spectra of the peaks were obtained using a stopped-flow UV scanning technique [16]. These spectra were scanned from 220 to 300 nm. To correct for the spectral background arising from changing optical properties of flow-cells and the monochromator, a blank spectrum was scanned over the same wavelength range and stored in the memory unit. This background was later subtracted from the scan of the compound under investigation. Next, the samples were chromatographed, the flow stopped at the top of the peak with retention time of adenosine, and the corrected spectrum scanned. These spectra were compared with the spectrum of standard adenosine. Good agreement between the spectra was taken as an indication of the purity of the peak.

RESULTS AND DISCUSSION

Because of the presence of adenosine deaminase in serum, adenosine is usually not found in detectable quantities. In order to ensure this, a blank serum sample was always chromatographed prior to the enzymatic assay. For the analysis of adenosine, a rapid, reversed-phase HPLC analysis developed by Hartwick and Brown [17] was used. This isocratic elution mode provides a fast separation (6—7 min) of adenosine from all the naturally occurring serum constituents.

Because of the presence of adenosine deaminase in serum, the adenosine which is produced from the AMP may be converted to inosine. Thus the levels of adenosine may be decreased by catalysis by adenosine deaminase. Therefore, experiments were conducted to determine the activity of adenosine deaminase at the two pH levels used in the assay of acid and alkaline phosphatase. Due to the low activity of adenosine deaminase in serum at pH 4.8 and 9.8 and the unfavorable kinetics at low concentrations of adenosine, it was found that the levels of inosine formed were insignificant. Therefore, no corrections for the adenosine deaminase activity were necessary.

Because of the presence of 5′-N in serum, an enzyme which specifically catalyzes the dephosphorylation of nucleotides having phosphate groups attached to the C_5 position on the ribose ring, Ni^{2+} ions were added to inhibit activity of 5′-N. Prior to the assay of acid and alkaline phosphatase, experiments were carried out to determine whether the presence of Ni^{2+} ions would have any effect on the activities. Therefore, solutions of 5′-N and acid and alkaline phosphatase were incubated with AMP in the absence and presence of Ni^{2+}, under the conditions of the assay. Incubation of 5′-N with AMP at a pH of 4.8 (sodium citrate buffer) in the absence (Fig. 1a) and in the presence of Ni^{2+} (Fig. 1b) indicated that this enzyme is completely inhibited in the presence of Ni^{2+}. At the same time, acid phosphatase assays in the absence (Fig. 1c) and in the presence of Ni^{2+} (Fig. 1d) showed no loss in the acid phosphatase activity in the presence of Ni^{2+} inhibitor.

The influence of Ni^{2+} ions on the enzymes under study was also tested at a pH of 9.8 (diethanolamine buffer). Results showed that 5′-N is completely inhibited by Ni^{2+} at pH 9.8. It should be noted that whereas the order of addition of reagents in the assay is not critical at pH 4.8, under alkaline conditions Ni^{2+} must be added to serum before the addition of diethanolamine buffer (pH 9.8), otherwise the Ni^{2+} ions would be removed in the form $Ni(OH)_2$. Under the same conditions at pH 9.8, incubation of alkaline phosphatase with AMP in the absence and presence of Ni^{2+} indicated no loss in the activity of this enzyme upon addition of the inhibitor of 5′-N.

To ensure that Ni^{2+} ions would have the same effect on serum enzymes, experiments were also conducted on serum samples. In testing the procedure, first a blank serum sample (no AMP added) was incubated for 25 min at 25°, the protein precipitated by heating, and the filtered sample chromatographed under the standard conditions (Fig. 2a). This procedure also indicated whether the sample itself contained any adenosine or any other compound with a similar retention time. Another blank mixture containing AMP and distilled water instead of serum was incubated under the same conditions. Fig. 2b

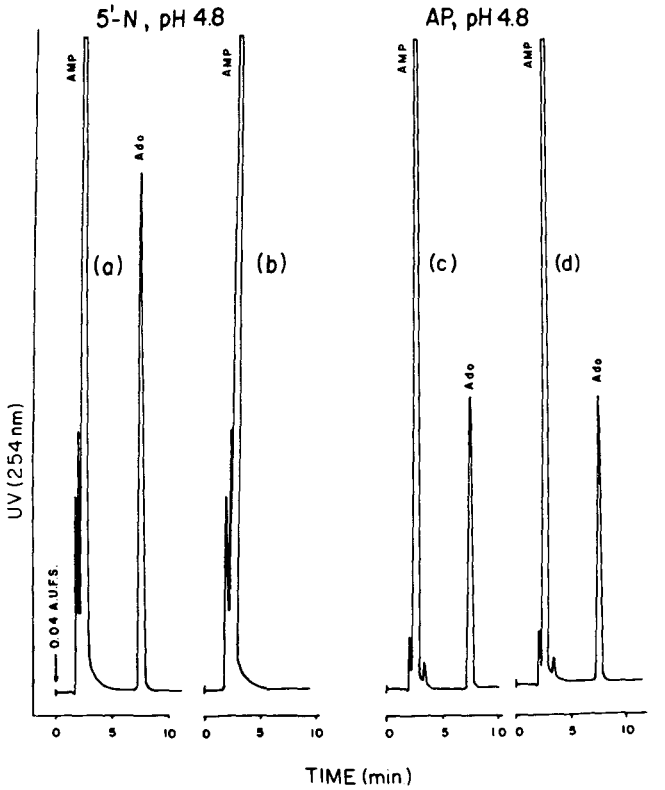

Fig. 1. Chromatograms of a mixture of AMP buffered to pH 4.8 and incubated (a) with 5'-N in the absence of Ni^{2+}, (b) in the presence of Ni^{2+}, (c) with AP in the absence of Ni^{2+}, and (d) in the presence of Ni^{2+}. Chromatographic conditions: mobile phase, anhydrous methanol—0.02 *M* KH_2PO_4 (pH 5.5); flow-rate, 1.5 ml/min; temperature, ambient. Sample volume, 75 µl.

shows a chromatogram of the mixture of AMP and a diethanolamine buffer (pH 9.8). The concentration of adenosine resulting from the hydrolysis of AMP under the alkaline conditions is negligible compared to the adenosine liberated from the alkaline phosphatase cleavage. Fig. 2c and d show the chromatograms of serum samples incubated with AMP in the absence and presence of Ni^{2+}, respectively. Little or no difference in the adenosine content of the two samples reflects the lack of 5'-N activity.

To confirm the spectral purity of adenosine resulting from the enzymatic cleavage of AMP, UV spectra of the adenosine peak in the incubated samples were obtained using the stopped-flow UV scanning technique. Fig. 3 shows a comparison of a UV spectrum of adenosine standard with that of the adenosine peak in serum sample incubated with AMP. Close agreement between the spectra confirms the identity and the purity of the peak in serum.

For the determination of serum acid and alkaline phosphatase, sera from seven healthy, normal subjects were incubated with AMP in buffer solutions of pH 4.8 and 9.8, respectively. Ni^{2+} ions were added to ensure the inhibition of the 5'-N activity. The activities were calculated using the formula

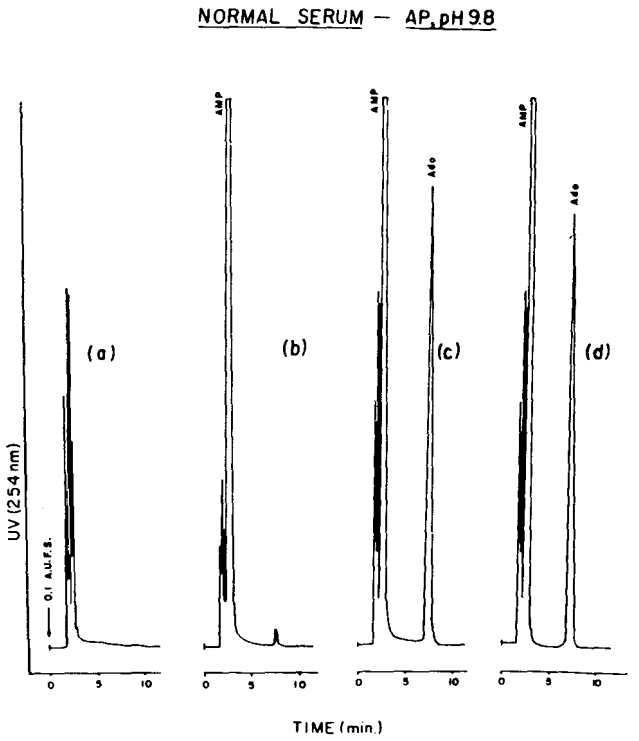

Fig. 2. Chromatograms of (a) normal serum sample blank, (b) AMP solution buffered to pH 9.8 and incubated for 25 min at 25° with AMP, (c) normal serum sample buffered to pH 9.8 and incubated with AMP in the absence of Ni^{2+} and (d) normal serum sample buffered to pH 9.8 and incubated with AMP in the presence of Ni^{2+}. Chromatographic conditions are the same as in Fig. 1; sample volume 25 μl in each case.

$$\text{activity (U/l)} = \frac{(\text{Ado}_{(s)} - \text{Ado}_{(b)}) \times \text{sample volume (ml)} \times \text{volume injected } (\mu\text{l})}{\text{serum volume (ml)} \times \text{response factor}_{(\text{Ado})}}$$

where $\text{Ado}_{(s)}$ and $\text{Ado}_{(b)}$ are the areas of adenosine peaks in the incubated serum sample and the blank, respectively, the blank containing AMP, buffer and water instead of serum; the response factor is area per μg of adenosine standard. The sample volume is the total volume of the incubation mixture, and serum volume is the volume of serum incubated.

Using the described procedure, the average of the seven normal sera gave consistent results of 5.11 ± 0.202 U/l for the acid phosphatase, and 150.16 ± 1.50 U/l for the alkaline phosphatase (Table I). The values compare well to the normal values obtained by other methods [18, 19]. Fig. 4 is a comparison of the chromatograms of three serum samples incubated with AMP in the presence of Ni^{2+} at pH 4.8; (a) is a chromatogram of a normal serum sample, (b) is of a serum sample from a patient suffering from cirrhosis, and (c) is of a serum sample from a patient with hepatitis. As can be seen from Fig. 4, there are small but definite differences in the activity of acid phosphatase in the three serum samples. Larger differences were noticed in the alkaline phosphatase activity in the same three samples. Fig. 5 shows the chromatograms

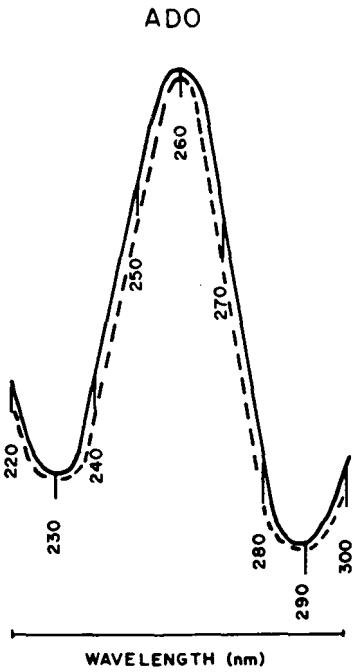

Fig. 3. Comparison of the corrected UV spectra of (——) adenosine standard and (— — —) adenosine peak in serum sample incubated with AMP, obtained using the stopped-flow UV scanning technique. Scanning rate, 100 nm/min; UV range, 0.1 a.u.f.s.

TABLE I

SERUM ACID AND ALKALINE PHOSPHATASE ACTIVITIES OF SEVEN HEALTHY SUBJECTS

Sample No.	Acid phosphatase activity (U/l)	Alkaline phosphatase activity (U/l)
1	4.92	150.1
2	5.01	151.5
3	5.20	148.9
4	5.02	150.5
5	5.20	148.6
6	5.19	151.2
7	5.20	150.3
Mean ± S.D.	5.11 ± 0.102	150.16 ± 1.50

of the three serum samples at pH 9.8, indicating alkaline phosphatase activity an order of magnitude higher in the serum from patients suffering from cirrhosis and hepatitis compared to the normal serum.

CONCLUSIONS

Results presented in this paper illustrate the utility of HPLC as a tool for the study of enzyme-catalyzed reactions. This assay has several advantages: first,

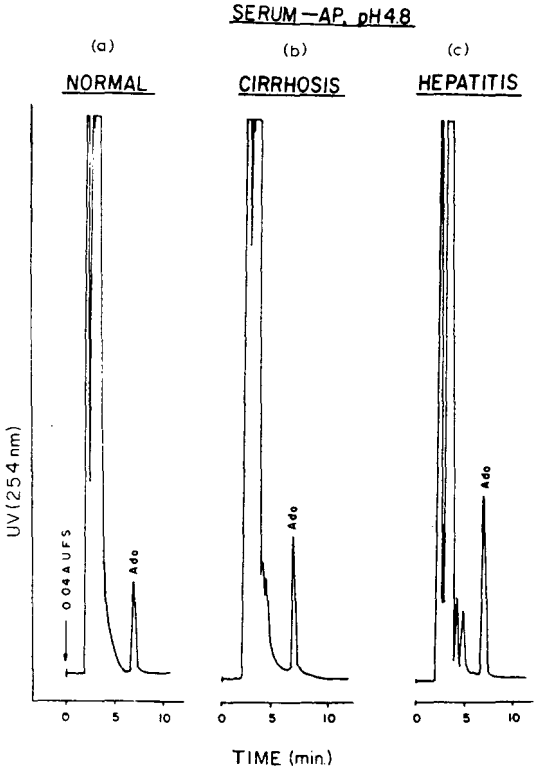

Fig. 4. Comparison of acid phosphatase activity in (a) normal serum, (b) serum sample from a patient with cirrhosis, and (c) serum sample from a patient with hepatitis. All three samples were incubated with AMP in the presence of Ni^{2+}. Chromatographic conditions are the same as in Fig. 1; sample volume, 75 μl in each case.

it is sufficiently sensitive to detect adenosine at the picomole level; secondly, the results are available within 6—7 min after the incubation and protein precipitation steps; thirdly, the reaction product, adenosine, is completely separated from the substrate or UV-absorbing serum constituents, thereby eliminating interferences often encountered in spectrophotometric or any other assays.

ACKNOWLEDGEMENTS

The authors thank Waters Associates Inc., and Kratos Inc., Schoeffel Instrument Division, for the use of their instruments, Unimetrics Corporation for their RP 8 column, Klaus Lohse for his valuable comments and Marion Barry for the preparation of the manuscript. This work was supported by Grant No. RO1 CA17603 from the National Cancer Institute of the United States Public Health Service.

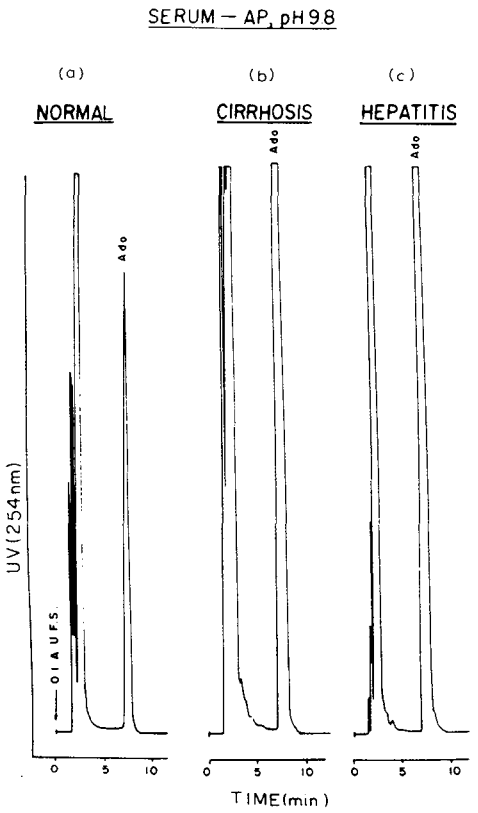

Fig. 5. Comparison of alkaline phosphatase activity in (a) normal serum sample, (b) serum sample from a patient with cirrhosis, and (c) serum sample from a patient with hepatitis. All samples were incubated with AMP in the presence of Ni^{2+}. Chromatographic conditions are the same as in Fig. 1; sample volume, 25 μl in each case.

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Journal of Chromatography, 163 (1979) 29—36
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CHROMBIO. 316

MEASUREMENT OF URINARY PYRIMIDINE BASES AND NUCLEOSIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received December 5th, 1978)

SUMMARY

A rapid procedure for the isolation, separation, identification and measurement of urinary pyrimidine bases and nucleosides by high-performance liquid chromatography (HPLC) is presented. The initial isolation of these compounds from urine was accomplished with small disposable ion-exchange columns. HPLC was performed on a silica gel column with a mobile phase composed of methylene chloride, methanol and 1 *M* aqueous ammonium formate buffer. Peaks were recorded at both 254 nm and 280 nm and the response ratio was used in conjunction with the elution volume for compound identification. The minimum detectable amount (signal-to-noise ratio = 2) ranged from 0.2 ng for uracil to 2.2 ng for cytidine. Linearity and recovery for thymine, uracil, uridine, pseudouridine, orotic acid and orotidine added to urine was demonstrated over almost a 10^3 concentration range. The potential application of this method for the study of inborn errors in the urea cycle is discussed.

INTRODUCTION

The ability to quantitatively measure the urinary excretion of certain pyrimi-

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dines is essential in the study and treatment of patients with primary or secondary hyperammonemia. The sequence of reactions leading to the formation of pyrimidines by the orotate pathway is the major source of pyrimidine components for DNA and RNA synthesis. This pathway is initiated by the enzyme carbamyl phosphate synthetase. When the urea cycle is either overloaded as a result of secondary hyperammonemia or is blocked as a result of an enzyme deficiency in the cycle itself, the excess carbamyl phosphate is available for extramitochondrial carbamyl aspartate synthetase. This is then shunted into the pyrimidine biosynthetic pathway resulting in increased urinary excretion of uracil, uridine, and orotic acid [1—3]. The availability of rapid quantitative procedures for measuring these urinary pyrimidines is useful, not only for research purposes, but also for monitoring the effectiveness of various forms of dietary therapy being used to treat patients with hyperammonemia [4].

A number of analytical methods have been used in the past to measure various urinary pyrimidines. Paper and ion-exchange chromatography combined with the measurement of UV absorption have been used for the determination of pseudouridine in normal urine [5, 6] and elevated levels of orotic acid, uracil and uridine in urine from patients with hyperammonemia [7]. Gas chromatography has been used for measuring urinary orotic acid in patients with ornithine transcarbamylase deficiency and orotic acid and orotidine in patients treated with azauridine [8]. Various colorimetric procedures have also been used for the measurement of orotic acid [9—11]. More recently various high-performance liquid chromatographic (HPLC) procedures have been reported for the separation of a number of pyrimidines and their nucleosides [12—14].

We describe here a simple procedure for the rapid separation and quantitation of a number of urinary pyrimidines using HPLC. These procedures were designed specifically for the study of urinary pyrimidines in patients with primary hyperammonemia.

EXPERIMENTAL

Instrumentation

The HPLC equipment used consisted of a Waters Assoc. (Milford, Mass., U.S.A.) Model M-6000A pump, a Model UK6 injector and a Model 440 dual wavelength, two-channel UV monitor. The UV monitor was used to simultaneously record chromatograms at both 254 nm and 280 nm on a Houston Instruments (Austin, Texas, U.S.A.) Model 5211-5 Omniscribe two-pen recorder.

Materials

All organic solvents used were distilled in glass (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Standard compounds were purchased from Sigma (St. Louis, Mo., U.S.A.) except for 2′-O-methylcytidine which was purchased from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Ion-exchange resins were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Isolation of pyrimidines from urine

A small disposable mixed-bed ion-exchange column was used to isolate a fraction containing neutral pyrimidines and another containing acidic pyrimidines from urine. The column was prepared by adding a slurry containing equal volumes of both AG 50W-X8 hydrogen form and AG 1-X8 acetate form (both 100—200 mesh) ion-exchange resins to a 5.75-in. disposable transfer pipette with a plug of glass-wool in its tip until a bed height of 1 cm was obtained. The transfer pipette was silylated before packing with 5% dimethyldichlorosilane in toluene. This was found useful for preventing the adhesion of the resin to the walls. The column was washed with 2 ml of 2 *M* acetic acid and 2 ml of distilled deionized water before use.

A 200-μl urine sample was applied to the column and collected in a screw-cap test tube. The column was eluted with two 1.0-ml portions of distilled deionized water. This fraction contained the "neutral" pyrimidines (uracil, uridine, pseudouridine, thymine, thymidine, and 5-hydroxymethyluracil) and could be used directly for HPLC analysis if elevated amounts of these pyrimidines are present. For the measurement of normal levels of uracil and pseudouridine further purification is required.

The column was next washed with two 1.0-ml portions of 10 *M* formic acid. This fraction was dried under a stream of nitrogen at 60°, redissolved in 1.0 ml of concentrated ammonium hydroxide, heated 10 min at 60° and again dried under a stream of nitrogen at 60°. The residue was dissolved in 200 μl of 20% 1.0 *M* ammonium formate buffer in methanol for injection into the HPLC column.

During recovery studies with orotidine, it was observed that when the sample from 10 *M* formic acid was dried at 60° under a stream of nitrogen, the recoveries were usually no more than 50%. It was also noted that a peak was present in the chromatograms of these samples with a retention time nearly identical to that of orotic acid but with a 254 nm to 280 nm ratio of 1.51. This was identical to the ratio for orotidine. Treatment of these samples with ammonia resulted in the disappearance of the new peak and markedly improved recoveries for orotidine. It appears that the new peak was a formate ester of orotidine. We have, therefore, incorporated the ammonolysis step into the isolation procedure in order to convert any formate ester back into the original orotidine.

Further purification of the "neutral" fraction collected from the mixed-bed ion-exchange column was accomplished in an anion-exchange column. The column was constructed as described above except that it was filled to a bed height of 0.5 cm with AG 1-XB (200—400 mesh) ion-exchange resin in the hydroxyl form. Concentrated ammonium hydroxide (200 μl) was used to adjust the previous collection to pH 11.0. This was applied to the column. The column was washed with two 1.0-ml portions of distilled deionized water and the pyrimidines eluted with two 1.0-ml portions of 1.0 *M* acetic acid. This fraction was dried at 60° under a stream of nitrogen and dissolved in 200 μl of 90% methanol for injection into the HPLC column.

High-performance liquid chromatography

Chromatography was performed in a Brownlee Labs. column (25 cm × 4.6

mm I.D.) packed with LiChrosorb SI-100, 5 μm average diameter silica gel particles. The mobile phase used for chromatography of the "neutral" fraction materials was composed of methylene chloride—methanol—1.0 *M* ammonium formate buffer pH 3.0 (75:22:3, v/v/v). This was designated mobile phase A. A solvent composed of the same components in a volume ratio of 64:30:6 was used for chromatography of the orotic acid containing fraction. This was designated mobile phase B. The flow-rate used was 2.0 ml/min (1900 p.s.i.).

RESULTS

Fig. 1 shows the separation achieved when a standard mixture of pyrimidines

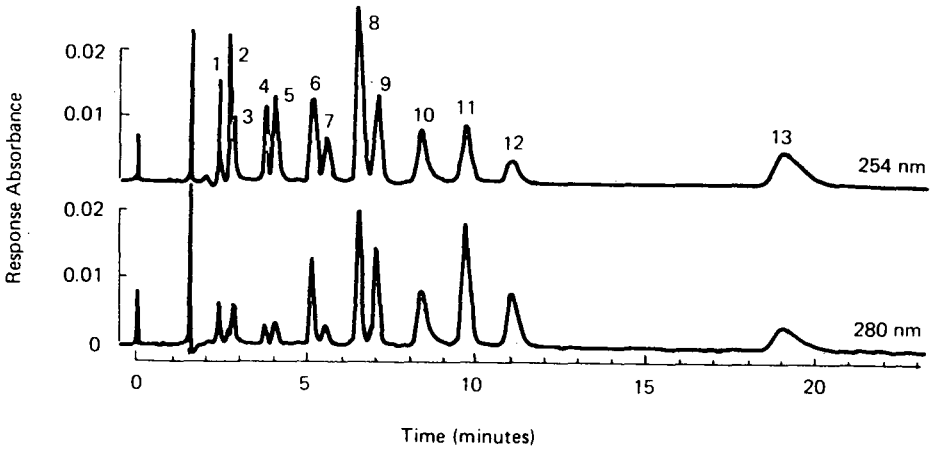

Fig. 1. Separation of a standard mixture of biologically interesting pyrimidines. Column, LiChrosorb SI-100 (25 cm × 4.6 mm I.D.). Mobile phase, methylene chloride—methanol—ammonium formate buffer (75:22:3). Flow-rate, 2 ml/min. 1, Thymine; 2, uracil; 3, thymidine; 4, 5-hydroxymethyluracil; 5, uridine; 6, 3-methylcytosine; 7, pseudouridine; 8, cytosine; 9, deoxycytidine; 10, 2'-O-methylcytidine; 11, cytidine; 12, orotic acid; 13, orotidine.

known to occur in biological systems was chromatographed with mobile phase A. Good separation of most of these pyrimidines could be achieved in less than 10 min. Orotic acid, however, eluted at 11 min and orotidine at 19 min. Mobile phase B was therefore developed to shorten the elution time and increase the sensitivity for orotic acid and orotidine. Fig. 2 demonstrates their separation with this solvent. Under these conditions they both elute completely in less than 8 min.

Table I lists the capacity factors (k') measured for the 13 biological pyrimidines chromatographed with mobile phase A. Orotic acid and orotidine gave k' values of 3.5 and 5.3, respectively, when chromatographed with mobile phase B. Table I also lists response ratios between 254 nm and 280 nm. This ratio is a useful identifying characteristic for each pyrimidine and can be used together with the retention time for peak identification and to detect the presence of interfering compounds in the peak. The presence of an overlapping UV absorbing peak normally will change the ratio significantly. The 254 nm to

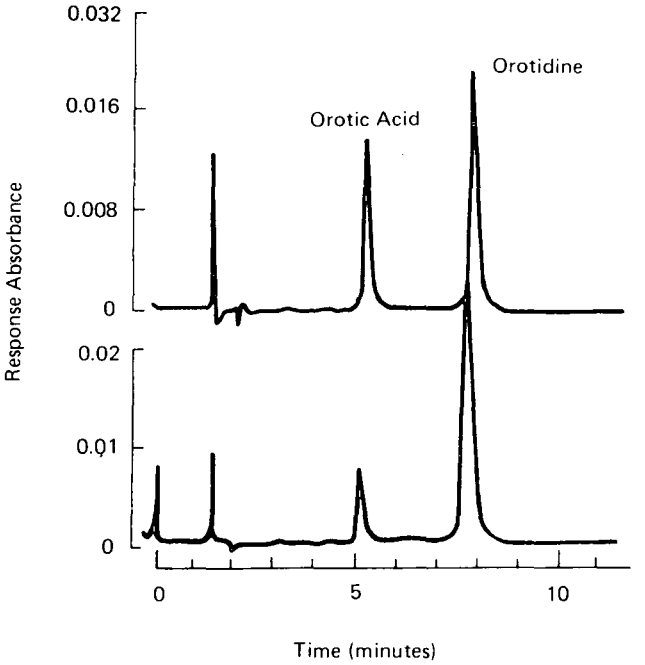

Fig. 2. Separation of orotic acid (0.2 μg) and orotidine (1.0 μg) standards. Column, LiChrosorb SI-100 (25 cm × 4.6 mm I.D.). Mobile phase, methylene chloride—methanol—ammonium formate buffer (64:30:6). Flow-rate, 2 ml/min.

TABLE I

CAPACITY FACTORS (k') AND 254 nm/280 nm RESPONSE RATIOS MEASURED WITH MOBILE PHASE A

No.	Compound	k'	254 nm/280 nm
1	Thymine	0.66	2.50
2	Uracil	0.88	9.10
3	Thymidine	0.98	1.70
4	5-Hydroxymethyluracil	1.59	3.70
5	Uridine	1.79	3.60
6	3-Methylcytosine	2.56	1.00
7	Pseudouridine	2.83	2.40
8	Cytosine	3.50	1.30
9	Deoxycytidine	3.86	0.96
10	2'-O-Methylcytidine	4.77	1.00
11	Cytidine	5.67	0.98
12	Orotic acid	6.61	0.51
13	Orotidine	13.10	1.50

280 nm ratios for orotic acid and orotidine are the same in mobile phases A and B.

The percent recovery for various pyrimidine standards added to urine are presented in Table II. These data were calculated by comparison of the peak

TABLE II

RECOVERY OF PYRIMIDINE STANDARDS ADDED TO NORMAL URINE

S = 8.9, 23.1, 5.45.

Amount added (μg/mg)	Percent recovery					
	Thymine	Uracil	Uridine	Pseudouridine	Orotate	Orotidine
1.6	108.7	118.1	97.2	*	**	**
8.0	101.4	96.2	96.4	*	91.3	99.6
40.0	101.9	95.8	100.6	100.8	93.3	90.8
200.0	96.1	89.8	92.6	92.8	94.3	100.5
1,000.0	97.1	88.7	94.1	95.4	89.3	95.9
Mean	101.0	99.2	96.2	98.8	92.1	96.7

*Recovery of pseudouridine was not measured at the 1.6 and 8.0 μg/ml addition levels because of the much higher endogenous level in urine.

**Measurement of orotic acid and orotidine at the 1.6 μg/ml level is not accurate due to the presence of small interfering peaks.

heights obtained from standards of known concentrations to peak heights obtained from injection of the urine extracts. The endogenous levels of uracil and pseudouridine in the normal urine were found to be 7.0 and 56.2 mg/ml respectively. Because of the potential effect on the accuracy of the measurements resulting from these high endogenous levels, the percent recovery of pseudouridine at 1.6 and 8.0 μg/ml was not measured. The high percent recovery (118%) for uracil at the 1.6 μg/ml level also appears to be a result of the high endogenous level of uracil in the urine.

Fig. 3 illustrates the pyrimidines found in the neutral fraction isolated from a normal urine specimen. Uracil and pseudouridine are the only pyrimidines in this fraction at levels detectable in our system. Table III shows the uracil and pseudouridine levels measured in six normal urines. The normal excretion of uracil ranged from 8.8 to 14.0 μg/ml creatinine with a mean of 12.2 μg/ml, and the normal excretion of pseudouridine ranged from 73 to 174 μg/ml with a mean of 108.5 μg/ml.

The reproducibility of the measurement of endogenous uracil and pseudouridine in a normal urine was determined by performing the isolation and measurement procedure on 10 separate portions of the same normal urine sample. The mean concentrations measured for uracil and pseudouridine were 14.9 mg/ml and 191 mg/ml of urine respectively, with relative percent standard deviations of 11.1 and 9.6 respectively.

DISCUSSION

The HPLC separation of the 13 pyrimidines studied here is rapidly and reliably accomplished on a LiChrosorb SI-100 column that provides high efficiencies of about 11,000 theoretical plates for all the compounds tested. This efficiency permits the use of relatively short analysis times while maintaining good resolution between the pyrimidines being measured and any interfering

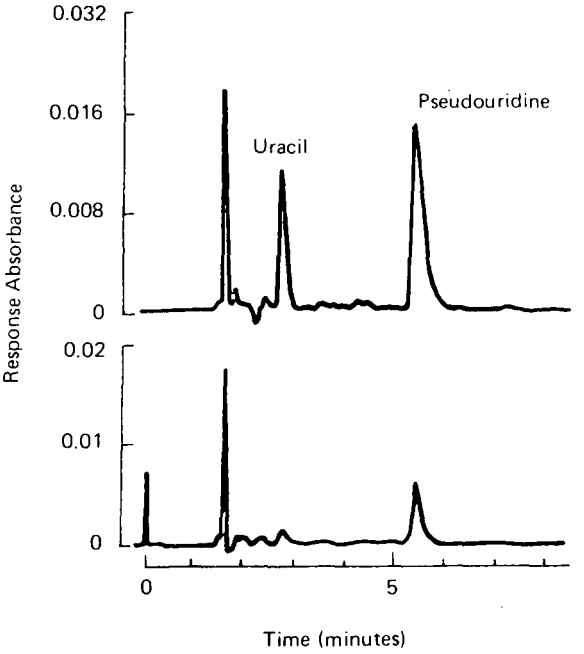

Fig. 3. Separation of pyrimidines from the "neutral" fraction of normal urine.

TABLE III

URACIL AND PSEUDOURIDINE LEVELS IN NORMAL URINES (μg/mg CREATININE)

	Uracil	Pseudouridine
	13.3	95.0
	14.0	124.0
	10.8	92.0
	12.6	73.0
	8.8	93.0
	13.5	174.0
Mean	12.2 (S.D. ± 2.0)	108.5 (S.D. ± 36.0)

compounds that may be present in urine samples. The ion-exchange procedures used for isolation of the pyrimidines from urine are fast and easy to use and provide good recoveries and suitable clean-up for HPLC analysis.

We have not attempted the isolation and analysis of basic pyrimidines (compounds 6 and 8—11 in Fig. 1) from urine samples but it is proposed that this could be accomplished with the use of small cation-exchange columns similar to those used for the neutral and acidic pyrimidines. It also appears feasible that these procedures could be applied to the study of pyrimidines in other biological fluids such as serum, cerebrospinal fluid, amniotic fluid, tissue extracts, nucleic acid hydrolysates, etc.

A rapid quantitative procedure for urinary pyrimidines is an alternative to

blood ammonia assays in studies of the effects of metabolic blocks in the urea cycle. Direct measurement of blood ammonia levels is subject to a number of technical pitfalls [15] and requires a considerable volume of venous blood from an infant. Increased urinary pyrimidine excretion is a reflection of the hyperammonemia and can be used for both diagnostic purposes and for the routine monitoring of patients undergoing dietary or α-ketoacid therapy. This rapid HPLC procedure is currently being used to measure pyrimidine levels in patients with various blocks in the urea cycle and will soon be routinely used for dietary monitoring.

ACKNOWLEDGEMENTS

This investigation was supported in part by Maternal and Child Health Services Projects No. 417 and No. 435, and by the National Institute of Child Health and Development Grant No. HD-03967.

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A RAPID, SENSITIVE METHOD FOR ACCURATE DETERMINATION OF THE LECITHIN/SPHINGOMYELIN RATIO BY THIN-LAYER CHROMATOGRAPHY AND REFLECTANCE SPECTROFLUOROMETRY

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(Received November 8th, 1978)

SUMMARY

A highly reproducible thin-layer chromatographic procedure has been developed for accurate determination of the lecithin/sphingomyelin ratio. Two interfering compounds, phosphatidyl inositol and phosphatidyl serine, have been investigated and eliminated by adsorption onto DEAE-cellulose. A uniform fluorescence staining procedure employing 2',7'-dichlorofluorescein has been developed. Accurate quantitation was performed by direct measurement of the reflected fluorescence intensity of the lecithin and sphingomyelin fluorophore spots with a spectrofluorometer equipped with a thin-layer scanning attachment. Stability and reproducibility studies are reported.

INTRODUCTION

Gluck and coworkers' [1] original lecithin/sphingomyelin (L/S) ratio procedure for the evaluation of fetal lung maturity has prompted much recent investigation of amniotic fluid phospholipids. Within the past few years, numerous thin-layer chromatographic (TLC) procedures have been developed for the determination of L/S ratios in amniotic fluid samples. Moreover, recent investigation has shown that phosphatidyl inositol (PI) and phosphatidyl serine (PS) may preclude accurate determination of the L/S ratio [2, 3]. Some of the techniques employed to achieve the separation of L and S from the aforementioned lipids include lengthy chromatographic separation time employing silica gel H chromatography plates containing 5% ammonium sulfate [4, 5], two-dimensional chromatography [3, 6], and adsorption of PI and PS onto diethylaminoethyl cellulose (DEAE-cellulose) [2].

Rapid TLC separation of L and S has been performed on silica gel impregnated glass microfiber chromatography sheets [3, 7—12]. A variety of solvent systems, visualization reagents, and quantitation methods have been employed

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TABLE I

TLC SEPARATION OF L AND S ON GLASS MICROFIBER CHROMATOGRAPHY SHEETS

Solvent system	Visualization reagent	Quantitation	R_F		Reference	Comment
			L	S		
$CHCl_3$—CH_3OH—NH_4OH (170:20:3, v/v)	I_2 vapor and/or bismuth subnitrate	Visual estimation	0.67	0.48	8, 9	
CH_2Cl_2—C_2H_5OH—H_2O (102:24:3, v/v)	Rhodamine B	Spot area (height × midpoint width)	0.27	0.12	7	
3.0 ml of CH_2Cl_2—C_2H_5OH—H_2O (34:8:1, v/v) plus H_2O optimization (2—4 drops of H_2O)	Rhodamine B	Spot area (height × midpoint width)	0.62	0.37	10	Miniature chromatography sheet (6.5 cm × 10 cm)
$CHCl_3$—CH_3OH—NH_4OH (170:20:3, v/v)	Malachite green	Densitometry			11	
$CHCl_3$—CH_3OH—NH_4OH (200:25:4, v/v)	Bismuth subnitrate	Visual comparison	0.63	0.44	12	
CH_2Cl_2—C_2H_5OH—H_2O (100:25:3, v/v), then			0.55	0.37	3	Two-dimensional chromatography
$CHCl_3$—CH_3OH—NH_4OH (170:20:3, v/v)	2′,7′-Dichlorofluorescein		0.53	0.38		

(Table I). Although most detection procedures can be adapted to visualize phospholipids separated on glass microfiber sheets, further development of sensitive and accurate in situ methods of quantitation is essential.

In this paper, a rapid and highly reproducible TLC procedure is described for the determination of L/S ratios. A previously reported procedure [10] has been modified as follows: A DEAE-cellulose treatment step has been added to eliminate the interference of PI and PS; the TLC application procedure has been improved; a sensitive 2′,7′-dichlorofluorescein (DCF) detection procedure has been incorporated; and quantitation has been performed by direct scanning of the chromatogram with a spectrofluorometer equipped with a thin-layer scanning attachment.

MATERIALS AND METHODS

The following Certified ACS grade solvents were purchased from Fisher Scientific (Winnipeg, Canada): chloroform, methanol, methylene chloride, and isopropanol. Absolute ethanol was obtained from Standard Chemicals (Winnipeg, Canada). Lecithin (L-α-phosphatidyl choline, from egg yolk), sphingomyelin (from bovine brain) and commercially prepared 1:1, 1.5:1, and 2:1 L/S ratio standards were purchased from Sigma (St. Louis, Mo., U.S.A.). The following phospholipids were obtained from Supelco (Bellefonte, Pa., U.S.A.): phosphatidyl serine (bovine) and phosphatidyl inositol (plant). Synthetic lysolecithin (LL) was purchased from Calbiochem (San Diego, Calif., U.S.A.). The DEAE-cellulose (DE32) was from North American Scientific Chemical (Calgary, Canada).

ITLC-type SG chromatography sheets (20 × 20 cm) were from Gelman (Ann Arbor, Mich., U.S.A.). The sheets were cut to size 9.9 × 6.4 cm, to fit the Gelman Seprachrom micro-chromatography chamber. A Gem hand-punch (McGill Metal Products Co., Marengo, Ill., U.S.A.) was used to excise round 3-mm chromatography discs. Holes were punched 2 cm from the bottom edge and at intervals of 1 cm from the left edge of each miniature chromatography sheet. The miniature sheets were heat activated in a 110° oven for 30 min. Activated sheets were stored in a desiccator above silica gel.

The chromatography solvent system, methylene chloride—ethanol—water (100:25:3, v/v), was prepared fresh weekly. The solvent system was stored at room temperature in a tightly stoppered brown bottle. Prior to use, the solvent system was vigorously mixed for 1 min.

A standard solution of lecithin was prepared to contain 1 mg of L per ml of chloroform—methanol (9:1, v/v). Standard solutions of S and LL were similarly prepared. PI and PS standards were prepared in chloroform. Three successive 1-μl volumes of the L standard solution were applied to a blank chromatography disc with a 10-μl syringe equipped with a Channey adaptor (Hamilton, Reno, Nev., U.S.A.). Standard discs were prepared in batches of ten or more, as required. Four dry standard L discs were inserted into a miniature ITLC sheet. Three milliliters of well-mixed solvent system were transferred to a Seprachrom chromatography trough. The chamber was assembled and the solvent system was allowed to migrate to within 1.5 cm of the top of the ITLC sheet. This required approximately 3.5 min. The developed chromatogram was

immediately removed and air dried for 5 min. The chromatogram was dipped for 10 sec into a solution containing 1.5 mg of DCF per 100 ml of isopropanol. Excess stain was allowed to drain onto the edge of the staining trough. The stained chromatogram was air dried for 15 min. Lecithin fluorophore spots were visualized under 375-nm ultraviolet light.

The reflected fluorescence intensity of the chromatogram spots was measured with a Farrand Mark I spectrofluorometer and recorded on a Farrand Model 100 strip-chart recorder (Model SR-204, Heath Co., Benton Harbor, Mich., U.S.A.). To improve instrument stability, the spectrofluorometer was modified as previously described [13]. Excitation and emission slit widths were 5 and 10 nm, respectively. The Color Specification numbers for the primary and secondary filters were 7-54 and 3-73, respectively. The area of the recorded L peaks was measured with a Koizumi Compensating Polar Planimeter purchased from Reliable Drafting Supplies (Regina, Canada). The above procedure was performed repeatedly. Fluorescence intensity studies were similarly performed for S, LL, PI, and PS. Relative fluorescence intensities were calculated with respect to the L fluorophore results.

Five microliters of a standard solution of PS (1 mg PS per ml of chloroform) were added to a blank chromatography disc. A second chromatography disc was prepared to contain L, S, and PS in 6-, 3-, and 5-μg quantities, respectively. For the preparation of a third chromatography disc 1.5 μl of the PS standard solution (50 mg PS per ml of chloroform) were added to a test-tube and the DEAE-cellulose adsorption procedure was performed as described by Gosselin and Foidart [2]. The residue was concentrated at the bottom of a pear-shaped flask and redissolved in 0.04 ml of chloroform—methanol (9:1, v/v). Three microliters were sequentially applied to the chromatography disc. For the preparation of the fourth chromatography disc, 40 μl of a 2:1 L/S standard and 1.5 μl of the PS (50 mg PS per ml of chloroform) standard were mixed in a test-tube, dried under nitrogen gas, and the DEAE-cellulose adsorption procedure was performed. The final residue was concentrated at the bottom of a pear-shaped flask with 0.04 ml of chloroform—methanol (9:1, v/v). Three microliters were applied to the chromatography disc. The four chromatography discs prepared above were inserted into a miniature ITLC sheet. Chromatographic separation and visualization were performed. The chromatogram was scanned from the origin to the solvent front for each test. A blank scan was obtained by scanning between two chromatogram discs. The above procedure was performed in duplicate. Similar testing of the DEAE-cellulose adsorption procedure was performed in the presence of 14 μl of a commercially prepared PI standard (10 mg PI per ml of chloroform). L/S ratio reproducibility studies were similarly performed for each of the following: a laboratory weighed in 2:1 L/S standard after DEAE-cellulose treatment; a commercial 2:1 L/S ratio standard with PS added and DEAE-cellulose treatment; and a commercial 2:1 L/S ratio standard with PI added and DEAE-cellulose treatment.

A fluorescence stability study was performed. A commercially prepared 2:1 L/S ratio standard was used to prepare a chromatogram disc which contained 6 μg of lecithin and 3 μg of sphingomyelin. Chromatographic separation and visualization were performed as described above. The fluorescence intensity of the chromatogram spots was recorded within 0.5 h after staining and after 3, 6,

9, 12, and 24 h. Results were calculated as percentages of the fluorescence intensity of the 6 μg lecithin fluorophore as measured within 0.5 h. L/S area ratios were also calculated. The above study was performed in triplicate. A fluorescence stability study using a weighed in 2:1 L/S ratio standard [2 mg of L and 1 mg of S per ml of chloroform–methanol (9:1, v/v)] was similarly performed.

Four chromatography discs were prepared to contain L and S in the following quantities: 2 μg of L and 1 μg of S; 4 μg of L and 2 μg of S; 6 μg of L and 3 μg of S; and 8 μg of L and 4 μg of S. Chromatographic separation, visualization, and measurement of the fluorescence intensity of the L and S fluorophore spots were performed as described above. Peak area measurements of L and S were plotted against μg of lipid applied. L/S area ratios were also calculated. The aforementioned study was similarly performed in duplicate.

A 1:1 L/S ratio standard was prepared by adding 2.0 mg of sphingomyelin and 20 μl of lecithin [1 g per 10 ml of chloroform–methanol (9:1, v/v)] to a 2-ml volumetric flask which was brought to volume with chloroform–methanol (9:1, v/v). Subsequent ratios of 1.5:1, 2.0:1, 2.5:1, 3.0:1, and 3.5:1 were similarly prepared by adding 30, 40, 50, 60, and 70 μl of the commercial lecithin solution, respectively. Chromatographic separation, visualization, and measurement of the reflected fluorescence intensity were performed as previously described. L/S area ratios were calculated and plotted against L/S weight ratios applied. The above study was performed in duplicate. The following commercially prepared L/S ratio standards were similarly tested: 1.0 mg L and 1.0 mg S per ml, 1.5 mg L and 1.0 mg S per ml, and 2.0 mg L and 1.0 mg S per ml of chloroform–methanol (1:1, v/v). The ratio results were calculated and plotted as described above. This study was similarly performed in duplicate.

RESULTS AND DISCUSSION

Rapid chromatographic separation of L and S has been achieved on unactivated ITLC-type SG chromatography sheets [7–10]. However, for accurate quantitation recent investigation in this laboratory has shown that ITLC sheets activated for 30 min in a 110° oven give smoother and more reproducible strip-chart recordings [13].

Uniform staining was achieved by dipping the chromatograms for 10 sec into a solution containing 1.5 mg of DCF per 100 ml of isopropanol. Excess stain was allowed to drain onto the edge of the staining trough. This procedure eliminated variation due to uneven spraying. The day-to-day chromatogram stain density also appeared to be more uniform.

Dry, powdered DCF reagent has been observed to discolor upon storage at room temperature for many months. With the present spectrofluorometer detection system, use of discolored DCF dye has resulted in increased background fluorescence and higher noise levels.

When the DCF-stained chromatograms were viewed under a long-wave ultraviolet lamp (375 nm), bright-green phospholipid spots were observed on a dark purple background. The intensity of the reflected fluorescence was measured with a spectrofluorometer equipped with a thin-layer scanning attachment and recorded on a strip-chart recorder. Relative fluorescence intensity results were

TABLE II

FLUORESCENCE INTENSITY OF 2',7'-DICHLOROFLUORESCEIN-STAINED PHOSPHOLIPIDS

Lipid	Quantity (μg)	Relative fluorescence* (%)	C.V.** (%)
L	3	100.0	5.2
S	3	77.8	4.8
LL	3	131.0	3.4
PS***	3	71.0	7.0
PI***	3	75.4	11.7

*Values are reported in percentages of the fluorescence of 3 μg L.
**Each of the reported C.V. were calculated from 10 test results.
***Approximate fluorescence intensity results were calculated from diffusely distributed fluorophore spots (see Fig. 1c). Refer to text for complete details.

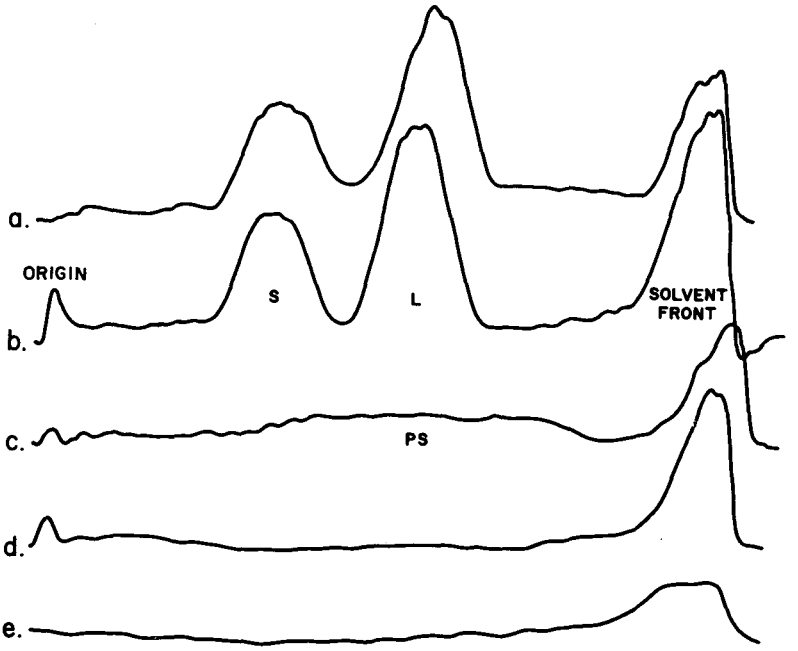

Fig. 1. Reflected fluorescence scans depicting: (a) L, S and PS; (b) L, S and PS after DEAE-cellulose treatment; (c) PS; (d) PS and DEAE-cellulose treatment; (e) blank chromatogram. Refer to text for complete details.

calculated for L, S, and LL (Table II). Very reproducible results were observed for the L, S, and LL standards. Although the fluorescence intensity of the LL fluorophore was more intense than that of the L or S, no interference was observed because of the excellent separation distances achieved; R_F values for L, S, and LL were 0.55, 0.37 and 0.23 respectively. In contrast, a similar investigation undertaken for PI and PS resulted in very elongated spot formation. A typical scan of a PS spot is depicted in Fig. 1c. In general, when L and S spots were scanned in the presence of PS or PI, the base-line intensity

increased, the noise-to-signal ratio increased, and an apparent incomplete separation was observed between the L and S spots (Fig. 1a). However, comparison of the scans depicted in Fig. 1 clearly shows that DEAE-cellulose removes the interference caused by the presence of PS. Similar results were observed for PI.

To maintain optimal reproducibility at least three rinse steps should be employed to concentrate the phospholipids at the bottom of the flask after rotary evaporation to dryness. Successive application of phospholipid material to blank chromatography discs was also observed to improve reproducibility of the L/S ratio results. In the present procedure, three successive 1-μl applications were made to the front side of blank discs.

The reproducibility of the presently developed L/S ratio procedure was repeatedly evaluated and a summary of the results is given in Table III. Very

TABLE III

L/S RATIO REPRODUCIBILITY STUDIES

n	L/S area ratio ($\bar{X}$)	S.D.	C.V. (%)	Comment***
40	1.92*	0.087	4.5	L and S, one batch per day for four days
10	2.11*	0.080	3.8	L, S, PS and DEAE-cellulose adsorption
13	2.03*	0.078	3.8	L, S, PI and DEAE-cellulose adsorption
10	2.27**	0.079	3.5	L and S
12	2.40**	0.108	4.5	L and S after DEAE-cellulose adsorption

*Commercial 2:1 L/S ratio standard.
**Laboratory weighed in 2:1 L/S ratio standard.
***Refer to text for complete details.

reproducible results were observed throughout all studies. However, the mean L/S ratio calculated for the commercial standard was consistently lower than the results calculated for the laboratory weighed in standards. Furthermore, a slight increase was observed for L/S ratio results after incorporation of the DEAE-cellulose adsorption step.

Fluorescence stability tests were conducted for a commercial L/S standard and a laboratory weighed in L/S standard. A total of six fluorescence stability tests were performed because of fluctuations encountered in the xenon arc lamp intensity during the 24-h studies; two studies are reported in Tables IV and V. In general, the L and S fluorophore intensity was observed to decrease only slightly over 24 h. During this time, highly reproducible L/S ratio results were observed (Tables IV and V).

Accessory attachments and modifications to improve stability of the Farrand Mark I Spectrofluorometer have been reported previously [13]. Excellent fluorometer stability has been observed; however, recently, large fluctuations in line voltage including power failures have caused fluctuations in the intensity of the xenon arc lamp. The increase in the relative fluorescence of the 3-h L and S fluorophore results reported in Table IV is attributed to a change in the intensity of the xenon arc lamp.

L/S ratio standards were applied to blank discs in the following quantities:

TABLE IV

FLUORESCENCE STABILITY OF A COMMERCIALLY PREPARED 2:1 L/S RATIO STANDARD

Time (h)	Relative fluorescence*		L/S ratio
	L (6 μg)	S (3 μg)	
0—0.5	100.0	48.7	2.05
3**	103.0**	56.1**	1.84
6	102.2	56.3	1.82
9	95.8	51.7	1.85
12	95.2	51.7	1.84
24	94.0	50.3	1.87

*Values are reported in percentages of the fluorescence intensity of 6 μg of lecithin as measured between 0 and 0.5 h.
**The relative fluorescence increase observed is attributed to spectrofluorometer instability. Refer to text for complete details.

TABLE V

FLUORESCENCE STABILITY OF A WEIGHED IN 2:1 L/S RATIO STANDARD

Time (h)	Relative fluorescence*		L/S ratio
	L (6 μg)	S (3 μg)	
0—0.5	100	45.9	2.18
3	99.3	46.4	2.14
6	97.9	46.4	2.11
9	96.4	45.4	2.12
12	97.1	46.2	2.10
24	96.4	45.4	2.12

*Values are reported in percentages of the fluorescence intensity of 6 μg of lecithin as measured between 0 and 0.5 h. Refer to text for complete details.

2:1, 4:2, 6:3, 8:4 μg of L and S, respectively. Corresponding L/S ratio results for each of the aforementioned tests were 3.2, 2.5, 2.3, and 2.3, respectively. The increased fluorescence intensity of the L fluorophore compared to the S fluorophore accounts for L/S ratio results above 2.0 (see Table II and Fig. 2). The L/S ratio results were particularly elevated at lower concentrations; for example, 2 μg of L:1 μg of S resulted in an L/S ratio of 3.2.

A linear relationship was obtained for L/S ratios between 1 and 3.5 when the L/S area ratios were plotted against the L/S weight ratios of the laboratory prepared standards (Fig. 3). The linear regression parameters for these results are: correlation coefficient, 0.999; y intercept, 0.467; slope, 0.920. The results were above the line of identity and converged toward the line of identity. The area ratio results above the line of identity are attributed to the greater fluorescence intensity of the lecithin fluorophore (Table II), while convergence of the results toward the line of identity is attributed to increased self-quenching of the L spots. The variation in slope between the commercial and the laboratory prepared standards is presently unexplained. Repeated testing of

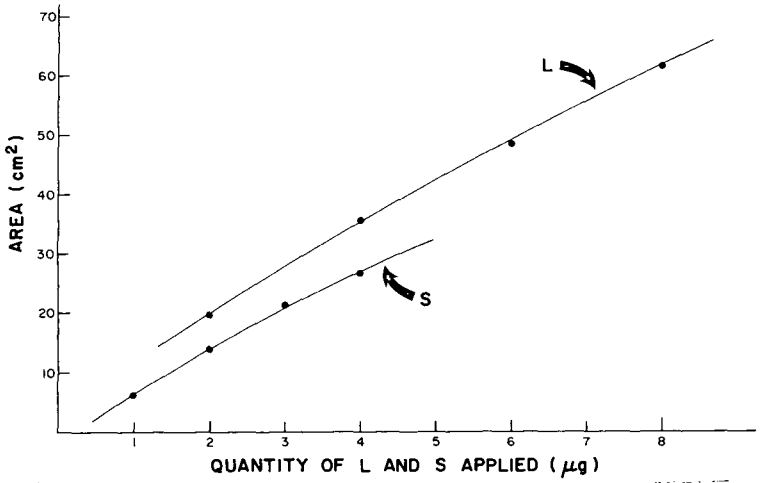

Fig. 2. L and S area measurements vs. quantity of lipid standard applied. The results depicted represent an average of two samples at each quantity studied. Refer to text for complete details.

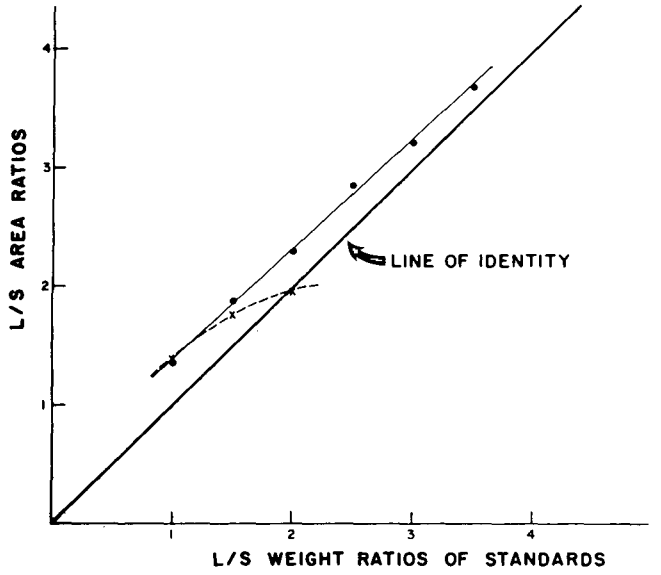

Fig. 3. Correlation of L/S area ratios vs. L/S weight ratios. The results depicted represent an average of two samples at each quantity studied. Refer to text for complete details. •——•, Laboratory prepared standards; x— — — x, commercial standards.

the 2:1 commerical standard has established a mean value of 1.92 which does not correlate with the laboratory weighed in standards (Table III). However, it should be noted that water contamination of the lecithin employed in preparation of the commerical standards is suspected.

The presently developed procedure is highly recommended for accurate and reproducible measurement of the L/S ratio.

ACKNOWLEDGEMENTS

The authors wish to thank the National Research Council of Canada for financial support of this work. A special thank-you is extended to Dr. S. Harold, Department of Clinical Chemistry, Regina General Hospital, for his constructive criticism during the preparation of this manuscript.

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Biomedical Applications

CHROMBIO. 310

GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF PLASMA LEVELS OF *d*-7,8-DIMETHOXY-3-METHYL-PHENYL-2,3,4,5-TETRAHYDRO-1H-3-BENZAZEPINE ACID MALEATE (SCH-12679) AND ITS MAJOR METABOLITES IN AGGRESSIVE MENTAL RETARDATES

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(Received October 2nd, 1978)

SUMMARY

A sensitive gas—liquid chromatographic technique for the quantitative analysis of SCH-12679 (*d*-7,8-dimethoxy-3-methyl-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine acid maleate) and its major metabolites in plasma of aggressive mental retardates receiving therapeutic doses of the medicament has been developed. The lower limits of detection are 20 ng/ml for SCH-12679, 0.5 ng/ml for 3-desmethyl SCH-12679 and 0.4 ng/ml for 7-desmethyl plus 8-desmethyl SCH-12679. SCH-12679 is estimated with a flame ionization detector. Its metabolites are quantitated using an electron-capture detector after conversion of the compounds to their heptafluorobutyryl derivatives by reaction with the ap-

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propriate anhydride. Data on plasma levels of unchanged SCH-12679, 3-desmethyl SCH-12679 and a combination of 7-desmethyl and 8-desmethyl SCH-12679 in fifteen patients treated with the medicament are presented.

INTRODUCTION

SCH-12679 (*d*-7,8-dimethoxy-3-methyl-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine acid maleate) is a novel chemical entity (Fig. 1A) with an interesting pharmacological profile. It exhibits a broad spectrum of activity in both laboratory animals [1] and humans [2—6]. Recently we have reported that SCH-12679 is an effective drug in the treatment of aggressive mental retardates [7]. The objective of this study was to develop a sensitive, selective, gas -liquid chromatographic (GLC) method for the quantification of unchanged SCH-12679 and its major metabolites (Fig. 1B, C and D) in plasma of mentally retarded patients after therapeutic doses of this medicament.

MATERIALS AND METHODS

SCH-12679, 3-desmethyl SCH-12679, 7-desmethyl SCH-12679 and 8-desmethyl SCH-12679 for chromatographic standards, as well as SCH-12679 capsules for oral administration to patients were supplied by Schering (Bloomfield, N.J., U.S.A.). Protriptyline was used as an internal standard for GLC analysis.

Reagents and materials

The following organic reagents were used: GLC—spectrometric quality

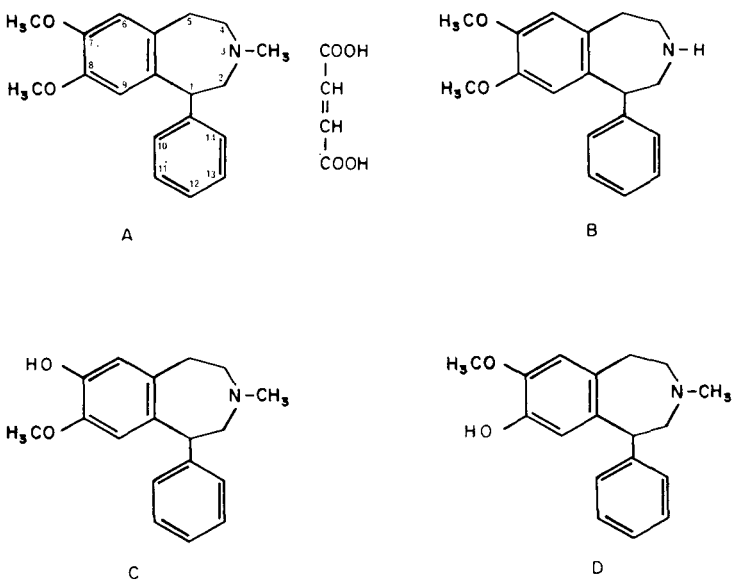

Fig. 1. Structural formulae of SCH-12679. (A) SCH-12679 maleate; (B) 3-desmethyl SCH-12679; (C) 7-desmethyl SCH-12679; (D) 8-desmethyl SCH-12679.

toluene and methanol (J.T. Baker, Phillipsburg, N.J., U.S.A.), MC/B "chromatoquality" isoamyl alcohol (Matheson Coleman and Bell, East Rutherford, N.J., U.S.A.), heptafluorobutyric anhydride (HFBA) (Eastman Kodak, Rochester, N.Y., U.S.A.) and β-glucuronidase type H-2 (Sigma, St. Louis, Mo., U.S.A.). The inorganic reagents were made up in doubly distilled water. The Dubnoff metabolic shaking incubator was purchased from Fisher Scientific (Montreal, Canada).

Gas—liquid chromatography

Analyses were performed with a Hewlett-Packard Model 5830A gas chromatograph equipped with dual flame ionization detectors (FID) and a ^{63}Ni (15 mCi) electron-capture detector (ECD). The apparatus had automation capability built into its digital processor (HP 18850 A) to operate the gas chromatograph throughout the analytical run, following precisely the instructions given on its keyboard. The processor continuously monitored detector output and reduced it to peak areas and retention times.

The chromatographic conditions for SCH-12679 and its major metabolites were as follows.

SCH-12679. A 1.8-m coiled glass column (O.D. 6.3 mm, I.D. 4 mm) was packed with 3% PC 3210 (50% SE-30 ultraphase + 50% OV-210) on 80—100 mesh Gas-Chrom Q (Chromatographic Specialties, Brockville, Canada). The column was conditioned at 275° for 24 h with a nitrogen carrier gas flow-rate of 45 ml/min. The column was operated at 200°, the FID at 260° and the injection port at 250° with the following gas flow-rates: nitrogen 45 ml/min, hydrogen 55 ml/min and air 180 ml/min. Under these conditions, the relative retention time of SCH-12679 to the internal standard (protriptyline) was 1.23 (Fig. 2B).

Major metabolites of SCH-12679. A 1.8-m coiled glass column, (O.D. 6.3 mm, I.D. 4 mm) was packed with 3% Apiezon L on 80—100 mesh Gas-Chrom Q (Chromatographic Specialties). The column was conditioned at 275° for 24 h with an argon—methane (95:5) carrier flow-rate of 50 ml/min. The column was operated at 220°, the ECD at 300° and the injection port at 250° with carrier flowing at 50 ml/min. The two isomers, namely 7-desmethyl and 8-desmethyl SCH-12679, could not be separated under the present GLC conditions. Therefore the assay yields the sum of both. The relative retention times of the isomers and 3-desmethyl SCH-12679 to the internal standard (protriptyline) were 0.416 and 0.814, respectively (Fig. 3B).

Hydrolysis of the conjugated compounds

In order to hydrolyse the conjugated metabolites of SCH-12679, pooled plasma of the patients who were treated with this drug was processed by the following methods.

(A) To 10 ml of pooled plasma was added 0.5 ml of β-glucuronidase type H-2. The mixture was adjusted to pH 5 with 0.1 *M* sodium acetate buffer and incubated at 37° for 24 h in a Dubnoff metabolic shaking incubator. After cooling at room temperature the mixture was brought to pH 10 with 10 *N* NaOH and extracted as described in the extraction procedure. It was found

that the hydrolysis was incomplete and the plasma extract gave several extraneous background peaks, which could interfere with the assay.

(B) To 10 ml of pooled plasma were added 10 ml of 1 *N* HCl and the mixture was heated at 95° for 30 min. After cooling at room temperature, the mixture was adjusted to pH 10 with 10 *N* NaOH and extracted according to the extraction procedure. It was noticed that substantial degradation of the parent drug and its metabolites occurred using the acid hydrolysis process.

(C) To 10 ml of pooled plasma were added 8 ml of 10 *N* NaOH and the mixture was heated at 95° for 30 min. After cooling at room temperature 3.5 ml of water were then added to the mixture, which was later extracted in the same way. It was observed that the hydrolysis was more effective without chemical degradation of the parent drug and its metabolites. This observation was confirmed by comparing the quantities of SCH-12679 and its metabolites in 10 ml of non-hydrolysed pooled plasma of the patients (same lot) with the hydrolysed sample. The quantity of 7-desmethyl and 8-desmethyl SCH-12679 was approximately three times more in the hydrolysed than in the non-hydrolysed pooled plasma. Huang and Kurland [8] also suggested that alkaline hydrolysis is preferable to acid hydrolysis for the phenothiazine compounds. For the hydrolysis of conjugated metabolites the alkaline hydrolysis was adopted in this study.

Extraction procedure

To 3 ml of plasma in a 13-ml glass-stoppered centrifuge tube were added 2.4 ml of 10 *N* NaOH. The mixture was heated at 95° for 30 min on an electric plate to hydrolyse the conjugated metabolites. The mixture was allowed to cool at room temperature. One milliliter of water was then added to the mixture, which was later totally transferred to a 50-ml centrifuge tube.

A 25-μl volume of the methanolic solution of the internal standard, protriptyline hydrochloride (100 μg/ml), were added to the mixture, which was then extracted twice with 5 ml of toluene containing 1.5% isoamyl alcohol. After each extraction the tubes were agitated in a mechanical shaker for 15 min and then centrifuged at 600 *g* for 10 min. The organic layer was transferred to a 13-ml centrifuge tube and the aqueous layer was discarded. The organic layer was extracted with 2 ml of 0.1 *M* phosphate buffer (pH 7.2) for 15 min and centrifuged for 10 min. The buffer layer was preserved for further extraction. The organic layer was back-extracted with 2 ml of 0.05 *N* HCl for 15 min. The sample was centrifuged as before and the organic layer was removed and discarded. The aqueous layer was made alkaline with 0.2 ml of 1 *N* NH_4OH solution and extracted with 1.5 ml of toluene—isoamyl alcohol mixture (98.5:1.5) for 15 min, then centrifuged as described above. The organic layer was transferred to a 5-ml conical glass-stoppered tube (A).

The phosphate buffer layer from the previous extraction was made alkaline with 0.2 ml of 1 *N* NH_4OH solution and extracted with 1.5 ml of toluene—isoamyl alcohol mixture (98.5:1.5) for 15 min. After centrifugation, the organic layer was transferred to the previous tube (A) and evaporated to dryness at 45° with a low stream of nitrogen. The aqueous layer was discarded. The walls of the tube were rinsed with 0.1 ml of methanol by vibrating with

a Vortex mixer for 1 min. The solution was evaporated to dryness as before and the residue was used further for quantification of the compounds.

By extracting the organic layer with 0.1 *M* phosphate buffer, the relatively polar, phenolic metabolites 7-desmethyl and 8-desmethyl SCH-12679 were separated from SCH-12679 and its less polar, 3-desmethyl, derivative. Thus, washing of the toluene extract with phosphate buffer (pH 7.2) selectively removed the phenolic metabolites. It was essential to use the phosphate buffer as the recovery of 7-desmethyl and 8-desmethyl SCH-12679 was substantially lower when these compounds were not separated before the back-extraction cleanup step.

Measurement of SCH-12679 and its major metabolites

SCH-12679 was directly estimated with FID. 3-desmethyl SCH-12679 as well as 7- and 8-desmethyl SCH-12679 were acylated with HFBA and the derivatives were determined by ECD.

The residue left after extraction was dissolved in 25 μl of methanol by vortexing for 1 min, and 3 μl of this solution were injected into the gas chromatograph using FID.

For the estimation of metabolites only 10 μl of the rest of the solution were evaporated to dryness as described in the extraction procedure. The residue was dissolved in 50 μl of toluene. To this solution were added 20 μl of 0.1 *M* triethylamine in toluene followed by 5 μl of HFBA. The mixture was vibrated with a Vortex mixer for 1 min and kept at room temperature for 10 min. Then 100 μl of phosphate buffer (pH 6.0) were added to the solution. The mixture was vortexed as before and centrifuged for 10 min. About 2 μl of the toluene phase were injected into the gas chromatograph using ECD.

Calibration curves

The calibration curves were established using the internal standardization method. The methanolic solutions of the maleate salts of SCH-12679, 3-desmethyl SCH-12679, 7-desmethyl and 8-desmethyl SCH-12679 in the range 0.750—7.500 μg, 0.050—0.400 μg, 0.025—0.125 μg and 0.025—0.125 μg, respectively, were added to 3 ml of fresh heparinized plasma and the extraction was carried out as described above. Quantitation was achieved using the ratio of the peak areas of the compounds to that of internal standard protriptyline. Peak area ratios were plotted against weight ratios to obtain the calibration curves. The response of the FID to SCH-12679 was linear from 0.250 to 2.50 μg/ml of plasma. The ECD response to 3-desmethyl SCH-12679 and to 7-desmethyl plus 8-desmethyl SCH-12679 was linear over the range 0.017—0.133 and 0.008—0.042 μg/ml of plasma, respectively.

Human studies

Fifteen out of forty-one hospitalized aggressive mental retardates participating in a large-scale double-blind clinical study of SCH-12679 vs. placebo and thioridazine were the subjects of this experiment. The patients received a total of 400 mg of SCH-12679 per day orally in four doses at 8.00 a.m., 12.00 p.m., 4.00 p.m. and 8.00 p.m. for a period of four weeks. Two 10-ml

blood samples were drawn 2 h after the first morning dose at the end of the fourth week of treatment. Blood samples were centrifuged immediately after collection. An equal volume of plasma was aspirated in two separate tubes, which were wrapped in aluminium foil and placed in a deep-freeze at −15° until analysis.

RESULTS AND DISCUSSION

Selectivity

Analytical studies indicate that extracts from blank human plasma do not show peaks that could interfere with the quantitative analysis of SCH-12679 and its major metabolites. Typical chromatograms are shown in Figs. 2 and 3. The stationary phase, 3% PC-3210 (50% SE-30 ultraphase + 50% OV-210), gave a good resolution for SCH-12679, 3-desmethyl SCH-12679 and the internal standard, protriptyline. Although 3-desmethyl SCH-12679 could be identified by FID without derivatization with HFBA, the detector response to this compound was very low (Figs. 2 and 3). The isomers 7-desmethyl and 8-desmethyl SCH-12679 were not eluted from the PC-3210 column using similar conditions.

With the exception of SCH-12679 all its three major metabolites reacted with HFBA forming the corresponding derivatives (Fig. 3). The following

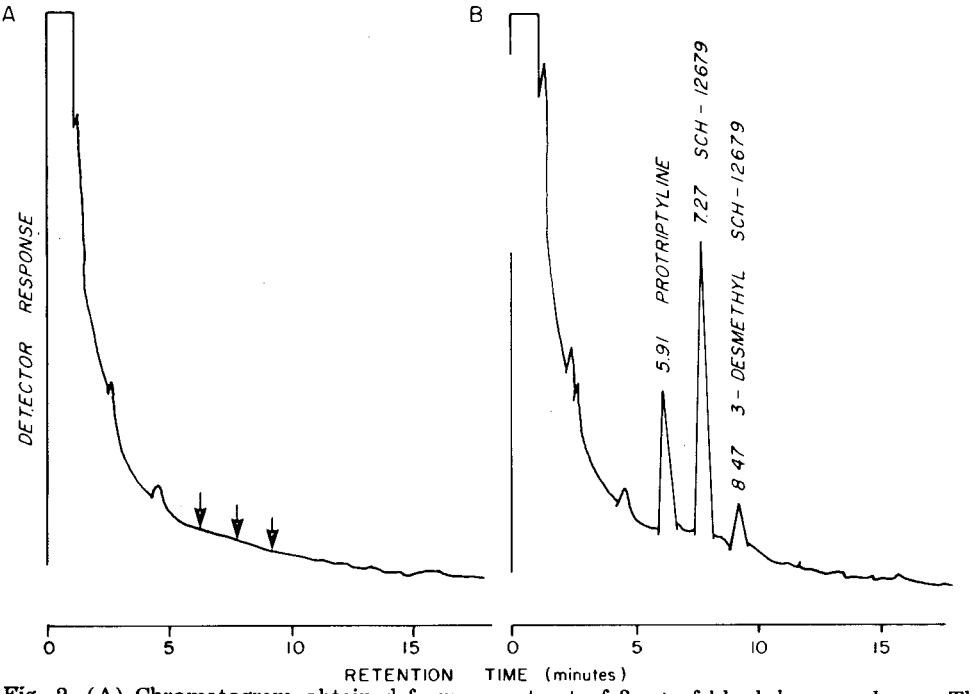

Fig. 2. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma. The arrows show the absence of signals at the retention times of the internal standard protriptyline, SCH-12679 and 3-desmethyl SCH-12679. (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the internal standard protriptyline, SCH-12679 and 3-desmethyl SCH-12679.

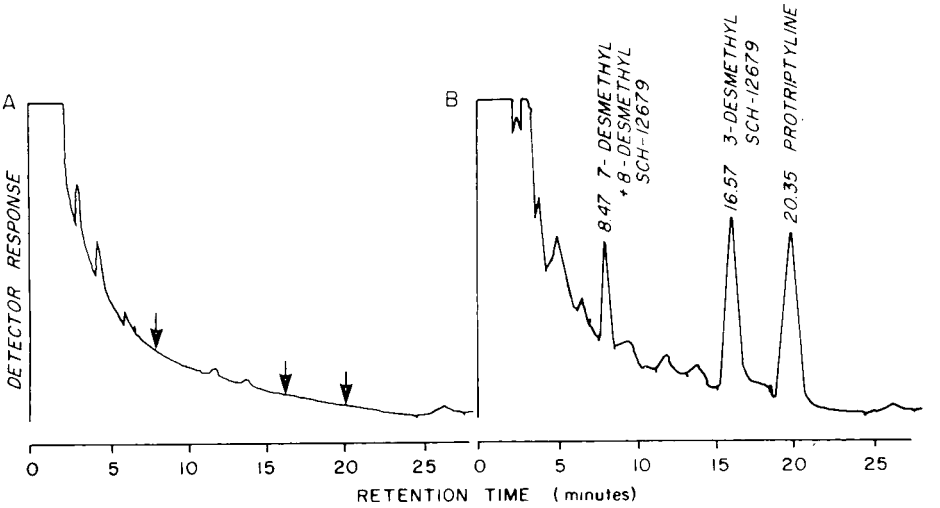

Fig. 3. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma derivatized with HFBA. The arrows show the absence of signals at the retention times of 7-desmethyl plus 8-desmethyl SCH-12679, 3-desmethyl SCH-12679 and the internal standard protriptyline. (B) Chromatogram obtained from an extract of 3 ml of human plasma containing 7-desmethyl plus 8-desmethyl SCH-12679, 3-desmethyl SCH-12679 and the internal standard protriptyline.

stationary phases were tried to resolve 7-desmethyl and 8-desmethyl SCH-12679: OV-7, OV-17, OV-210, SE-52, Dexsil 300, QF-1, Carbowax 20M, PC-3210 and Versamid 900. None of these phases could separate the two isomers. Apiezon L was selected as the stationary phase as it gave shorter retention times for the compounds assayed. Since all patients were kept under rigorous control as to the drug regimen during the four-week treatment period, the interference of other drugs and their metabolites was eliminated.

Recovery studies

The recoveries of SCH-12679, 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 from 3 ml of spiked plasma were determined using the same internal standardization method as described previously. The peak area ratio of each compound and the internal standard protriptyline was used as the index of detector performance and overall efficiency of the analytical procedure. The reproducibility and recovery results of SCH-12679 and its major metabolites are given in Table I. The overall coefficient of variation is below 10%.

Sensitivity

The lower limits for accurate determination of SCH-12679 and its metabolites were established by spiking 3 ml of blank plasma with dilute methanolic solutions of SCH-12679, 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 in the range of 60—600 ng, 1.5—50 ng, 1.2—25 ng and 1.2—25 ng, respectively, followed by the previously described extraction procedure using protriptyline as an internal standard. The lower detection limit was fixed to the minimum response of FID and ECD to the respective com-

TABLE I

GLC ESTIMATION OF SCH-12679, 3-DESMETHYL SCH-12679 AND A COMBINATION OF 7-DESMETHYL AND 8-DESMETHYL SCH-12679 ADDED TO PLASMA

Compound	Compound added (ng)	Compound recovered (ng)	Recovery* (%)	C.V. (%)
SCH-12679	67.7	63.8	94.2	4.8
	135.5	120.7	89.1	2.9
	270.9	251.4	92.8	3.5
	485.5	421.9	86.9	1.1
	677.3	591.3	87.3	2.3
3-Desmethyl SCH-12679	0.81	0.73	90.1	6.8
	2.43	2.08	85.6	2.5
	6.48	6.07	93.7	3.7
	9.70	8.61	88.8	5.1
	16.20	13.60	83.9	4.3
7-Desmethyl plus 8-desmethyl SCH-12679	0.405	0.292	72.0	9.7
	1.21	0.811	67.0	4.1
	2.00	1.55	77.5	6.8
	4.00	2.70	67.5	3.4
	6.00	4.16	69.3	5.2

*Each value is the mean of four determinations.

pounds with peak areas up to 40000 counts at an attenuation of × 128. It was found that the lowest detection limit of SCH-12679 with FID was 20 ng/ml of plasma and the ECD response to 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 was limited to 0.5 and 0.4 ng/ml of plasma, respectively.

Application of the method to human studies

The plasma levels of unchanged SCH-12679 and its major metabolites in psychiatric patients are presented in Table II. The plasma concentrations of SCH-12679 and its major metabolites measured in patients after four weeks of treatment show a marked individual variation. The plasma concentrations of SCH-12679 varied from 61 to 875 ng/ml, those of 3-desmethyl SCH-12679 and 7-desmethyl plus 8-desmethyl SCH-12679 from 34 to 534 ng/ml and 12 to 222 ng/ml, respectively. The interpatient variations in the steady-state plasma levels of antidepressants and neuroleptics in patients treated with these drugs had already been observed in earlier reports [9—14]. The therapeutic levels of SCH-12679 and its metabolites are not yet well defined. Of the three metabolites, only 8-desmethyl SCH-12679 has a significant behavioral activity in animals, whereas its isomer 7-desmethyl SCH-12679 is relatively inactive [15]. As 7-desmethyl and 8-desmethyl SCH-12679 could not be separated under the GLC conditions used, the two isomers were quantitated together in the ratio of 1:1. When the two isomers were analyzed separately, the ECD response to the heptafluorobutyryl derivative of each compound was different. If the two isomers were mixed in the ratio of 1:1, the resulting peak area was the sum of each heptafluorobutyryl derivative of the metabolites. This fact

TABLE II

PLASMA LEVELS OF SCH-12679, 3-DESMETHYL SCH-12679 AND A COMBINATION OF 7-DESMETHYL AND 8-DESMETHYL SCH-12679 IN PATIENTS RECEIVING SCH-12679 THERAPY

Patient	Sex	Age (years)	Weight (kg)	Plasma level of SCH-12679 (ng/ml)	Plasma level of 3-desmethyl SCH-12679 (ng/ml)	Plasma level of 7-desmethyl + 8-desmethyl SCH-12679 (ng/ml)
1	F	40	60	143	172	210
2	M	27	48	97	372	47
3	F	31	40	528	216	168
4	F	26	53	69	47	67
5	M	30	50	144	401	44
6	M	36	55	478	142	222
7	F	31	45	319	191	39
8	F	30	42	194	213	25
9	M	33	61	506	95	26
10	M	29	52	200	99	57
11	M	29	54	208	34	65
12	F	22	50	61	88	59
13	M	21	45	306	177	52
14	M	50	43	875	534	53
15	M	23	41	131	458	12

showed that the derivatization of the isomers with HFBA in the mixed ratio of 1:1 was complete and comparable to the separate analysis of each heptafluorobutyryl derivative of the respective metabolites.

To our knowledge, the present method is the first report for the assay of SCH-12679 and its major metabolites in human plasma. It can be useful in bioavailability studies in humans as well as in clinical and toxicological monitoring of patients.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Schering Corporation, Montreal, Canada, for its financial support in carrying out this project as well as providing them with SCH-12679 capsules and authentic standards of SCH-12679 metabolites for both clinical and laboratory use. They also thank Merck Frosst Laboratories, Montreal, Canada, for supplying an authentic sample of protriptyline hydrochloride. Finally, they acknowledge the technical assistance of Miss Angelique Bordeleau.

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Journal of Chromatography, 163 (1979) 57—63
Biomedical Applications

CHROMBIO. 318

CIMETIDINE ASSAY IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY

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(First received October 16th, 1978; revised manuscript received January 19th, 1979)

SUMMARY

An assay for the determination of cimetidine in human plasma is described. Cimetidine was extracted from alkalized plasma with ethyl acetate, washed once over hydrochloric acid, re-extracted into ethyl acetate, and the organic phase was evaporated to dryness. The residue was dissolved in ethanol and injected into a liquid chromatograph.

In vitro sulphoxidation was found to occur in whole blood, for which reason the assay was performed in plasma. The accuracy of the method was found to be within 3% and the lower limit for sensitivity was demonstrated to be 0.1 mg/l using 750 μl plasma.

Five volunteers received 1 g cimetidine perorally per day given in four doses with various intervals. Blood samples were drawn hourly, five dose intervals over two days. The average minimum concentration of plasma cimetidine was found to correlate significantly with the mean value of the area under the time/concentration curve over a period of three dose intervals ($r = 0.96$).

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INTRODUCTION

Cimetidine (CMT) (Tagamet^R SKF), a non-thiourea H_2-receptor antagonist, was recently introduced as a potent inhibitor of gastric acid secretion; for a complete review, see Brogden et al. [1]. It has accordingly proved to be highly efficient in the treatment of patients with peptic ulcer. The degree of inhibition of the acid secretion has been shown to be dose-dependent [2], and measurements of CMT plasma levels may therefore be clinically important, as these may function as a more reliable expression of the dose. However, a prerequisite is that a correlation has been established between the effect (e.g. measured as the reduction of gastric acid secretion) and the amount of CMT in plasma, which again requires a dependable chemical assay of CMT.

Procedures for measurements of the plasma concentration of CMT and its metabolite cimetidine sulphoxide have been published recently [3, 4]. This paper reports a liquid chromatographic method for measuring CMT in plasma using a simplified extraction procedure, as compared to the above mentioned methods [3, 4]. Furthermore, a schedule for blood sampling which provide representative areas under time/concentration curve in plasma is described.

EXPERIMENTAL

Reagents

Ethyl acetate from E. Merck (Darmstadt, G.F.R.) was of analytical-reagent grade. Sodium hydroxide (6 *N*), hydrochloric acid (0.02 *N*) and a saturated sodium chloride solution were all prepared in our laboratory.

Reference substances

Stock solutions (1 g/l) in distilled water of CMT; of the internal standard, metiamide, and of cimetidine sulphoxide, all from Smith, Kline & French (Welwyn Garden City, Great Britain) were prepared. The solutions were kept in a refrigerator and stored in this way, they were stable for at least one year.

Extraction procedure

To a centrifuge tube containing 750 μl plasma, 2 μg of the internal standard were added. To the sample were added 100 μl 6 *N* sodium hydroxide and this was extracted with 6 ml of ethyl acetate by mixing for 5 min in a rotary mixer (20 rpm). After centrifugation for 5 min, the organic phase was transferred into a 10-ml glass-stoppered tube containing 1 ml of 0.02 *N* hydrochloric acid and 1 ml of saturated sodium chloride. The compounds were extracted into the aqueous phase by mixing for 10 min. After centrifugation the organic phase was discarded. The aqueous phase was made alkaline by adding 100 μl 6 *N* sodium hydroxide solution. The compounds were extracted into 3 ml of ethyl acetate by mixing for 5 min. After centrifugation the organic phase was transferred into a tapered tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of ethanol, and 25 μl of this solution were injected into the chromatograph.

Liquid chromatography

A liquid chromatograph (Pye Unicam, Cambridge, Great Britain) type LC 3 equipped with an ultraviolet detector type LC 3 was used. The column (25 cm × 4.6 mm I.D.) was filled with Partisil^{R} 10-ODS, particle size 10 μm. The mobile phase was acetonitrile—water—ammonia (1000:50:1) with a flow-rate of 2.5 ml/min. The detection was carried out at 228 nm.

Calculations

The plasma concentrations were read from standard curves constructed from chromatograms of plasma samples containing varying, but known amounts of CMT giving concentrations from 0.50 to 4.00 mg/l. The peak height ratios between CMT and metiamide were plotted against the concentrations.

Volunteers

Five subjects (all males, age 18—24 years) consented to participate in a short term CMT study after careful information. They received 1 g CMT daily starting 24 h prior to the study. Blood samples were drawn hourly for two days during a total of five dose intervals (cf. Fig. 3).

RESULTS

Assay

Fig. 1 illustrates chromatograms of two plasma samples containing CMT in a concentration equal to 1.0 mg/l (left) and a blank plasma sample (right). The peak representing CMT appeared 3.2 min after the injection.

Table I gives the ratio (R) between the CMT peak height and the metiamide peak height together with the corresponding concentration of CMT.

Accuracy and recovery tests for CMT were performed on seventy plasma samples with concentrations from 0.5 to 4.0 mg/l. No concentration deviated more than 3% from the mean value (Table II). The calibration graph was constructed from ethanolic solutions containing CMT in known concentrations. The recovery was almost 100%. The lower limit for safe quantitation (sensitiv-

TABLE I

RATIO (R) BETWEEN THE PEAK HEIGHTS OF CIMETIDINE AND METIAMIDE FOR DIFFERENT CIMETIDINE PLASMA CONCENTRATIONS

For each concentration the number of samples was ten.

Concentration added (mg/l)	R*
0.50	0.16 ± 0.01
1.00	0.31 ± 0.02
1.50	0.48 ± 0.01
2.00	0.63 ± 0.02
2.50	0.81 ± 0.01
3.00	0.96 ± 0.01
4.00	1.28 ± 0.02

*Mean ± S.D.

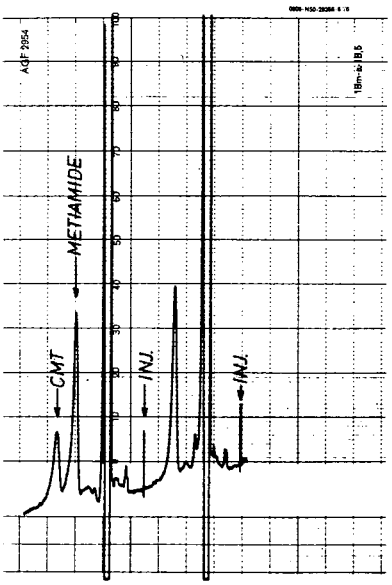
TABLE II

ACCURACY TEST FOR CIMETIDINE FROM PLASMA

The plasma volume extracted was 750 μl. For each concentration the number of samples was ten.

Concentration added (mg/l)	Calculated concentration (mg/l)*
0.50	0.51 ± 0.01
1.00	1.00 ± 0.03
1.50	1.49 ± 0.03
2.00	2.02 ± 0.01
2.50	2.49 ± 0.03
3.00	3.01 ± 0.02
4.00	4.02 ± 0.01

*Mean ± S.D.



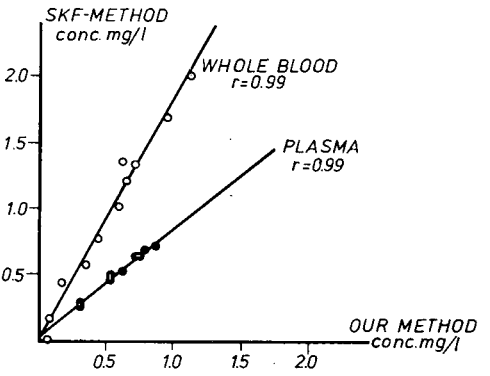


Fig. 1. Chromatograms of two plasma samples. The chromatogram to the right illustrates a blank plasma sample with added internal standard. The left one illustrates a plasma sample with added CMT in a concentration equal to 1.0 mg/l.

Fig. 2. Correlation of the Smith, Kline & French method and our method, carried out using plasma as well as whole blood samples.

ity) was found to be 0.1 mg/l, with a reproducibility of 10%, when 750 μl plasma was used. Plasma samples from 30 patients not taking CMT did not contain compounds interfering with the assay.

Plasma and whole blood determinations

Fig. 2 demonstrates the correlation between measurements made from iden-

tical samples analyzed at the Smith, Kline & French research laboratory [3] and in our laboratory. Ten whole blood samples were obtained from patients treated in England. The only difference between the handling procedures up to the measurements was that the samples had thawed some time before arrival in Copenhagen, while the samples analyzed in England were thawed immediately before measuring. Our method gave systematically lower results, approximately 40%. Ten plasma samples from Danish patients were also analyzed in both laboratories and the results were identical. Therefore, known amounts of CMT were added to samples of blank whole blood and stored at room temperature for at least four hours. This procedure resulted in two peaks. The first peak represented CMT while the second peak occurring 21 min after injection was found to be identical with that seen after injection of CMT sulphoxide. The ratio we found between the retention times for CMT sulphoxide and CMT was similar to that found in the earlier investigation [4]. No attempt was made to measure the rate with which CMT was metabolized into the sulphoxide.

Plasma levels and areas under the time/concentration curves

The plasma CMT concentration found in the samples drawn in the morning of the first day of the investigation (9 h after a 400-mg dose) was on average 0.34 mg/l with no great difference between the five individuals (Fig. 3).

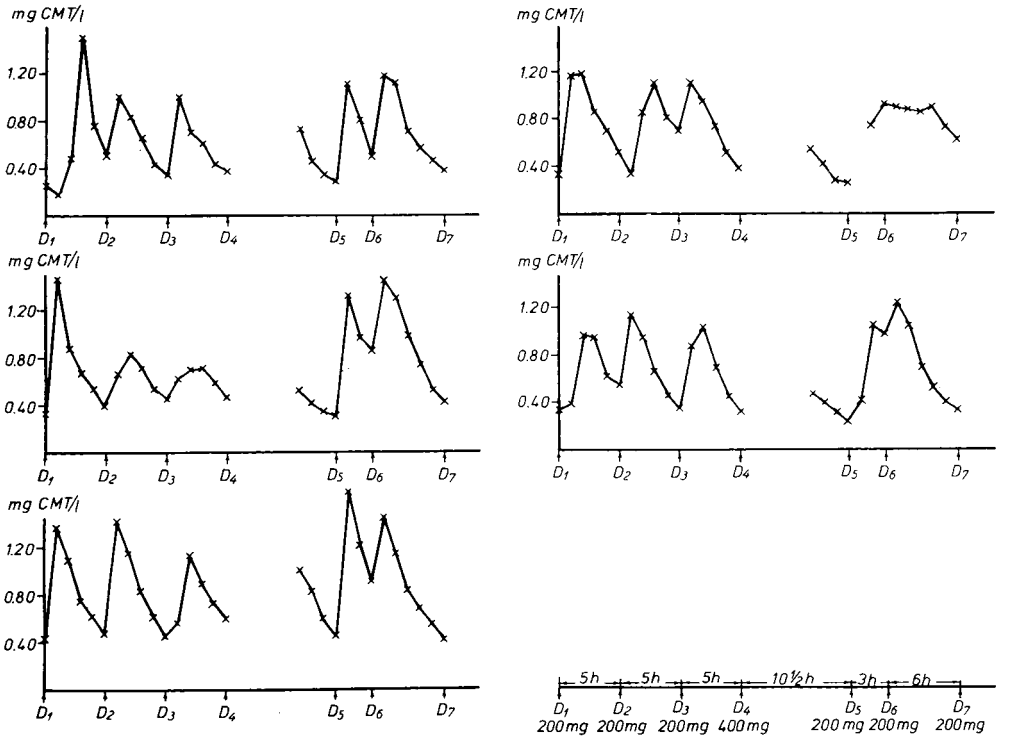


Fig. 3. Individual time/concentration curves for five volunteers after one day of peroral cimetidine ingestion prior to D_1. The dose 9 h before D_1 was 400 mg, the D_4 dose was also 400 mg, the other doses were 200 mg each.

The subsequent minimum concentrations on the first day (always 5 h after a 200-mg dose) came almost invariably very close to the morning value ($C_{D_1} = C_{D_2} = C_{D_3} = C_{D_4}$). This result indicates that steady-state conditions (in the pharmacokinetic meaning of this term) have been obtained. The peak concentrations, in contrast, showed great variations within the same individual and between individuals. Fig. 4 demonstrates that the average minimum concentration [$\overline{C}_{\text{min.}}$ (day 1) i.e. ¼ × ($C_{D_1} + C_{D_2} + C_{D_3} + C_{D_4}$)] was significantly correlated to the average area under the time/concentration curve (AUC), i.e. 1/3 × ($AUC_{D_1-D_2} + AUC_{D_2-D_3} + AUC_{D_3-D_4}$).

On the following day the five subjects continued on a total dose of 1 g CMT per day, but the dose fractions (200 mg) were given at non-equidistant intervals. Fig. 3 demonstrates considerable variations in the plasma concentrations, particularly with respect to the minimum concentrations. Consequently, a poor correlation was found between the average minimum concentrations and the average AUC.

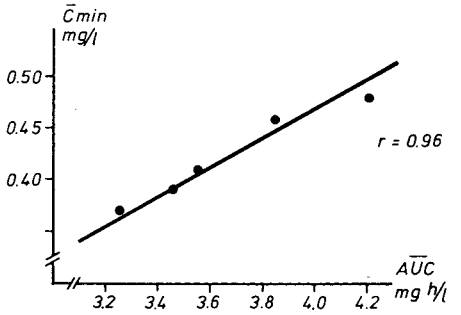


Fig. 4. Correlation between $\overline{C}_{\text{min.}}$ and $\overline{AUC}$ for cimetidine, given at equidistant dose intervals during one day.

DISCUSSION

A chemical method for determination of CMT in plasma may be of considerable clinical importance. The assay described in this paper has proved to be sufficiently sensitive and reliable for clinical use.

The problem of whether plasma or whole blood should be used has also been solved in this study. A liquid chromatographic method was early developed in the Smith, Kline & French (SKF) research laboratory [3], and the present assay is similar to the SKF method concerning the chromatographic system and the mode of detection. However, the procedures for extraction and isolation of the compound are quite different, but the specificity seems to be the same. Samples analyzed by both assays showed identical results for plasma (Fig. 2), whereas whole blood samples differ systematically probably due to in vitro sulphoxidation at room temperature in the presence of erythrocytes, a phenomenon previously described for other sulphur-containing compounds [5]. In order to avoid erroneous results CMT analysis should therefore be carried out only in plasma.

Marked individual variations in the plasma concentrations between two doses (reflected in AUC) as well as some variations in the minimum values might be foreseen. The resulting individually variable dose/concentration ratio would constitute a good reason for plasma level monitoring. The necessary pharmacokinetic calculations, based on steady-state conditions presume equal dose intervals. This schedule was used during the first day. However, in the calculations applied in Fig. 4 the C_{D_1} value was included, as the night dose (given 9 h earlier) was twice the normal dose.

The amount of drug on receptor site (in the secretory cells of the mucosa) is probably correlated to the amount of drug in the plasma, particularly during steady-state conditions. This amount can be expressed as the area under the time/concentration curve in plasma (AUC) between two doses. Determinations of the AUC during some dose intervals would, however, not be practical in daily clinical work. Another expression must therefore be sought. However, the peak concentration after each dose was not usable, as it was calculated on varying times and poorly reproduced from dose to dose. It is therefore of importance that in further clinical pharmacological investigations with CMT, the AUC covering three 5-h intervals of equal doses during the steady state, can be replaced simply by the average of the four concentrations measured just before each dose (Fig. 4).

On the second day, in which different dose intervals were used, as expected no correlation could be demonstrated between $\overline{C}_{\text{min.}}$ and $\overline{AUC}$.

ACKNOWLEDGEMENTS

Pure cimetidine, cimetidine sulphoxide, and metiamide were kindly provided by Smith, Kline & French, Welwyn Garden City, Great Britain. We are grateful for the skilful and accurate technical assistance of Mrs. Mariann Laustsen.

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Journal of Chromatography, 163 (1979) 64—71
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CHROMBIO. 315

Note

Reversed-phase high-performance liquid chromatography of prostaglandins — biological applications

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(Received November 23rd, 1978)

The isolation and purification of prostaglandins is increasingly important to studies of their physiologic or pharmacologic effects as the number of known compounds and variety of their actions increase. In the more commonly used thin-layer systems, resolution and recovery of prostaglandins is seldom optimal. The application of high-performance liquid chromatography (HPLC) to the isolation and purification of a large number of related prostaglandins in high yields has been shown to be uniquely suited to problems in this field of research from the view point of high resolution, short retention times, and good sample recovery [1, 2].

We recently reported HPLC methods using silicic acid columns for isolation of prostaglandins of purity suitable for gas chromatographic—mass spectrometric (GC—MS) analysis [1]. However, these methods are not suitable for analysis of a wide spectrum of prostaglandins since we have found that compounds such as PGE_1, PGE_2, 6-keto $PGF_{1\alpha}$, and thromboxane B_2 co-chromatograph in the solvent system reported.

Conventional reversed-phase chromatography has previously been applied to prostaglandin analysis [3], but adequate resolution has been hard to achieve and retention times are excessively long. Reversed-phase HPLC, however, offers both excellent resolution and short retention times. We have therefore extended the usefulness of HPLC methods by using reversed-phase chromatography in combination with adsorption chromatography for the isolation of several prostaglandins from the same biological matrix. These techniques have allowed us to investigate more thoroughly the products of arachidonic acid metabolism in various tissues and to analyze a wider range of prostaglandins in a single sample.

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MATERIALS AND METHODS

All solvents were glass distilled reagents from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). HPLC was done using two Waters Assoc. (Milford, Mass., U.S.A.) solvent delivery systems (Model 6000A) coupled to a solvent flow programmer (Model 660). The reversed-phase column and silicic acid columns were pre-packed μBondapak Fatty Acid Analysis and μPorasil columns respectively manufactured by Waters Assoc. (both column materials were 10-μm particles). The packing material for the Fatty Acid Analysis column is proprietory information (Waters Assoc.).

Unlabeled prostaglandins were obtained from Upjohn (Kalamazoo, Mich., U.S.A.). Labeled prostaglandins other than [^{3}H]6-keto-PGF$_{1\alpha}$, [^{3}H]PGD$_2$ and [^{3}H]thromboxane B$_2$ (TxB$_2$) were obtained from Amersham Searle (Arlington Heights, Ill., U.S.A.). Tritiated 6-keto-PGF$_{1\alpha}$, PGD$_2$ and TxB$_2$ were synthesized in our laboratory from tritiated arachidonic acid obtained from Amersham Searle [4]. [1-^{14}C]Arachidonic acid (55 mCi/mmole) was obtained from New England Nuclear (Boston, Mass., U.S.A.).

Liquid scintillation spectrometry was done using a Mark III instrument from Searle Analytic (Des Plaines, Ill., U.S.A.). GC—MS analysis was done on a Hewlett-Packard dodecapole Model 5980A mass spectrometer as previously described [5, 6] using glass columns (3 ft. × 2 mm I.D.) packed with 3% OV-1 (Supelco, Bellefonte, Pa., U.S.A.) operated isothermally at 250°. Prostaglandins were derivatized as previously described [6].

The PGE$_2$ methyl ester methoxime trimethylsilyl ether and PGF$_{2\alpha}$ methyl ester trimethylsilyl ether were quantitated by selected ion monitoring (SIM), using tetradeutero internal standards (Upjohn), at *m/e* 508 vs. 512 or *m/e* 494 vs. 498 respectively. PGE$_1$ was quantitated against hexadeutero-PGE$_1$ (Upjohn) as previously reported [7]. For analysis of 6-keto-PGF$_{1\alpha}$, the methylester methoxime trimethylsilyl ether was prepared and measured by SIM at *m/e* 598 vs. 605 against heptadeutero-6-keto-PGF$_{1\alpha}$ synthesized from 5,6,8,9,11,12,14,15-octadeuteroarachidonic acid [4].

Prostaglandin synthesis was studied in renal papillary slices prepared from normal male rats. The tissue was incubated with [^{14}C]-20:4 (Na^+ salt, 1 μCi, 5.5 μg) in 2 ml of Krebs—Ringer bicarbonate buffer (pH 7.4) containing 2 g/l of glucose under an atmosphere of oxygen—carbon dioxide (95:5). After 30 min the incubation mixture plus tissue was acidified and homogenized in 20 ml of chloroform—methanol (1:2). The mixture was filtered, evaporated to dryness (room temperature), redissolved in chloroform and purified for GC—MS by HPLC using both adsorption and reversed-phase chromatography.

For analysis of urinary prostaglandins, normal female Sprague Dawley rats were placed in metabolic cages. The urine was collected in vessels maintained at 0° and immediately a known amount of internal standards for SIM analysis was added. Prostaglandins were extracted from the acidified urine with chloroform, purified by HPLC (adsorption and reversed-phase), derivatized and quantitated by SIM.

RESULTS AND DISCUSSION

The silicic acid (Fig. 1) and reversed-phase (Fig. 2) columns were standardized using a mixture of tritiated prostaglandin standards (1—2 μg of each plus 0.05—0.1 μCi of the tritiated compound). The separation achieved is primarily due to differences in polarity of the prostaglandins; however, other effects are important on the silicic acid column since prostaglandins of dissimilar polarity co-elute in the organic solvents used. Fractions containing unresolved prostaglandins from the silicic acid column were subsequently run on the reversed-phase column which allowed the complete separation and isolation of a wide range of prostaglandins. Through both columns the recovery is normally 70—80% of the initial sample. Table I gives a summary of the average retention volumes of prostaglandins on each column.

These chromatographic techniques were employed in experiments designed to study prostaglandin synthesis in rat renal papillary slices. In Fig. 3A, the chromatographic pattern of ^{14}C-labeled compounds synthesized from [^{14}C]-20:4 is shown. In these experiments approximately 5% of the labeled arachidonic acid was converted to prostaglandins by 50 mg of papillary slices. Again

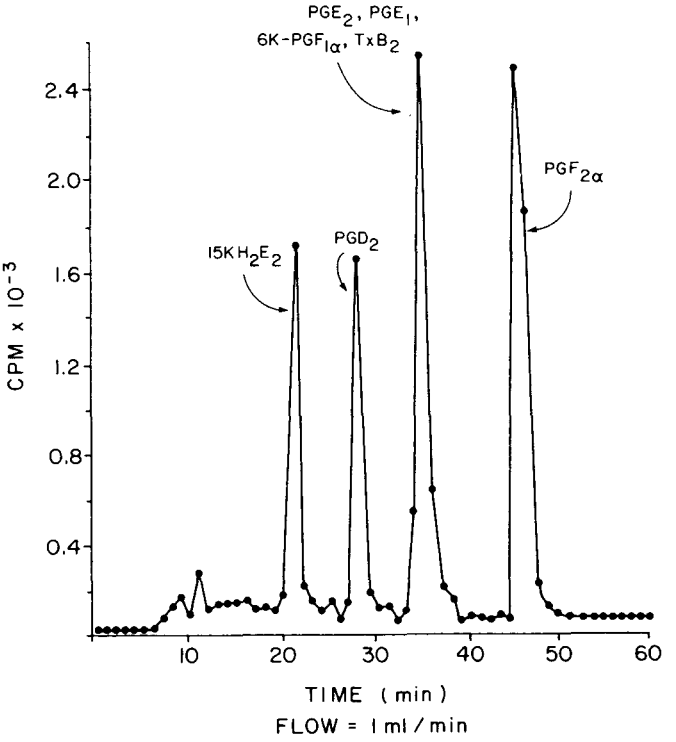

Fig. 1. Chromatogram produced by HPLC of prostaglandins (1—2 μg or 0.05—0.1 μCi of tritiated compound) as free acids on a silicic acid column (μPorasil). The sample was dissolved in 0.25 ml of chloroform, applied to the column and eluted by a 60-min linear gradient from chloroform to 6% methanol and 0.6% acetic acid in chloroform. The flow-rate was 1 ml/min and 1-min fractions were collected and assayed for radioactivity. In other experiments, the identity of eacn compound was confirmed by GC—MS.

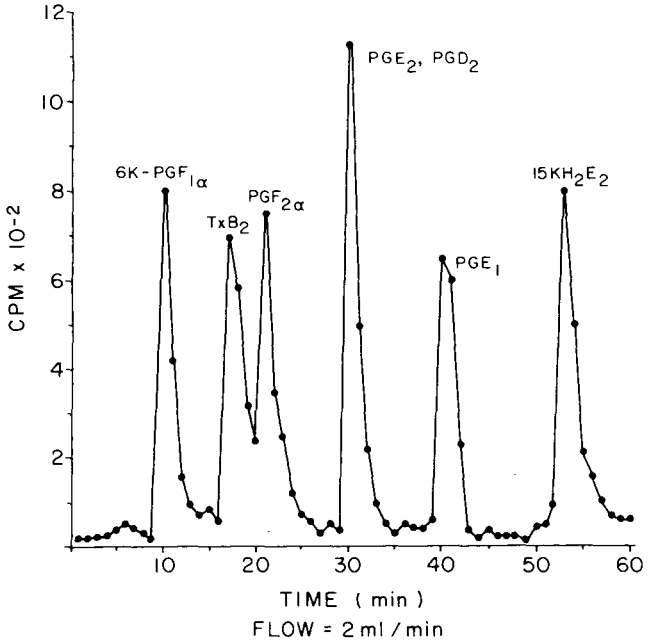

Fig. 2. Chromatogram produced by HPLC of prostaglandins (1—2 μg of 0.05—0.1 μCi of tritiated compound) as free acids on a reversed-phase column (μBondapak Fatty Acid Analysis). The sample was dissolved in 0.5 ml of the column solvent and eluted isocratically using a mixture composed of water—acetonitrile—benzene—acetic acid (76.7:23:0.2:0.1). The flow-rate was 2 ml/min and 1-min fractions were collected and assayed for radioactivity. In other experiments, the identity of each compound was confirmed by GC—MS.

TABLE I

AVERAGE RETENTION VOLUME OF PROSTAGLANDINS ON HPLC

Prostaglandins were chromatographed on silicic acid (μPorasil) columns as free acids or methyl esters using a linear gradient of chloroform to 6% methanol and 0.6% acetic acid in chloroform in 60 min or a linear gradient of chloroform to 5% methanol in chloroform in 50 min respectively, with a flow-rate of 1 ml/min in both cases. Reversed-phase (Fatty Acid Analysis column) chromatography of prostaglandins as the free acid was done isocratically using the solvent system acetonitrile—benzene—acetic acid—water (23:0.2:0.6:76.7) and the methyl esters were run isocratically using acetonitrile—benzene—water (28:0.2:71.8). Flow-rate for reversed-phase chromatography was 2 ml/min. Data represent peak center mean ± range of 3 runs. The peak center for compounds chromatographed on silicic acid varied less than ± 1 fraction while the center varied less than ± 2 fractions on the reversed-phase column.

Prostaglandin	Average retention volume (ml)			
	Silicic acid		Reversed-phase	
	Free acid	Methyl ester	Free acid	Methyl ester
15-keto-H_2E_2	22	19	106	106
PGD_2	28	29	60	60
PGE_2	35	38	60	60
PGE_1	35	38	82	82
TxB_2	35	38	36	36
6-keto-$PGF_{1\alpha}$	35	38	20	20
$PGF_{2\alpha}$	45	49	42	42

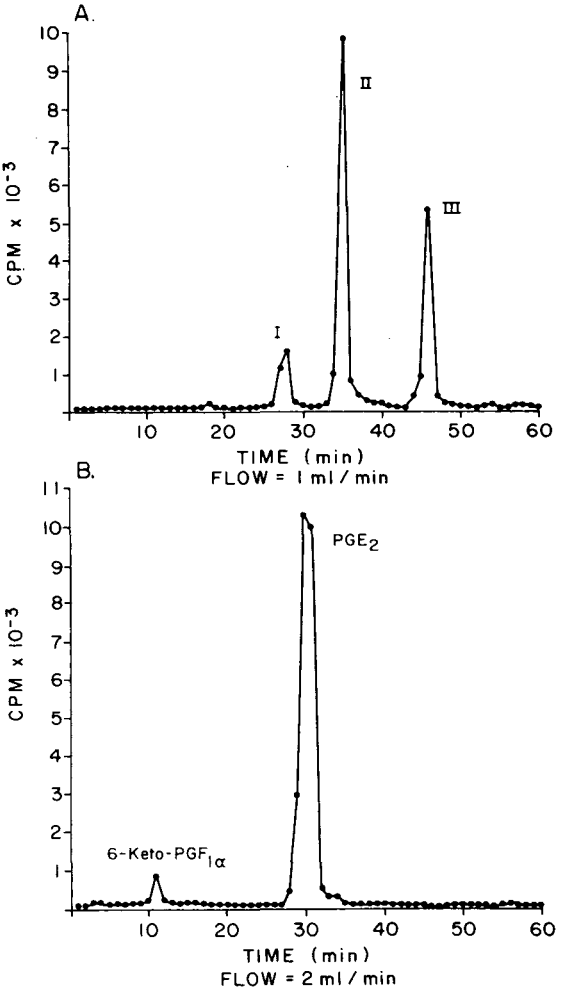

Fig. 3. (A) Silicic acid HPLC of labeled prostaglandins isolated from incubations of [^{14}C]-20:4 with rat renal papillary slices. Incubations were as described in the text. Labeled prostaglandins were isolated and chromatographed as in Fig. 1. The fractions (34—36) containing prostaglandin E_2 were collected and re-chromatographed as in Fig. 3B. (B) Reversed-phase HPLC of the peak containing prostaglandin E_2 from the silicic acid column (Fig. 3A). Fractions 29—32 contained prostaglandin E_2 and fractions 9—11 contained 6-ketoprostaglandin $F_{1\alpha}$. Conditions were as in Fig. 2.

the fractions containing PGE_2 were further analyzed for 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 on the reversed-phase column (Fig. 3B). Under these experimental conditions 54 ± 4% of the prostaglandins produced was PGE_2, 30 ± 3% was $PGF_{2\alpha}$, 13 ± 1% was PGD_2, and 2 ± 1% was 6-keto-$PGF_{1\alpha}$ (mean ± S.D., n = 4). No thromboxane B_2 was detected in these experiments. The combination of adsorption and reversed-phase chromatography using these methods allows the complete resolution of all the prostaglandins studied.

Analysis of biological samples requiring isolation and derivatization of prostaglandins is improved with the combination of these two chromatographic

columns, particularly for the analysis of PGE_2 and $PGF_{2\alpha}$. Fig. 4A shows a SIM tracing at *m/e* 508 vs. 512 for PGE_2 isolated from normal rat urine by silicic acid alone. As can be seen, several other peaks are present in addition to those due to urinary PGE_2. Another aliquot of the same sample was run over both silicic acid and reversed-phase columns. Only the major and minor isomers of the PGE_2 derivative are seen in the SIM tracing of the latter sample (Fig. 4B). demonstrating the improved selectivity of these methods as judged by GC—MS. With these techniques, normal female rat urines were analyzed for PGE_2, $PGF_{2\alpha}$, PGE_1 and 6-keto-$PGF_{1\alpha}$. Using the silicic acid column, the

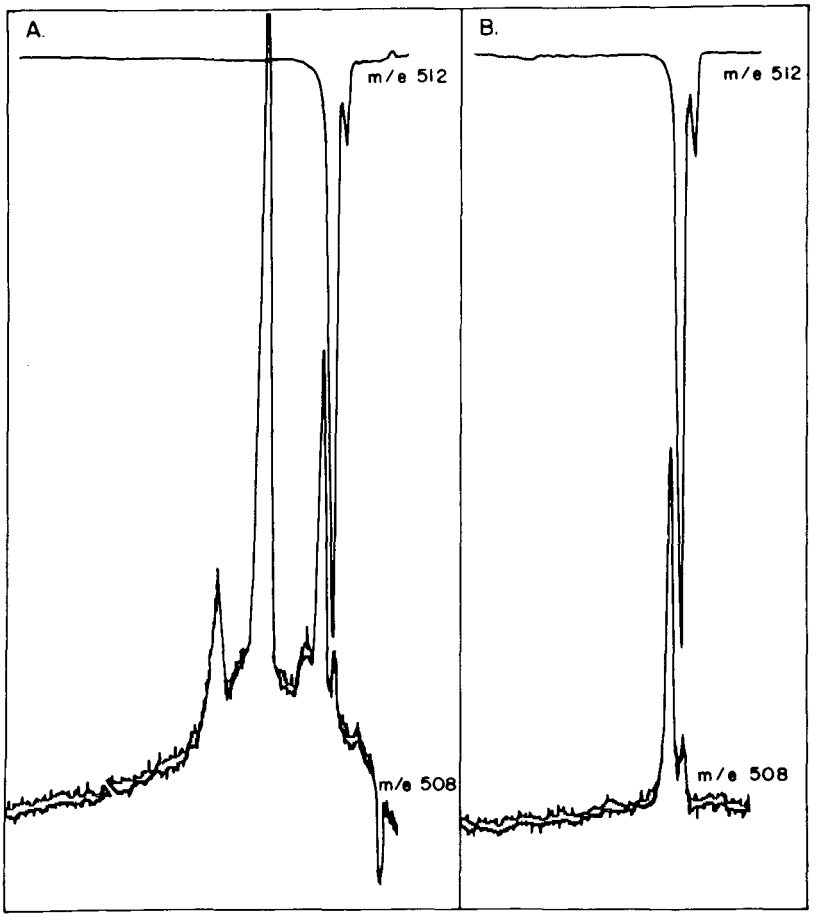

Fig. 4. SIM tracings of PGE_2 methyl ester methoxime trimethylsilyl ether from 6 ml of rat urine. Upper tracings represent the intensities of the ion *m/e* 512 ($M^+ -31$ for the tetra-deutero-PGE_2 derivative) and the lower tracings represent the intensities of the ion at *m/e* 508 ($M^+ -31$ for the endogenous PGE_2 derivative). Tracings run from right to left with the *m/e* 508 channel being 20-fold more sensitive than the 512 channel. The intensity of the peak *m/e* 512 corresponds to 1.86 μg of deuterated PGE_2; thus, the 6-ml urine sample contained 53 ng of PGE_2. (A) SIM tracing of the urine sample chromatographed on a silicic acid column as in Fig. 1. (B) SIM tracing of another aliquot of the identical sample chromatographed on a silicic acid column (Fig. 1) followed by re-chromatography on a reversed-phase column (Fig. 2).

urine extract (tritiated and deuterated internal standards added) gave two peaks of radioactivity. One contained endogenous $PGF_{2\alpha}$ plus the labeled internal standard, while the other peak contained endogenous PGE_2, PGE_1 and 6-keto-$PGF_{1\alpha}$ plus their corresponding labeled internal standards. Both peaks were collected and re-chromatographed on the reversed-phase column from which individual prostaglandins were collected, derivatized and quantitated by SIM. Measurable levels of each of these prostaglandins were found in this group of rats and are given in Table II. The major prostaglandins in rat urine were PGE_2 and $PGF_{2\alpha}$. Smaller amounts of PGE_1 and 6-keto-$PGF_{1\alpha}$ were also measured.

TABLE II

URINARY PROSTAGLANDINS FROM NORMAL FEMALE RATS

Urine was collected at 0° from normal female rats. Deuterated and tritiated prostaglandin standards were added and the sample was extracted, purified by HPLC and quantitated by SIM. Data represent mean ± S.E.M.

Prostaglandin	*n*	ng/24 h
PGE_2	9	130 ± 15
$PGF_{2\alpha}$	6	71.9 ± 3.5
6-keto-$PGF_{1\alpha}$	6	15.0 ± 2.6
PGE_1	8	7.5 ± 2.7

The importance of these combined techniques to the analysis of a variety of prostaglandins from biological samples can be readily seen. Processing samples through both columns results in the complete resolution of all prostaglandins studied. Methylation of the prostaglandins results in a greater difference in polarity between the prostaglandin of interest and interfering compounds. Isolation of prostaglandins based on the differing characteristics of these two chromatographic materials results in a relatively pure sample, free from the overwhelming quantity of contaminants frequently found after adsorption chromatography. Thus, these methods are particularly applicable to quantitative analysis by GC—MS and should improve quantitation by radioimmunoassay techniques by removing compounds which may interfere.

ACKNOWLEDGEMENTS

The authors wish to thank Christiana Ingram and Margaret Cameron for technical assistance. The work was supported by NIH grants HL-14192 and GM-15431. Prostaglandins (unlabeled and deuterated) were kindly supplied by Dr. J. Pike of the Upjohn Company.

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Journal of Chromatography, 163 (1979) 72—76
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Note

Rapid analysis of tryptophan metabolites using reversed-phase high-performance liquid chromatography with fluorometric detection

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(First received July 18th, 1978; revised manuscript received December 13th, 1978)

The importance of tryptophan and its metabolites is a subject of increasing interest. The role of tryptophan as a precursor of serotonin and its implications in mental disturbances [1, 2], as well as in the mode of action of anti-rheumatic non-steroidal, anti-inflammatory drugs has been discussed in the literature [3, 4].

Tryptophan is metabolized via two major pathways: the kynurenine pathway and the serotonin pathway. An abnormal metabolism of tryptophan has been observed in patients suffering from a wide variety of diseases; altered levels of its metabolites have been found in patients with cancer of the breast [5, 6], bladder [7] and in Hodgkin's disease [8]. Patients afflicted by these diseases have higher than normal amounts of several metabolites along the kynurenine pathway while sub-normal levels of tryptophan metabolites have been detected in serum from uremic patients [9].

The tremendous physiological importance of tryptophan and its metabolites has stimulated the development of many analytical methods [10—13], most of which are either laborious or of inadequate sensitivity. In addition, the close structural and chemical similarity of these compounds has often resulted in poor separations.

Recent developments in microparticulate, chemically-bonded nonpolar phases have made possible rapid and reproducible high-performance liquid chromatographic (HPLC) analyses. Because of the complexity of the retention mechanism, it is possible to separate simultaneously compounds of a wide polarity range. The determination of biologically-important compounds at levels at which they occur naturally places great demands on HPLC detection systems. Fluorometric measurements offer great advantages over other commonly used detection systems in terms of sensitivity and selectivity. In addition, since few naturally-occurring compounds possess native fluorescence, interferences are not encountered as often as with the less selective detection systems.

Reported in this paper is the use of a reversed-phase partition mode of HPLC, coupled with a fluorometric detection system in the analysis of tryptophan and its metabolites.

EXPERIMENTAL

A Model 6000 A solvent delivery system, Model 660 solvent programmer and Model U6K universal injector, all from Waters Assoc. (Milford, Mass., U.S.A.), were used in all determinations. An FS 970 fluorescence monitor and an SF 770 Spectroflow monitor (Kratos Inc., Schoeffel Instrument Division, Westwood, N.J., U.S.A.) were connected in series and used for monitoring column effluents. The fluorescence monitor was equipped with an SFA 339 wavelength drive and an MM 700 memory module which were used for obtaining the excitation spectra.

A prepacked, stainless-steel column, μBondapak C_{18} (10 μm), was purchased from Waters Assoc.

Reagents

All reagents used were of the highest purity (A.C.S. certified grade). Reference compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.), and methanol, distilled in glass, from Burdic & Jackson (Muskegon, Mich., U.S.A.).

Chromatographic conditions

The low concentration eluent was a 0.02 *M* KH_2PO_4, pH 3.7, and the high concentration eluent was a mixture of anhydrous methanol and water (6:4, v/v). Eluents were filtered through Millipore membrane filters, Type HA, pore size 0.45 μm (Millipore, Bedford, Mass., U.S.A.) and degassed before use. A 35-min linear gradient from 0 to 100% of the high concentration eluent was used. The flow-rate was 1.5 ml/min, and the temperature was ambient in all cases.

Preparation of the serum samples

Freshly drawn human blood was collected in a tube without anticoagulant. The blood was allowed to clot spontaneously for 10—15 min at room temperature. The serum sample was deproteinated by the addition of 1 ml of cold, 6% (w/w) trichloroacetic acid (TCA) to each ml of serum. Samples were vortexed at moderate speed for 1 min and centrifuged at 630 *g* for 5 min. Excess acid was neutralized with solid tris(hydroxymethyl)aminomethane.

Identification of peaks in serum sample

Initial identification of chromatographic peaks in the serum sample was based on retention times and co-chromatography with the reference compounds. Further proof of the peak identity was obtained by comparing the stopped-flow excitation spectra of the reference compounds and the peaks under study [14].

RESULTS AND DISCUSSION

The reversed-phase HPLC separation of the synthetic mixture of tryptophan metabolites, detected by measuring their native fluorescence and UV absorbance is shown in Fig. 1. In spite of the fact that the fluorescence signals were highly attenuated, the enhancement in sensitivity compared to the UV absorption is obvious. Only kynurenine does not fluoresce naturally under the conditions used, and UV monitoring is mandatory for its detection.

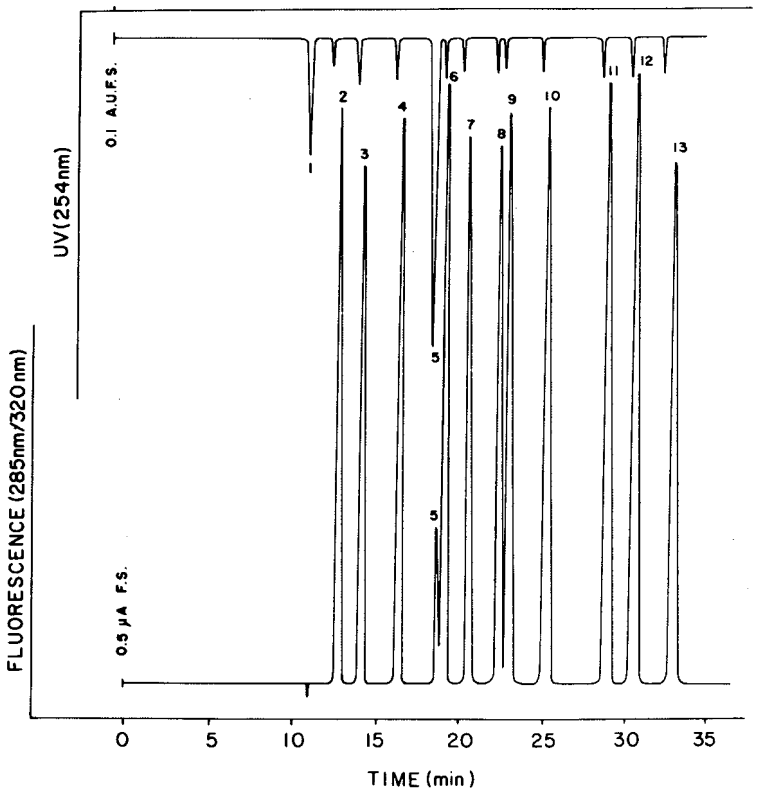

Fig. 1. Separation of tryptophan metabolites detected by a native fluorescence with an excitation wavelength of 285 nm and the emission cut-off filter of 320 nm. Column: μBondapak C_{18} (Waters Assoc.). Mobile phase: low concentration eluent, 0.02 *M* KH_2PO_4, pH 3.7; high concentration eluent, amhydrous methanol—water, 6:4 (v/v). Gradient: linear from 0 to 100% of the high concentration eluent in 35 min. Flow-rate, 1.2 ml/min at ambient temperature. Detection: fluorescence, 285 nm excitation, 320 nm cut-off filter; UV, 254 nm. Peaks: 1 = kynurenine (1.37 nm); 2 = 5-hydroxytryptophan (1.11 nm); 3 = serotonin (0.026 nm); 4 = tryptophan (1.73 nm); 5 = kynurenic acid (1.37 nm); 6 = tryptamine (0.995 nm); 7 = 5-hydroxyindole-3-acetic acid (2.766 nm), 8 = anthranilic acid (6.33 nm), 9 = indoleacetamide (1.62 nm), 10 = indole-3-lactic acid (1.36 nm), 11 = indole-3-acetic acid (1.74 nm), 12 = indole (2.10 nm) and 13 = indole-3-propionic acid (1.15 nm).

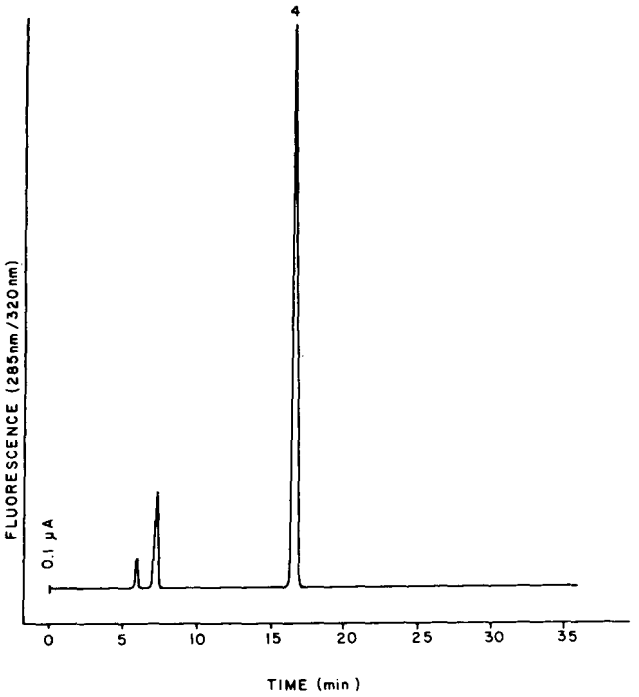

Fig. 2. Chromatogram of a serum sample from a normal subject. Chromatographic conditions and peak identity same as in Fig. 1. Volume of the TCA extract injected: 50 μl (25 μl of serum).

The described separation and detection methods were tested in the analysis of a serum sample from a normal subject (Fig. 2) and a patient with bladder cancer (Fig. 3). It should be noted that because of the use of an acidic protein precipitant (TCA), the total, rather than the free tryptophan was monitored.

This assay was also tested in the analysis of rat brain constituents where no interference with the other naturally-fluorescing compounds were observed.

In conclusion, the described HPLC method is fast, quantitative and the detection is sensitive and selective. The assay is well suited for routine testing of tryptophan metabolites in biological samples, and we believe that it will circumvent many of the problems commonly encountered in current methods of analysis.

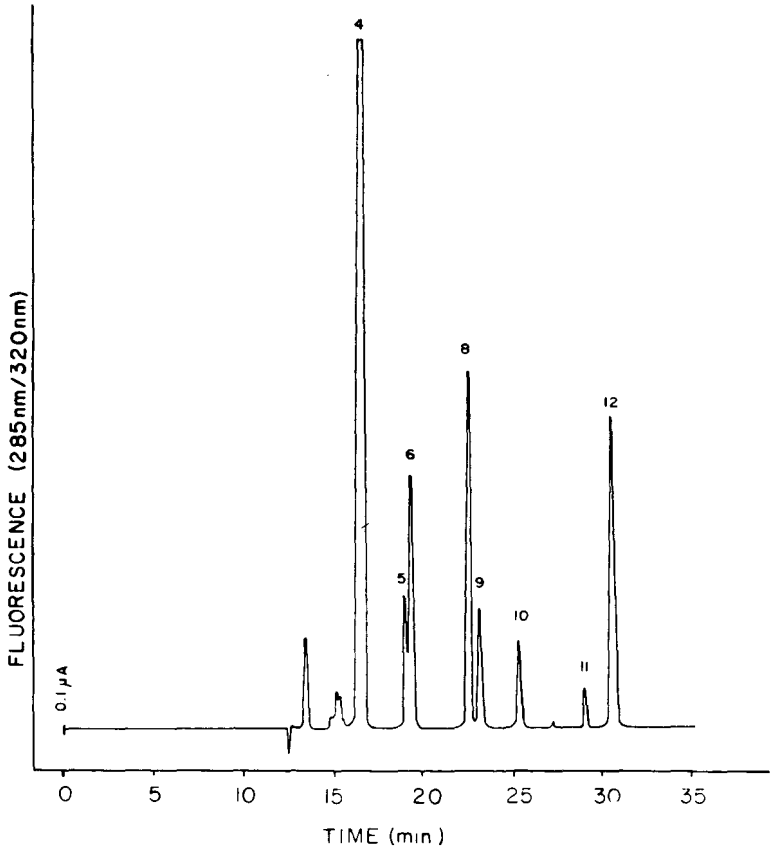

Fig. 3. Chromatogram of a serum sample from a patient with bladder cancer. Chromatographic conditions and peak identity as in Fig. 1. Volume of the TCA extract injected = 50 μl (25 μl of serum).

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Journal of Chromatography, 163 (1979) 77—80
Biomedical Applications

CHROMBIO. 306

Note

Simple and rapid separation of metanephrine and normetanephrine by reversed-phase high-pressure liquid chromatography

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The involvement of catecholamines in cardiovascular, psychiatric and neurological disease states has been the subject of growing interest during recent years [1]. Knowledge in these areas has increased following the development of radioenzymatic techniques sensitive to the picogram quantities found in biological fluids [2]. These methods depend on the enzymatic methylation of catecholamines using tritiated S-adenosyl-L-methionine as methyl donor and either catechol-O-methyl transferase or phenylethanolamine-N-methyl transferase as enzyme. Subsequently, the methylated derivatives are separated by thin-layer chromatography (TLC). The enhanced speed and accuracy of high-pressure liquid chromatography (HPLC) has resulted in attempts to interpose an HPLC, rather than a TLC, step in the assay methodology. One method uses a cation-exchange column [3] but the mobile phase has a pH considerably in excess of that recommended for this type of column and in our experience the column life is very short. Another technique employs ion-pair chromatography but requires critical column preparation, equilibration and temperature control [4].

We describe a simple and rapid method for the separation of normetanephrine and metanephrine using reversed-phase HPLC.

EXPERIMENTAL

Reagents and materials

All reagents necessary to perform the radioenzymatic assay of catecholamines were supplied in the form of a commercial kit (CAT-A-KIT™, Upjohn Diagnostics, Kalamazoo, Mich., U.S.A.). This kit includes a stopping solution containing unlabelled metanephrine, normetanephrine and methoxytyramine in the concentration of 4 m*M* to act as carrier for the radiolabelled reaction products. The water used for chromatography was glass distilled. Methanol used in the chromatography was "distilled in glass" quality, purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.). Heptane sodium sulphate was purchased from Eastman Kodak (Rochester, N.Y., U.S.A.). Omnifluor was purchased from New England Nuclear (Los Angeles, Calif., U.S.A.). All other solvents and reagents were reagent-grade quality. The amyl alcohol was prewashed with 5% phosphoric acid and rinsed with water.

Sample preparation

Only 20 μl of plasma are required for this assay. Norepinephrine and epinephrine are radioenzymatically converted to normetanephrine and metanephrine using tritiated S-adenosyl-L-methionine as the methyl donor and catechol-O-methyl transferase as the enzyme. The reaction is allowed to proceed for 1 h at 37° in the presence of buffer containing 2-amino-2(hydroxymethyl)-1,3-propanediol (tromethamine), ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) and magnesium chloride. The reaction is terminated with stopping solution containing borate buffer, unlabelled metanephrine, normetanephrine and methoxytyramine. Extraction is performed into 2 ml of a mixture of toluene in amyl alcohol (3:2) with back extraction into 20 μl 0.1 *N* sulphuric acid. The entire acid phase was injected into the chromatograph.

Chromatography

A Varian 8500 dual-pump high-pressure liquid chromatograph was used, fitted with a Varian Micro-Pak MCH-10 (Monomeric C18 bonded phase) reversed-phase column (25 cm × 2.0 mm I.D.). Pump A contained a 0.01 *M* solution of 1-heptanesulphonic acid sodium salt in water adjusted to pH 3.5 with glacial acetic acid. Pump B contained the same concentrations of salt and acid in methanol. Both solvents were filtered through Whatman No. 2 paper using a vacuum. An isocratic mixture containing 10% B with a flow-rate of 50 ml/h and a precolumn pressure of 1500 p.s.i. was used for analysis. A Vari-Chrom® detector was used to measure absorbance at 280 nm using an 8-nm band width. Chromatograms were recorded on a Varian 9196 recorder with a 100 mV span set at a chart speed of 1.0 cm/min. Effluent was collected under each peak from the outlet of the detector.

Subsequent analysis

The samples were brought to pH 10 with 0.05 *M* ammonium hydroxide and then oxidised with sodium meta periodate [2]. Following acidification with 0.1 *M* acetic acid the oxidation products were extracted into 8 ml of toluene. The aqueous phase was frozen in an acetone—dry-ice bath and the toluene

decanted into a scintillation vial containing 0.6 ml Omnifluor^R in the concentration 10 g of Omnifluor per 100 ml toluene. The scintillating fluid was brought to a volume of 15 ml with additional toluene before counting.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the normetanephrine, metanephrine and methoxytyramine peaks occur at 3.8, 6.0 and 9.5 min. As shown in Fig. 1, the chromatographic separation is excellent and allows accurate collection of effluent.

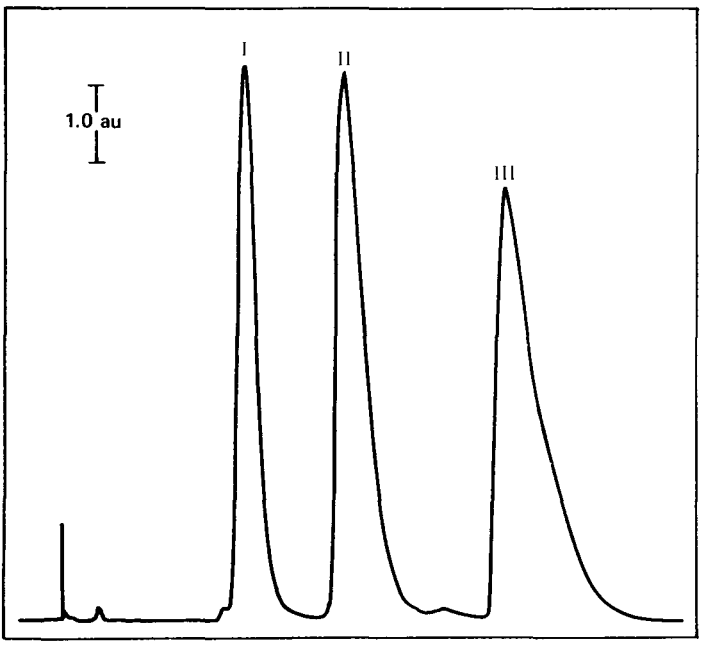

Fig. 1. Chromatogram of methylated catecholamines under the conditions described in the text. I, Normetanephrine 3.8 min; II, metanephrine 6 min; III, methoxytyramine 9.5 min.

Although the methoxytyramine peak is well defined, an unidentified radioactive product of the enzymatic reaction contaminates the effluent collected under this peak and prevents the accurate measurement of very low concentrations of dopamine. However, this method has given reliable results for both epinephrine and norepinephrine when used to analyze plasma containing known concentrations (Table I).

The disadvantage of using TLC in this as in other assays rests on the relatively poor resolution obtained because of tailing between the zones. This leads to coefficients of variation as high as 15% for interassay data [2]. HPLC has the advantage of high resolution which in our experience has resulted in an interassay coefficient of variation of 4%.

The enhanced accuracy of HPLC has led to several attempts at interposing an HPLC step in the radioenzymatic assay of catecholamines. The method we describe has the advantage of simplicity and reliability.

TABLE I

DATA OBTAINED USING METHOD DESCRIBED IN THE TEXT

	Normetanephrine (pg/ml)	Metanephrine (pg/ml)
Actual	840	580
Calculated	810	579
Actual	680	485
Calculated	662	463

ACKNOWLEDGEMENTS

We wish to thank Linda Halloran for preparation of the manuscript. Dr. Rubin is a Research Fellow of the American Heart Association. This work was supported by NIGMS grant GM22209 and NIH grant 1RO1-HL-21221.

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Note

Separation of bilirubin azopigments from bile by high-performance liquid chromatography

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(First received July 25th, 1978; revised manuscript received January 19th, 1979)

Recent reports from several laboratories have revealed that bilirubin is excreted in bile as a heterogenous group of relatively polar derivatives which arise by hepatic conjugation of bilirubin with a variety of carbohydrates and possibly other compounds as well [1—7]. Factors which influence the formation of bilirubin conjugates are of clinical interest with regard to a number of conditions which result in jaundice. Investigations of the nature of such factors should be materially aided by the ability to rapidly and specifically determine the various bilirubin conjugates in body fluids.

I have developed a high-performance liquid chromatography (HPLC) method for the resolution of *p*-iodophenylazo derivatives of bilirubin and its conjugates from dog bile. The method employs ion-pair chromatography on a reversed-phase column and is performed directly on the derivatized samples without intervening extractions or other preliminary purification steps.

EXPERIMENTAL

Chemicals

Reagent grade solvents were obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and were filtered through an 0.5-μm Fluoropore filter (Millipore) before use. β-Glucuronidase (type B-10 from bovine liver) and saccharic acid 1—3 lactone were obtained from Sigma (St. Louis, Mo., U.S.A.). Bilirubin standards were from American Monitor (Indianapolis, Ind., U.S.A.). All other chemicals were the best grade commercially obtainable and were used without further purification.

Apparatus

All experiments described herein employed an ALC 200 Series liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) equipped with a Model U6K injector and a Model 440 absorbance detector operating at a fixed wavelength

of 546 nm. The reversed-phase column (30 cm × 4 mm I.D.) was obtained prepacked with μBondapak C_{18} (10 μm) from Waters Assoc.

Preparation of samples

Bile was collected from the gall bladders of anesthetized dogs via a hypodermic needle and immediately expelled into a foil-covered, screw-capped tube and frozen. The frozen bile was stored at -20°C until used. Preparation of *p*-iodophenylazo derivatives of bilirubin was accomplished by mixing 100 μl of bile with 100 μl of acetonitrile followed by 200 μl of methanol—ethyl acetate (1:1) and 50 μl of diazo reagent (diazotized *p*-iodoaniline). After allowing the reaction to proceed on ice for 30 min, 25 μl of ascorbic acid (100 mg/ml) were added to destroy excess diazo reagent. The samples were centrifuged for 5 min in an Eppendorf micro centrifuge to remove suspended matter, and aliquots of the supernatant were chromatographed. The diazo reagent was prepared as follows [8]: 75 μl of sodium nitrite (100 mg/ml) was mixed with 100 μl of 2 *M* *p*-toluenesulfonic acid, and 0.5 ml of *p*-iodoaniline (21 mg/ml in glacial acetic acid) was added. This mixture was allowed to stand for 2 min then diluted with 2.5 ml of water followed by 50 μl of 1.5 *M* ammonium sulfamate. The reagent was used after being allowed to stand on ice for 5 min.

β-Glucuronidase hydrolysis

In order to identify which components were susceptible to hydrolysis by β-glucuronidase, bile samples were incubated with the enzyme before derivatization. The reaction mixtures consisted of 100 μl of β-glucuronidase (2 mg/ml), 100 μl of 0.2 *M* citrate—phosphate buffer (pH 5.6) and 75 μl of bile. Samples were incubated for varying periods of time at 30°, then aliquots of the incubation mixtures were derivatized and chromatographed as described above. To demonstrate inhibition by saccharic acid 1—3 lactone, a buffered 1 m*M* solution of the inhibitor was substituted for the buffer in the incubation mixtures. Each sample incubated with β-glucuronidase was accompanied by a control incubated without enzyme.

HPLC operating conditions

Separations were performed at ambient temperature with a flow-rate of 2.0 ml/min (column inlet pressure 1500—3000 p.s.i.). The solvent was a solution of acetonitrile—ethyl acetate—methanol—water (1.0:1.1:1.5:3.5) containing 1.4 ml of tetrabutyl ammonium phosphate solution (Waters Assoc. PIC-A) per 100 ml of solvent. The volume of sample injected was generally 5 μl.

RESULTS

HPLC of azopigments from dog bile yielded 9 major peaks (Fig. 1A). Fig. 1B is a chromatogram of a commercial bilirubin standard treated in the same manner as the bile sample. Bilirubin yielded two azopigment peaks whose retention times were the same as peaks 4 and 5 from bile.

As a first step toward establishment of the identities of the other components separated, their susceptibility to hydrolysis by β-glucuronidase was investigated.

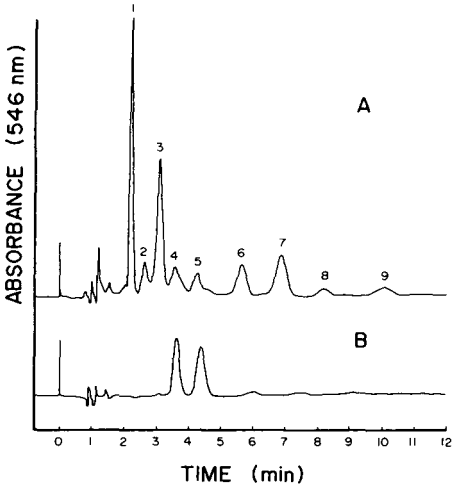
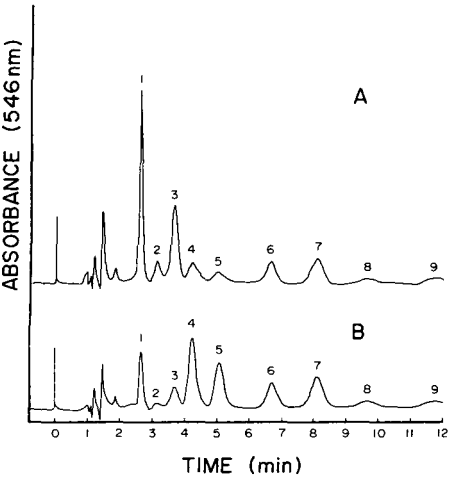


Fig. 1. (A) Separation of bilirubin azopigments from dog bile. (B) Bilirubin standard (20 mg/dl) treated in same manner as bile. Column packing μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s.; eluent, acetonitrile—ethyl acetate—methanol—water (1.0:1.1:1.5:3.5) with 1.4 ml of tetrabutyl ammonium phosphate (Waters PIC-A) per 100 ml; flow-rate, 2.0 ml/min.

Figs. 2 and 3 show the results of these investigations. The sample depicted in Fig. 2A was a control incubated for 2 h without enzyme and Fig. 2B shows the results obtained from bile incubated for 30 min in the presence of β-glucuronidase. It is apparent that the quantity of azopigments represented by peaks 1, 2,



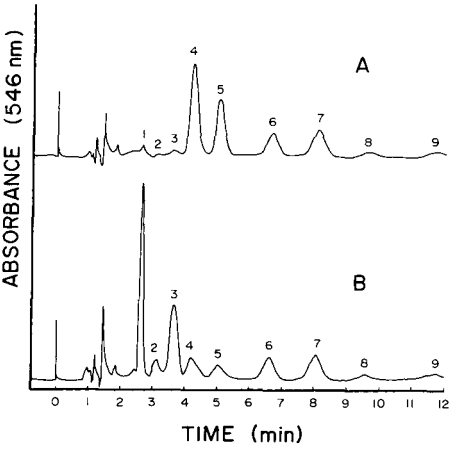


Fig. 2. (A) Control bile sample incubated at 30° for 2 h without enzyme. (B) Bile sample incubated with β-glucuronidase for 30 min at 30°. Other conditions same as in Fig. 1.

Fig. 3. (A) Bile sample incubated with β-glucuronidase for 2 h at 30°. (B) Bile sample incubated with β-glucuronidase plus saccharic acid 1—3 lactone for 2 h at 30°. Other conditions same as in Fig. 1.

and 3 was diminished while components 4 and 5 were increased. Incubation with β-glucuronidase for 2 h resulted in the virtual disappearance of peaks 1, 2, and 3 with a further increase in the magnitude of peaks 4 and 5 (Fig. 3A). When bile was incubated for 2 h with β-glucuronidase plus saccharic acid 1—3 lactone (Fig. 3B) no changes were observed relative to the control.

DISCUSSION

It is customary in investigations of bilirubin conjugates to couple the bile pigments with diazonium salts to produce azopigments of greatly enhanced stability. While this approach has the disadvantage of being unable to distinguish monoconjugated from diconjugated bilirubin, it does permit identification of the various moieties with which bilirubin is conjugated [9]. Previous investigators have employed solvent extraction combined with column chromatography and thin-layer chromatography to separate the various azopigments [2, 3]. Such multistep approaches inevitably result in incomplete yields making quantitation unreliable, and increase the risk of introducing artifacts. By means of the HPLC method reported here, the azopigments are separated in a single step requiring no preliminary fractionation.

In the coupling of bilirubin with diazonium salts the molecule is cleaved into two isomeric dipyrroles [9]. The present system is capable of resolving these isomers as shown in Fig. 1B. Peaks 6—7 and 8—9 probably represent isomeric conjugated azo dipyrroles. The nature of the conjugating moieties is presently unknown. The findings of Fevery et al. [3] suggest that they are probably glucose and xylose.

The predominant form in which bilirubin is excreted in bile is the diglucuronide, with lesser amounts of bilirubin monoglucuronide being present [4, 10]. Components 1, 2, and 3 in our system were susceptible to hydrolysis by β-glucuronidase. This does not unequivocally establish their identity as simple glucuronides. However, since peaks 1 and 3 are quantitatively the major constituents they probably represent the vinyl and isovinyl isomers of azodipyrrole glucuronic acid [1, 4—6]. Peak 2 is quantitatively minor. Since it is susceptible to the action of β-glucuronidase, constituent 2 may be the same as pigment B6 reported by Kuenzle [2], a pseudoaldobiouronide. Experiments designed to unequivocally identify all of the components separated are currently in progress.

ACKNOWLEDGEMENTS

The expert technical assistance of Mrs. Janice C. Brady is gratefully acknowledged. This research was supported by a Basil O'Connor Starter Research Grant from the National Foundation March of Dimes.

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Note

Eine rationelle, quantitative Bestimmungsmethode dünnschichtchromatographisch getrennter Lipide des Serums

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(Eingegangen am 13. Oktober 1978)

Zur Diagnostik von Stoffwechselerkrankungen aus dem Serum gewinnt, trotz einer grossen Zahl an automatisierbaren chemischen und enzymatischen Methoden zur Bestimmung einzelner Lipidparameter, die Dünnschichtchromatographie (DC) als sehr leistungsfähige Methode an Bedeutung [1—4]. Die Entwicklung hat ihre Ursache in einer verbesserten Gerätetechnik zur densitometrischen in-situ-Auswertung [2, 5] der entwickelten Chromatogramme, einer vereinfachten Lipidextraktion [1], einer verbesserten DC [6] sowie einer besseren Richtigkeit durch Einführung objektiver Korrekturfaktoren [3, 7].

Als wesentlicher Vorteil der DC gegenüber chemischen bzw. enzymatischen Einzelbestimmungen gilt, dass die Bestimmung aller wesentlichen Lipidparameter in einem Arbeitsgang mit der gleichen Präzision erfolgt.

VERSUCHE ZUR METHODE

Extraktion

Die allgemein übliche Lipidextraktion nach Folch et al. [8] wird aus zweierlei Gründen abgelehnt, die Mischungslücke zwischen Extraktionsmittel und wässriger Phase führt zu Verlusten an wasserlöslichen Lipiden [9] und durch die Verwendung von Methanol besteht die Gefahr chemischer Umsetzungen, wie Reduktion und Umesterung [10].

Die von Segura und Gotto [1] vorgeschlagene Extraktion mit Isopropanol vermeidet diese Nachteile. Wir fanden in Übereinstimmung mit Dittmer et al. [11] bei dem Vergleich einer chemischen Cholesterinbestimmung nach Isopropanolextraktion [12] mit der enzymatischen Bestimmung [13] niedrigere Werte für die chemische Bestimmung (Heissextraktion: $y_{enzym.} = 6.74 + 0.88x$, $r = 0.847$ [11]. Kaltextraktion: $y_{enzym.} = 6.40 + 0.97x$, $r = 0.950$ [14]. Bei DC-Untersuchungen von Isopropanol-Lipidextrakten konnte ein Verlust an Cholesterinestern nachgewiesen werden. Dieser Verlust konnte durch Extraktion des

Serums mit dem fünfzehnfachen Volumen eines Isopropanol—*n*-Hexan-Gemisches (2.75:1, v/v) vermieden werden. Nach Eindampfen des Extraktes mit einem Vakuumrotationsverdampfer wurde der verbleibende Rückstand in Chloroform gelöst.

DC und Detektion

Von Van Gent [6] wurde eine leistungsfähige Zweistufenmethode auf Kieselgel mit verschiedenen Laufmitteln im Mikroformat beschrieben, die von Egge et al. [15] durch eine weitere chromatographische Entwicklung zur Entfernung der Phospholipoide vom Start modifiziert worden ist.

Abgesehen davon, dass zumindest bei bandförmigem Auftragen, eine Entfernung der Phospholipoide vom Start nicht notwendig ist, konnte auch nicht die angegebene Qualität dieser Trennungen erreicht werden. Eine sehr gute Trennung aller im Serum üblichen Lipidklassen wurde durch eine eindimensionale Zweifachenentwicklung mit unterschiedlicher Trennstrecke (Gesammtlaufstrecke: 5—6 cm), zuerst in Benzol—Dioxan (94:6, v/v), dann nach Zwischentrocknen in Tetrachlorkohlenstoff erhalten. Durch das zweite Laufmittel trennen sich Triglyceride und Cholesterinester, wobei es zu einer teilweisen Aufspaltung der Cholesterinesterbanden kommen kann. Als Sorbens wurden mit 10%iger Ammoniumsulfatlösung imprägnierte Kieselgelfolien der Fa. Merck (Darmstadt, B.R.D.; Art. Nr. 5553) verwendet. Fig. 1 zeigt ein solches Chromatogramm im Punkt- und Strichauftrag mit unterschiedlichen Mengen an Lipidextrakt nach Verkohlung mit 10% methanolischer Schwefelsäure bei 180—200°. Monoglyceride, die im Serum normalerweise nicht auftreten, liegen dabei — vom Start aus gesehen — kurz vor dem Cholesterinfleck.

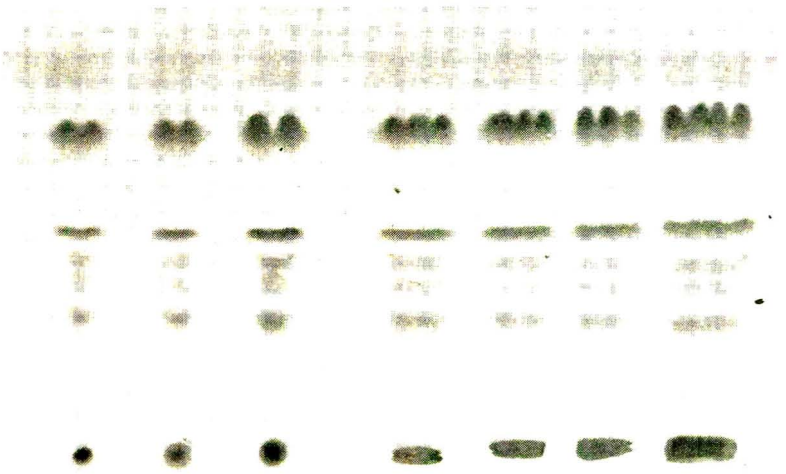

Fig. 1. Chromatogramm der Auftrennung eines Serumlipidextraktes im Punkt- und Strichauftrag mit jeweils steigenden Mengen (1, 2, 3 bzw. 3, 6, 8, 12 µl Extrakt, 1 µl Extrakt entspricht 3 µl Serum) nach Verkohlung mit Schwefelsäure (Flecken vom Start aus: Phospholipoide, Cholesterin, Fettsäuren, 1,2-, 1,3-Diglyceride, Triglyceride, Cholesterinester).

Auswertung

Die Auswertung erfolgt in Remission mit dem Elektrophoreseauswertegerät ERI 65 m der Fa. Carl Zeiss (Jena, D.D.R.) bei 530 nm (Spaltbreite: 0.3 mm). Da der nach der Sulfovanillinphospho-Reaktion (SVP) bestimmte Gesamtlipidwert als Bezugsgrösse dient, erübrigt sich das Auftragen definierter Lipidextraktmengen zur Chromatographie. Die von Chobanov et al. [16] beschriebene Empfindlichkeitssteigerung des Densitometers durch Verzicht auf eine Wellenlängenselektion wurde, obwohl dadurch die Sensitivität der Methode erheblich gesteigert wird, nicht angewendet, da spektrale Veränderungen der Lichtquelle zu nicht reproduzierbaren Ergebnissen führen können. Die aus der Integrationskurve ermittelten Fleckenflächenwerte der einzelnen Lipidklassen werden wegen ihres unterschiedlichen Kohlenstoffgehaltes auf Cholesterin als einheitliche Lipidfraktion korrigiert [7] und ihre Absolutbeträge aus den korrigierten Relativflächenwerten und dem Gesamtlipidwert berechnet. Fig. 2 stellt das Schema eines Chromatogramms mit dazugehöriger Remissionsgradorts- und Integrationskurve dar.

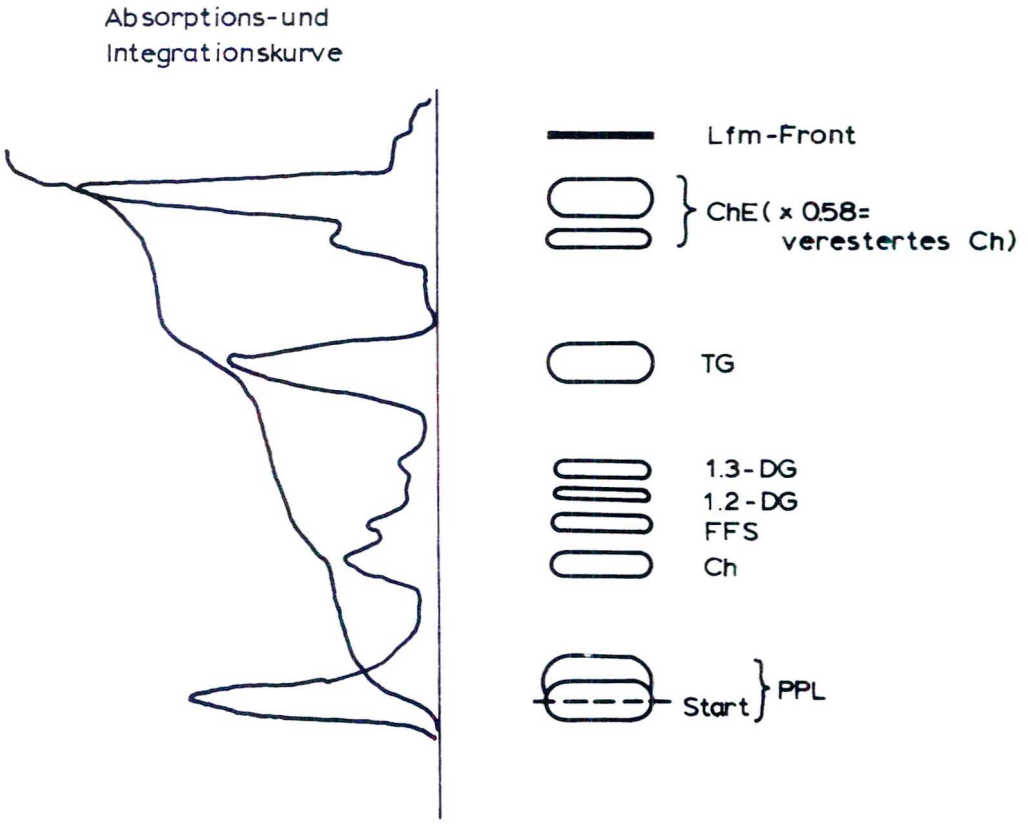

Fig. 2. Schema des Serumlipidchromatogramms mit dazugehöriger Absorptions- und Integrationskurve.

METHODE

0.2 ml Serum werden mit 3 ml eines Isopropanol —*n*-Hexangemisches (2.75: 1, v/v) extrahiert. Nach schonendem Eindampfen des Extraktes im Vakuum wird der Rückstand in etwa 0.5 ml Chloroform gelöst und Anteile davon strichförmig auf ammoniumsulfatimprägniertes Kieselgel aufgetragen. Die Chromatographie erfolgt in Stufentechnik zuerst 4.5 cm in Benzol—Dioxan (94:6, v/v) und anschliessend 6 cm in Tetrachlorkohlenstoff, die Detektion durch Besprühen mit 10%iger methanolischer Schwefelsäure und 10 min langes Erwärmen auf 180—200°. Nach Densitometrie und Korrektur der integrierten Werte für die Remissionsgrad—Ortskurven [7] erfolgt die Berechnung der Absolutbeträge der einzelnen Lipidfraktionen aus den korrigierten Einzelflächenwerten und dem Gesamtlipidwert (gleich Summe der korrigierten, integrierten Einzelflächenwerte).

ERGEBNISSE UND DISKUSSION

Als Kriterium für die Brauchbarkeit, dem auch die Praktikabilität untergeordnet werden muss, ist die Richtigkeit anzusehen. Dazu wurden 31 Proben eines Humanserumpools nach vorstehend beschriebener Methode aufgearbeitet und die Mittelwerte der bei den Lipidklassen ermittelten Konzentrationen mit denen nach chemisch bzw. enzymatisch analytischen Methoden (n = 30) gefundenen Mittelwerten verglichen.

Die Tabelle I gibt den Vergleich der dünnschichtchromatographisch ermittelten Lipidwerte nach Verkohlung mit den einzelanalytisch gefundenen Werten, die jeweiligen Variationskoeffizienten und das relative Richtigkeitsmass des DC-Verfahrens in Bezug auf die einzelanalytischen Bestimmungen wieder. Sie zeigt, dass die DC-Bestimmung des Lipidstatus den chemischen bzw. enzymatischen Bestimmungen ebenbürtige Werte, mit geringerer, vom Densitometer abhängender Präzision liefert. Andere in-situ-Auswerteverfahren wie die Messung der in den Lipidklassen enthaltenen Doppelbindungen bei 196 nm [2] oder auch fluorimetrische Messungen [1] erfordern einen grossen gerätetechnischen Aufwand. Während sich der Kohlenstoffgehalt kaum oder gar nicht durch Lipidstoffwechselstörungen ändert [7], sind im Falle der Remissionsmessungen bei 196 nm Unterschiede bis ca. 10% festzustellen [2] . Aus Fig. 1 ist ersichtlich, dass sich im ersten Laufmittel wahrscheinlich eine β-Front ausbildet. Das bedeutet eine Ausbildung scharfer Lipidbanden aber auch eine Abhängigkeit der R_F-Werte von der Laufstrecke.

Hohe Konzentrationen an freien Fettsäuren, wie sie in den untersuchten Seren niemals auftraten, können eine Verschiebung des Fettsäurenfleckes zwischen die Di- und Triglyceride bewirken. Aus praktischen Gründen wurde als Bezugswert für die relativen Flächenwerte der Gesamtlipidwert gewählt.

Weitere Vorteil dieser DC-Methode ist, dass sich ein Auftragen definierter Lipidextraktmengen erübrigt. Die Richtigkeit der Bestimmung des Gesamtlipidwertes ist eng an die Wahl des Standards gebunden. Die Verwendung von 860 mg Cholesterin/100 ml entsprechend 1000 mg Gesamtlipid pro 100 ml Serum als Standard entspricht wie aus der Tabelle I und aus Vergleichen mit Referenzlaboratorien (INSTAND, Düsseldorf, Berlin, B.R.D.) hervorgeht, weitgehend den "richtigen" Werten.

TABELLE I

VERGLEICH DER LIPID-EINZELBESTIMMUNGEN MIT DEM DÜNNSCHICHTCHROMATOGRAPHISCH BESTIMMTEN LIPIDSTATUS

Parameter	Vergleichsbestimmung (n = 30)			DC-Bestimmung (n = 31)		
	Methode	x (g/l)	Variationskoeffizient (%)*	x (g/l)	Variationskoeffizient (%)**	Relatives Richtigkeitsmass (%)***
Gesamtlipid	SVP§	7.91	2.65			
Triglyceride	Halbenzymatisch§§	1.61	3.98			2.48
	Vollenzymatisch§§§	1.50	1.90	1.65	8.17	10.0
Gesamtcholesterin	ZAK-Reaktion [12]	1.98	5.32			1.01
	ZAK-Reaktion direckt [12]	2.08	1.96	2.00	8.80	3.85
	Enzymatisch+	2.02	2.18			1.00
Freies Cholesterin	ZAK-Reaktion [13]	0.554	3.90			1.41
	Enzymatisch+	0.548	2.27	0.564	11,1	2.04
Verestertes Cholesterin	ZAK-Reaktion [13]	1.42	3.80			1.41
	Enzymatisch	1.47				2.04
Phospholipoide	Zilversmit++	2.66	4.05	2.71	7.48	1.88

*Seriel ermittelt.

** Von Tag zu Tag ermittelt.

*** $\frac{\bar{x}_{chem.} - \bar{x}_{DC}}{x_{chem.}} \cdot 100\%$.

§Testkombination der Fa. Boehringer (Mannheim; B.R.D.) Best. Nr. 124 303.

§§Boehringer -Testkombination Best. Nr. 125032.

§§§Boehringer-Testkombination Best. Nr. 126 012.

+Boehringer-Testkombination Best. Nr. 124 087.

++Boehringer-Testkombination Best. Nr. 124 974.

Die hier beschriebene DC-Methode ermöglicht eine schnelle Bestimmung aller Lipidklassen mit hinreichender Genauigkeit bei geringem arbeitstechnischen Arbeitsaufwand auf rationellem Wege. Weitere methodische Vereinfachungen, wie eine direkte Serumextraktion auf Dünnschichtplatten mit Konzentrierungszonen [17] sollten möglich sein.

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CHROMBIO. 301

Note

Rapid gas chromatographic method for the determination of nalidixic acid in plasma

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(First received September 26th, 1978; revised manuscript received December 19th, 1978)

Nalidixic acid is mostly used for the treatment of urinary tract infections, but in some cases it is administered intravenously for the treatment of general infections [1]. In those cases it is necessary to determine the plasma concentration of nalidixic acid, preferably by means of a rapid, simple method. In the literature, fluorimetric [2, 3] and liquid chromatographic [4, 5] methods have been described. The fluorimetric methods have the disadvantage of not being specific and the recently described high-performance liquid chromatographic (HPLC) method involves a time consuming derivatization step.

In this paper we shall describe a gas chromatographic (GC) method that is specific and sensitive enough to determine therapeutic levels in a 1 ml plasma sample within 30 min of receipt of the sample.

EXPERIMENTAL

Apparatus

A Packard-Becker Model 419 gas chromatograph, equipped with flame ionisation detectors was used. The glass column (1.5 × 6 mm O.D.) was packed with 10% OV-17 on Chromosorb W HP, 80—100 mesh (Chrompack, Middelburg, The Netherlands). The operating conditions were: injection port temperature, 290°; column temperature, 270°; detector temperature, 290°; carrier gas (nitrogen) flow-rate 20 ml/min; hydrogen flow-rate 30 ml/min; air flow-rate 300 ml/min.

Quantitation was carried out both by means of peak height measurements and peak area measurements with the chromatography Data Analyser System IV (Spectra Physics, Santa Clara, Calif., U.S.A.).

Chemicals

Toluene and dimethylacetamide (analytical grade) were obtained from Merck (Darmstadt, G.F.R.), nalidixic acid and butyliodide from Fluka (Basel, Switzerland), phenprocoumon from Hoffman-La Roche (Basel, Switzerland), flufenamic acid from Parke-Davis (Detroit, Mich., U.S.A.) and tetramethylammoniumhydroxide solution (20% in methanol) from Aldrich Europe (Beerse, Belgium). Hydroxymethylnalidixic acid was a friendly gift from Winthrop Labs. (New York, N.Y., U.S.A.).

Procedure

To 1 ml of plasma in a centrifuge tube was added 50 μl of 4 *M* hydrochloric acid and 2 ml toluene, containing flufenamic acid (2 mg/ml) and phenprocoumon (25 μg/ml) as internal standard. After shaking on a vortex mixer for 30 sec. and subsequent centrifugation at 2500 g for 2 min the toluene phase was transferred into another centrifuge tube. To this solution was added 25 μl of a 20% solution of tetramethylammoniumhydroxide in methanol and after thorough mixing and centrifugation 10 μl of the bottom layer was transferred into a glass capillary tube (5 cm × 3 mm I.D.) to which was added 25 μl dimethylacetamide and 7 μl butyliodide. After mixing and standing for 5 min the tubes were centrifuged for 5—10 min at 2500 *g* and 1—2 μl of the clear supernatant was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Injection of solutions of underivatized nalidixic acid on various columns revealed that it was necessary to alkylate this drug before GC in order to obtain reasonably symmetrical peaks. Methylation proved to be unsatisfactory. Probably the methyl ester is still too polar a compound. The butyl ester gave better results. On a 3% OV-17 column there was still a significant tailing, which was also the case on 10% SE-30, so a 10% OV-17 column was used. Multiple injections of the butyl ester on this column revealed that there were still losses in the first injections. Therefore, flufenamic acid was added to the solution in a rather high concentration, resulting in a deactivation of the column. In this manner reproducible results were obtained.

At pH values below 4, nalidixic acid, flufenamic acid and phenprocoumon are well extracted with toluene, the back extraction into a small volume of tetramethylammoniumhydroxide solution is an easy way to obtain a very clean blank chromatogram (Fig. 1a) and to concentrate the sample.

Pre-column butylation based on the method described by Greeley [6] has some advantages over on-column butylation, for instance there is less risk of decomposition of the compounds due to the high temperature and the strongly alkaline medium during the alkylation process, the peaks are somewhat narrower and higher and column life is prolonged, because a neutral instead of a strongly alkaline solution is injected.

Under the described chromatographic conditions phenprocoumon and nalidixic acid give well resolved symmetrical peaks (Fig. 1b) with retention times of 7 and 8 min respectively. Flufenamic acid gives a peak at 1.5 min, so it is eluted in the solvent peak.

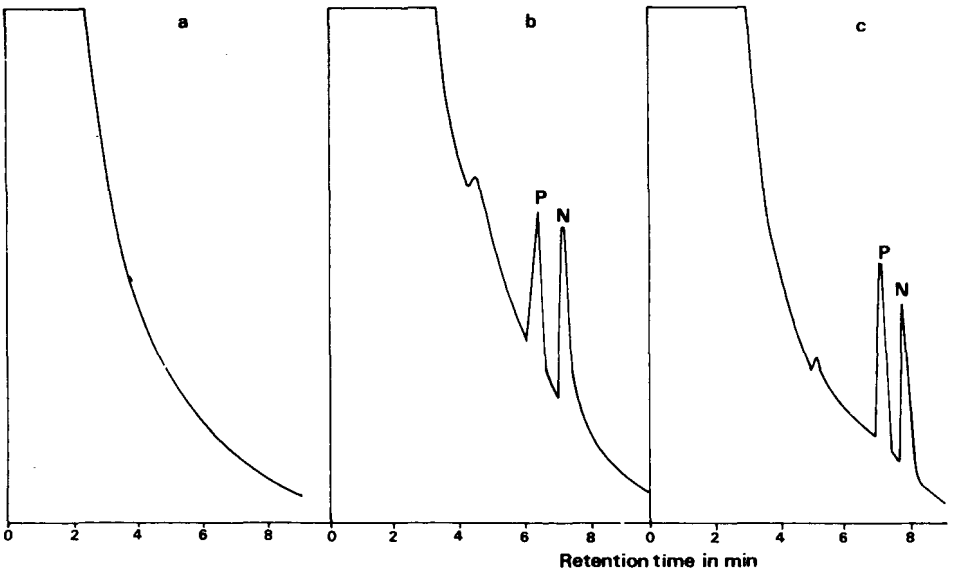

Fig. 1. Chromatogram of plasma samples (for conditions, see text). a = Blank plasma. b = Blank plasma spiked with nalidixic acid to a concentration of 26 μg/ml; N = nalidixic acid, P = phenprocoumon. c = Plasma sample obtained from a patient, treated with nalidixic acid.

Calibration curves were constructed by adding known amounts of nalidixic acid to blank plasma samples and treating these samples as described. They were straight lines in the range studied (5—100 μg/ml), which covers the therapeutic range of 10—60 μg/ml; there was a small negative intercept of 0.1—0.3 μg/ml.

The standard deviation was determined at 13 μg/ml and at 50 μg/ml by analyzing six plasma samples at each concentration and calculated to be 3.5%.

Recovery studies showed that with toluene, nalidixic acid is extracted completely from the plasma, the recovery of the back extraction with tetramethylammonium hydroxide is 77%, so the overall recovery of the method is 77%.

In order to evaluate the potential interference of the most important metabolite of nalidixic acid (hydroxymethyl nalidixic acid) this compound was treated as described for nalidixic acid. GC showed two very small peaks at longer retention times, so there is no interference of this metabolite with the quantitative determination of nalidixic acid. Analysis of a plasma sample of a patient, treated with nalidixic acid, using this method (Fig. 1c) and our previously described HPLC procedure [5] gave essentially the same result (23.8 ± 3.5% versus 26.4 ± 4% μg/ml).

Although the GC method is less sensitive than the HPLC method and needs a larger plasma volume, its speed and ease of operation make it attractive for routine monitoring of plasma levels.

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Note

Rapid gas chromatographic determination of carbamazepine for routine therapeutic monitoring

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(First received June 27th, 1978; revised manuscript received December 29th, 1978)

The tricyclic drug carbamazepine (Tegretol, Geigy, Summit, N.J., U.S.A.) is widely prescribed for the treatment of trigeminal neuralgia and of several forms of epilepsy. Its pharmacology, particularly with respect to epilepsy, has been reviewed [1—3].

Several methods for its determination have been published; the spectrophotometric and fluorometric methods have been reviewed [1, 3]. More recently, gas—liquid chromatographic [4—13] and high-pressure liquid chromatographic [14] methods have been introduced, as well as enzyme immunoassay [15]. Each technique has its advantages and disadvantages with respect to sensitivity, potential interference, speed, and cost and availability of reagents and equipment. At present, gas—liquid chromatographic methods using flame ionization detection appear to be most widely used.

Several such procedures have been described for determining carbamazepine along with other anticonvulsant drugs [4—6, 8, 10]. Many of these procedures are lengthy and fairly complicated, mainly because of carbamazepine, which differs chemically from the other generally administered anticonvulsants. In most laboratories, phenobarbital, primidone, and phenytoin determinations are requested much more frequently, and more urgently in many cases, than is that of carbamazepine. It appeared, therefore, that a more practical approach was to use a quicker routine methodology for the determination of the former three drugs and do a separate rapid determination of carbamazepine when that analysis was required.

Several methods are available for chromatography of carbamazepine in the underivatized form [7, 8], or after conversion to trimethylsilyl [9—11] or methyl [4—6] derivatives. Underivatized carbamazepine is prone to decomposi-

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tion during chromatography. Procedures involving use of the other derivatives generally are lengthy, requiring several extraction and evaporation steps and, in the case of the trimethylsilyl derivatives, rigid exclusion of moisture.

Perchalski and Wilder [12, 13] introduced the use of dimethylformamide dimethyl acetal as a carbamazepine derivatizing reagent. The reagent appeared to have good potential in the determination of this drug and of other compounds as well. The procedure, however, presented several problems. Cyheptamide [9] subsequently had been withdrawn from commercial availability (it has only very recently become available again), and we preferred to use a column packing that is more generally useful and already in routine use for other analyses. The use of carbon disulfide in the final step and as injecting solvent, while minimizing the size of the solvent front, also was considered objectionable.

The method described here has been used successfully in this laboratory for over two years. In addition to avoiding many of the problems discussed, the present method allows: (1) the use of reagents, glassware, and chromatography column common to other analyses a laboratory may be called upon to do; (2) the preparation in advance of extraction tubes containing internal standard; and (3) the reporting of results promptly after receipt of specimens with a minimum number of extraction and derivatization steps.

EXPERIMENTAL

Materials

Chemicals used in the routine method, and their sources, were: carbamazepine, a generous gift from Geigy Pharmaceuticals (Summit, N.J., U.S.A.), and used as a stock solution (50 μg/ml ethanol); *p*-amino-N-(2-dipropylaminoethyl)-benzamide hydrochloride, generously provided by E.R. Squibb and Sons, Inc. (New Brunswick, N.J., U.S.A.), and used as internal standard as a stock solution (100 μg/ml ethanol); N,N-dimethylformamide diethyl acetal, from Aldrich Chemical Co. (Milwaukee, Wisc., U.S.A.). Glass distilled benzene and ethyl acetate were from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.).

Sodium phosphate buffer (0.2 *M*) was prepared with Na_2HPO_4 and the pH adjusted to 12.5 with NaOH.

Other chemicals used in the development of the method were phenytoin (grade I) and phenobarbital (crystalline), purchased from Sigma (St. Louis, Mo., U.S.A.), mephenytoin from Sandoz Pharmaceuticals (Hanover, N.J., U.S.A.), primidone from Ayerst Labs. (New York, N.Y., U.S.A.), and ethotoin and trimethadione from Abbott Labs. (North Chicago, Ill., U.S.A.). The latter four compounds were the generous gifts of their manufacturers.

Glassware included 12-ml heavy-duty conical centrifuge tubes with PTFE-lined screw caps (Pyrex) and 5-ml Reacti-Vials (Pierce, Rockford, Ill., U.S.A.). A Lab-Line Temp-Blok heater with a Pierce Reacti-Blok (5-ml vial heating block) were used for heating. A laboratory-improvised multiple evaporator with adjustable-height glass capillary tubes was used for solvent evaporation with nitrogen.

Gas chromatography

A Hewlett-Packard 7611A flame-ionization gas chromatograph was used with a 6 ft. × 2 mm I.D. silanized glass column packed with 3% OV-1 on Gas-Chrom Q, 100—120 mesh (Applied Science Labs., State College, Pa. U.S.A.). Nitrogen (20 ml/min) was the carrier gas. Injector and detector temperatures were 240° and 245°, respectively. The analysis was run isothermally at 230°. Any residual material on the column could be eluted by raising the oven temperature to 240° after completion of a run.

The method was developed using peak height ratios for quantitation. Subsequently, a Hewlett-Packard 3352B Laboratory Data System was used for this purpose. Statistical analyses also were done with this system.

METHOD

Batches of extraction tubes were prepared in advance by adding 100 μl of internal standard solution to 12-ml screw-capped centrifuge tubes; the solvent was carefully evaporated with nitrogen. These tubes, each containing 10 μg internal standard, were capped and stored until used.

Serum, 1.0 ml or 0.5 ml, was added to an extraction tube, followed by 0.5 ml phosphate buffer. Each addition was followed by brief vortex mixing. Five milliliters of benzene—ethyl acetate (4:1, v/v) were then added and the tube contents vortex-mixed for 2 min. (Pipetting and evaporation of the solvent mixture were done in a fume hood.) After centrifugation in an IEC clinical centrifuge for 5 min at 550 *g*, the organic layer was transferred to a 5-ml Reacti-Vial. The solvent was evaporated to dryness under a gentle stream of nitrogen and 30 μl of dimethylformamide diethyl acetal were added, followed by brief mixing. The vial was then heated at 100° for 10 min. A 1-μl aliquot was injected into the chromatograph.

RESULTS

A representative chromatogram of a serum extract is shown in Fig. 1. Retention times of carbamazepine and internal standard were 6 and 8.5 min, respectively. No peak at the retention time of carbamazepine was observed when serum containing no carbamazepine was extracted and derivatized.

Calibration curve

A calibration curve was constructed by adding varying amounts of carbamazepine, in duplicate, to drug-free serum and processing in the usual manner. A plot of carbamazepine:internal standard peak height ratio vs. carbamazepine concentration was linear to at least 15 μg/ml.

Recovery

Varying amounts of carbamazepine were added to drug-free serum and processed. The results were compared with those obtained with a derivatized carbamazepine—internal standard mixture. As shown in Table I, average recoveries ranged from 94 to 100%.

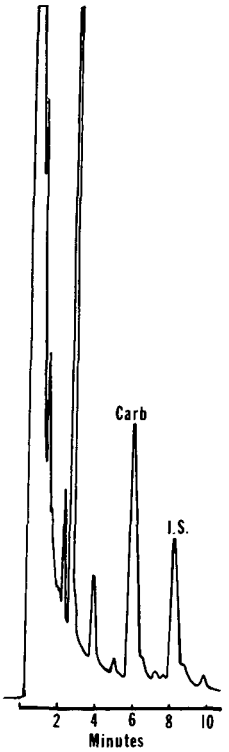

Fig. 1. Chromatogram of an extracted, derivatized serum specimen. Chromatographic conditions are as described in the text. Times shown are minutes after injection. Carb = carbamazepine, I.S. = internal standard, *p*-amino-N-(2-dipropylaminoethyl)benzamide, both as derivatives with dimethylformamide diethyl acetal. Chart speed, 0.25 in./min. Range and attenuation, 10^2, 4.

TABLE I

RECOVERY OF CARBAMAZEPINE ADDED TO SERUM

Amount added (μg/ml)	Amount recovered (μg/ml)	Recovery (%)
0	0	—
2.5	2.4 (2.3—2.6)	98 (92—104)
5.0	4.7 (4.7—4.7)	94
10.0	9.8 (9.6—10.0)	98 (96—100)
15.0	15.0 (15.0—15.1)	100.4 (100—100.7)

Interference

Since a patient's drug regimen frequently includes several anticonvulsant drugs, some of the more frequently administered potentially interfering drugs were tested in the assay procedure. Phenobarbital, primidone, phenytoin, mephenytoin, ethosuximide, ethotoin, and trimethadione were added to drug-free serum in amounts corresponding to their respective therapeutic ranges. None interfered with the analysis.

Precision

To establish the relative response of the flame ionization detector to carbamazepine and to the internal standard, a mixture of equal amounts of the two was extracted, derivatized, and injected. For ten determinations, the mean peak height ratio was 2.7, the standard deviation 0.024, and the coefficient of variation 0.9%.

To determine the within-assay and between-assay reproducibility of the method, carbamazepine was added to drug-free serum which was then mixed, divided into portions, and frozen. Twenty samples were processed simultaneously and fifteen were processed at different times. The mean level determined and the standard deviation and coefficient of variation are given in Table II.

TABLE II

PRECISION OF METHOD: WITHIN-RUN AND RUN-TO-RUN VARIATION

Variation	n	X (μg/ml)	s	C.V. (%)
Within run	20	17.2	0.31	1.8
Run-to-run	15	17.2	0.59	3.5

DISCUSSION

The method described has been in use in this laboratory for over two years and has given reliable results with the patient specimens received from a large neurology service.

The use of prepared extraction tubes containing dried internal standard, an approach we also follow with our other anticonvulsant drug analyses, appears to have several advantages over the more conventional procedures. The internal standard can be dispensed accurately and uniformly to a large batch of tubes whenever it is convenient to do so, not necessarily during the analysis. By gentle evaporation with nitrogen, the internal standard dries as a thin film in the lower part of the tube, and is readily dissolved when the added sample is mixed on a vortex-type mixer.

Tubes can be kept for long periods and are ready for use whenever routine specimens are received, as well as day or night stat tests, at which time only one accurate pipetting, that of the specimen, is required. The 100-μl volumes of internal standard solution can be dispensed with greater accuracy and precision than can the 10—20 μl or smaller volumes used in some methods in which the internal standard is added to a serum aliquot just before extraction. Initial solution of the internal standard in the specimen allows a more uniform extraction of both drug and internal standard than is the case when the standard is not present in the sample at all but is already dissolved in the extracting solvent. In these latter procedures, preparation of large volumes of the internal standard solution is necessary, and accurate pipetting of solvent volumes of several milliliters is essential.

Cyheptamide, the internal standard used in several published methods [7, 9, 12], had been withdrawn from commercial availability before this method was

developed; only very recently has it again become available from another source. Synthesizing an internal standard by chemically modifying a limited amount of carbamazepine did not appear to be a reasonable long-term solution for most clinical laboratories. We tested several possible internal standard solutions already in use for other analyses and found *p*-amino-N-(2-dipropylaminoethyl)benzamide hydrochloride, used in the determination of procainamide [16], to be satisfactory for this determination as well.

In the present method, the internal standard is dissolved in the sample aliquot at the outset, is extracted with the drug, and gives a peak well separated from potentially significantly interfering peaks, yet close to that of carbamazepine under the chromatographic conditions employed. Decomposition of the two compounds does not appear to be a problem, and removal of the derivatizing reagent and solution in another solvent before chromatography was found to be unnecessary. The use of carbon disulfide, which, while minimizing the solvent front in the chromatogram, is extremely unpleasant to use, is thereby avoided.

The use of the same internal standard solution in several analyses and of a generally useful column such as OV-1 has also been found economical and advantageous in a laboratory doing many different types of analyses. The use of the diethyl acetal of dimethylformamide gave a "cleaner" chromatogram than did the dimethyl acetal. Both acetals give the same derivative [17]. The less satisfactory performance of the dimethyl acetal may be due only to the particular reagent lot used.

Because of the convenience of saliva determinations in assessing levels of the non-protein bound, pharmacologically active form of anticonvulsant drugs [18], the present method also was tested for its suitability for saliva analyses. Using a 1:5 dilution of internal standard and varying concentrations of carbamazepine added to pooled, drug-free saliva, the method appears applicable to such analyses. Due to an insufficient number of saliva specimens, the clinical application of the method to saliva assays has not yet been thoroughly evaluated.

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Note

Quantitation of acenocoumarol in plasma by reversed-phase high-performance liquid chromatography

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(First received September 25th, 1978; revised manuscript received December 15th, 1978)

Acenocoumarol (Sintrom) is a short-acting synthetic oral anticoagulant belonging to the mono-coumarin class. It is structurally related to warfarin and phenprocoumon which are relatively longer-acting oral anticoagulants [1]. Acenocoumarol is used clinically in the prophylaxis and treatment of venous thrombosis and pulmonary embolism and as an adjunct in the treatment of coronary occlusion and transient cerebral ischemic attacks. Recently, there has been considerable interest in the anticoagulant activity [2] and metabolism [3] studies of acenocoumarol. For the investigation of acenocoumarol pharmacokinetics, it is desirable to have a simple, sensitive and specific quantitation procedure. In 1968, a pharmacokinetic study in man was performed using a photometric assay [4] which apparently lacked specificity [3]. In 1977, a gas—liquid chromatographic procedure [5] for quantitating acenocoumarol was reported, but the technique contained a lengthy extraction procedure and diazomethane derivatization of the sample prior to chromatographic analysis. More recently, a sensitive and specific thin-layer chromatographic method for the quantitative and qualitative analysis of acenocoumarol was reported [6]. The technique required the reduction of the nitro group and derivatization in the formation of a fluorophore.

High-performance liquid chromatography (HPLC) has been shown to be a useful tool for the separation and quantitation of coumarin anticoagulants because of its simplicity, specificity and sensitivity [7—10]. Previously, we have reported a simple and specific HPLC procedure for the quantitation of warfarin in human plasma [11]. The technique was found to be generally applicable to the mono-coumarins (warfarin, acenocoumarol, phenprocoumon) and in this paper, we describe an adaptation for the analysis of acenocoumarol. With the incorporation of a highly sensivity UV detector (Vari-

Chrom; Varian, Palo Alto, Calif., U.S.A.), as low as 125 ng/ml of the drug in plasma can be quantitated with a coefficient of variation (C.V.) of 4.7%. Methylated warfarin [(3-α-acetonylbenzyl)-4-methoxycoumarin] is used as an internal standard.

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Varian-Aerograph Model 4100, equipped with a positive displacement pump capable of developing a pressure up to 5000 p.s.i., a stop-flow injection port and a variable-wavelength UV absorbance detector (Vari-Chrom, Varian) operated at 305 nm and attenuated to 0.05 a.u.f.s. The column (25 cm × 2.2 mm I.D., stainless steel) was packed with 10 μm LiChrosorb RP-2 using a high-pressure (5000 p.s.i.) balanced-density slurry technique [12]. A pre-column (5 cm × 2.2 mm I.D.) packed with Vydac-RP (30—44 μm) was used to trap eluent insoluble materials. The mobile phase contained 0.75 g ammonium acetate per 100 ml of a mixture of acetonitrile—water—acetic acid (37:62:1, v/v). The system was operated at a flow-rate of 40 ml/h at ambient temperature.

Materials

Methylated warfarin was synthesized by reacting warfarin (Aldrich, Milwaukee, Wisc., U.S.A.) with an ethereal alcoholic solution of diazomethane (Diazald; Aldrich) giving quantitative yield of the product [13]. A solution of methylated warfarin (240 ng/ml) was prepared in *n*-butyl chloride. Acenocoumarol (Ciba-Geigy, Basle, Switzerland) was neutralized with 0.1 *N* NaOH and a stock solution (40 μg/ml) in distilled water was prepared and appropriate dilutions were made for the construction of standard curves. Ammonium acetate was obtained from BDH (Toronto, Canada). Glass distilled acetonitrile, *n*-butyl chloride and acetone were supplied by Caledon Labs. (Georgetown, Canada). LiChrosorb RP-2 was purchased from Aviation Electric (Montreal, Canada).

Plasma level study

Acenocoumarol (neutralized with 0.1 *N* NaOH) dissolved in water was administered orally to two rabbits at a dose of 1 mg/kg. Blood samples (3—4 ml) were drawn from the ear vein, using heparinized syringes, at 0, 1, 3, 5, 7, 10 and 24 h after drug administration. The blood samples were centrifuged and the plasma collected and stored at −20° and analyzed within three weeks.

Extraction and analysis procedure

To 0.5—1.0 ml of plasma (spiked or from dosed animals) in a conical glass-stoppered centrifuge tube (15 ml) were added 1 ml of 3 *N* HCl and 5 ml of *n*-butyl chloride solution containing methylated warfarin as internal standard. The sample was agitated at slow speed in a horizontal shaker for 10 min. After centrifugation at 2000 *g* for 10 min, about 4 ml of the organic layer was transferred into another conical glass-stoppered centrifuge tube (5 ml) and the solution evaporated to dryness under a stream of dry nitrogen at room

temperature. The side of the tube was washed with 1 ml of acetone and evaporated to dryness. The residue was dissolved in 30 μl of acetonitrile or dioxane, mixed in a Vortex mixer for 15 sec, and aliquots (5—10 μl) were chromatographed. Drug concentration was estimated by comparing the drug:internal standard peak height ratio to that of a standard constructed from pooled human plasma spiked with 0.125, 0.25, 0.5, 1.0 and 2.0 μg/ml of acenocoumarol.

Evaluation of extraction efficiency

Plasma was spiked with acenocoumarol to give concentrations of 0.25, 0.5, 1.0 and 2.0 μg/ml, extracted with *n*-butyl chloride containing methylated warfarin (1.2 μg) and chromatographed as described above. Care was taken to recover all of the organic extract. The extraction efficiency was evaluated by comparing peak height obtained after extraction of spiked plasma to those obtained from corresponding concentrations of standard solutions.

RESULTS AND DISCUSSION

With a few modifications, the HPLC technique reported earlier for the quantitation of warfarin [11] was adapted to the analysis of acenocoumarol in plasma. A mobile phase of ammonium acetate in acetonitrile—water—acetic acid was used in place of a dioxane—water mixture because: (a) it gave a more stable column peak retention over a long period of use, and (b) it eliminated the use of dioxane which had to be redistilled prior to use. *n*-Butyl chloride (5 ml) was used for extraction instead of ethylene chloride (25 ml) as it gave a cleaner extract and, because of its greater volatility, was easily evaporated at room temperature. The sensitivity of the method was greatly improved by the incorporation of a highly sensitive variable wavelength UV detector.

Fig. 1A shows a chromatogram obtained from blank plasma and Fig. 1B from plasma to which acenocoumarol and methylated warfarin had been added. The retention times for acenocoumarol and methylated warfarin were 5.5 and 9 min, respectively. The extraneous peaks from the plasma had retention times which were <4.5 min and therefore did not interfere with the assay. Fig. 1C shows a chromatogram of a plasma extract containing 170 ng/ml of acenocoumarol from a 10-h sample (1 ml) of a rabbit after receiving a single oral dose (1 mg/kg) of the drug.

The efficiency of the extraction procedure is depicted in Table I. The average mean recoveries of acenocoumarol and methylated warfarin from plasma were 79.74 ± 9.6 and 81.31 ± 5.7%, respectively (0.25 to 2 μg/ml range). The accuracy of the analysis is shown in Table II. Results are based on at least 4 determinations of each concentration (0.125 to 2 μg/ml). The peak height ratio of acenocoumarol and methylated warfarin was used as an index for quantitation. A linear response was obtained with a mean slope of 1.68 ± 0.05. The overall C.V. over the range was 4%. A larger C.V. was obtained when estimating below 0.125 μg/ml.

Application of the HPLC method for the estimation of acenocoumarol plasma levels in the rabbit is described in Fig. 2. The figure shows the plasma

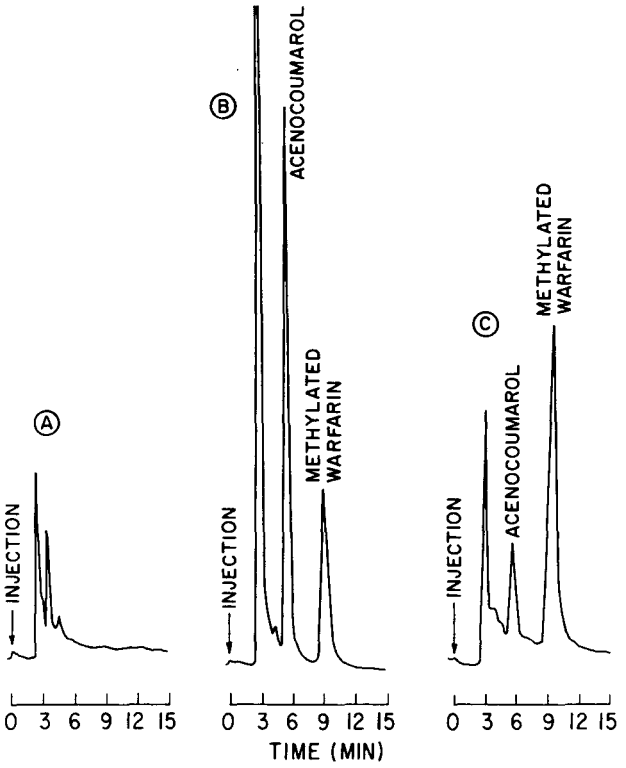

Fig. 1. Chromatographic separation from (A) control human plasma, (B) pooled human plasma spiked with acenocoumarol, and (C) 10 h plasma sample from a rabbit given 1 mg/kg of per oral acenocoumarol. HPLC system: column, 25 cm × 2.2 mm I.D., packed with LiChrosorb RP-2; mobile phase, 0.75 g ammonium acetate per 100 ml acetonitrile—water—acetic acid (37:62:1); flow-rate, 40 ml/h.

TABLE I

RECOVERY OF ACENOCOUMAROL AND METHYLATED WARFARIN FROM PLASMA

Values in brackets are percentage recoveries; $n = 3$ for each estimation.

Acenocoumarol added to 1 ml plasma (μg)	Acenocoumarol recovered Mean ± S.D. (μg)	Methylated warfarin added to 1 ml plasma (μg)	Methylated warfarin recovered Mean ± S.D. (μg)
0.25	0.17 ± 0.03 (68.8)	1.2	0.92 ± 0.18 (76.7)
0.5	0.38 ± 0.03 (75.1)	1.2	0.93 ± 0.08 (77.2)
1.00	0.90 ± 0.03 (90.4)	1.2	1.07 ± 0.06 (88.9)
2.00	1.69 ± 0.16 (84.7)	1.2	0.99 ± 0.18 (82.5)

TABLE II

HPLC ESTIMATION OF ACENOCOUMAROL ADDED TO PLASMA

Acenocoumarol added to 1 ml plasma (μg)	*n*	Mean peak height ratio of acenocoumarol:methylated warfarin	S.D.	C.V.	Slope*
0.125	4	0.21	0.01	4.7	1.68
0.250	5	0.44	0.01	2.3	1.76
0.500	5	0.84	0.03	3.5	1.68
1.000	5	1.61	0.07	4.3	1.61
2.000	5	3.33	0.17	5.1	1.67

*Peak height/concentration.

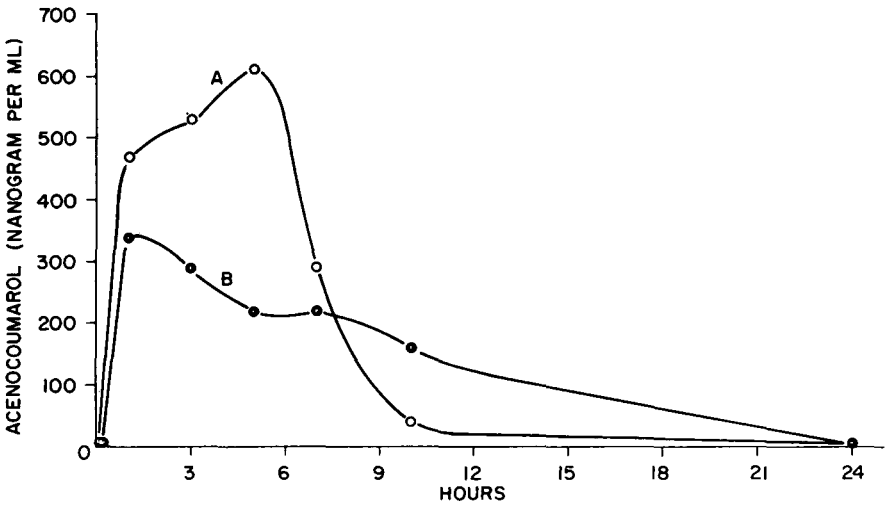

Fig. 2. Plasma acenocoumarol levels in two rabbits during 24 h following a single oral dose of 1 mg/kg acenocoumarol.

profile over 24 h of two rabbits after each received a single oral dose (1 mg/kg) of acenocoumarol. The peak acenocoumarol plasma level in rabbit B (340 ng/ml) was attained within 1 h, but that of rabbit A (610 ng/ml) was reached only after about 5 h. The technique is of sufficient sensitivity for the estimation of acenocoumarol plasma levels in humans after therapeutic doses since Dieterle et al. [3], using ^{14}C-acenocoumarol, reported plasma concentrations in the mid-nanogram range in human subjects who had received a low therapeutic dose of the drug.

In summary, a simple and sensitive HPLC procedure for the estimation of acenocoumarol in plasma has been developed. Previous quantitation of the coumarin in biological samples relied mainly on the use of gas—liquid chromatography [5] which required an elaborative purification step and a derivatiza-

tion procedure. The use of reversed-phase HPLC provides a simpler means of analysis for the anticoagulant, with a simple and direct cleanup procedure, no derivatization of sample prior to chromatographic analysis, and a sensitivity limit in the nanogram range.

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Biomedical Applications

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Note

Determination of 1-hexylcarbamoyl-5-fluorouracil and its metabolites in biomedical specimens by high-performance liquid chromatography

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(Received October 5th, 1978)

1-Hexylcarbamoyl-5-fluorouracil (HCFU), a recently discovered anticancer agent [1], was shown to have more favorable therapeutic ratios than its parent compound, 5-fluorouracil (FU), by oral administration. This compound, a masked form of FU [2], is metabolized in a mammalian body to give FU and other derivatives such as 1-ω-carboxypentylcarbamoyl-5-fluorouracil (CPEFU), and 1-ω-carboxypropylcarbamoyl-5-fluorouracil (CPRFU) [3]. Although HCFU and all its metabolites have an anticancer activity, the main activity is considered to reside in FU. The adverse side-effect of HCFU is suspected to be connected with CPEFU and/or CPRFU [4].

Detailed pharmacokinetic studies could provide the necessary information for the optimal drug administration schedule, which offers maximum therapeutic response with minimum toxicity. A rapid, sensitive method of assay is required for this purpose, since the microbiological method does not give enough accuracy. In our laboratory, high-performance liquid chromatography (HPLC) is used for monitoring the anticancer agents such as FU, 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT) and mitomycin C. This paper describes the HPLC analysis of HCFU, FU, CPEFU and CPRFU in biomedical specimens.

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EXPERIMENTAL

Reagents

HCFU, CPEFU and CPRFU used in this investigation were kindly supplied by Mitsuiseiyaku (Tokyo, Japan) and FU by Kyowahakkokogyo (Tokyo, Japan). All solvents for HPLC and chemicals were certified grade and products of Wakojunyaku (Osaka, Japan).

HPLC instrumentation

A Waters Assoc. liquid chromatograph equipped with a Model 6000 solvent delivery system, a Model U6K universal injector, a Model 440 UV detector operated at 254 nm and a μBondapak C_{18}/Porasil (particle size, 8—10 μm; 300 × 3.9 mm I.D.) column was used. The flow-rate of the mobile phase was 1 ml/min, at a pressure of about 1500 p.s.i.

Eluent

Although a number of mobile phases were tested during the investigation, no single solvent system afforded simultaneous resolution of HCFU, its metabolite and endogenous components of serum and tissues. Solvent systems used for the separation and determination of HCFU and the metabolites are described in the Results section.

Biomedical sample preparation

Human serum for analysis was prepared in the usual way. Tumors or other tissues obtained at operation or biopsy were homogenized to a 20% suspension in distilled water at 0°, and centrifuged at 7000 *g* for 30 min. To 0.5 ml of serum or the homogenate supernatant, 0.1 ml of 1 *N* HCl and 4.0 ml of ethyl acetate were added, and the sample was extracted with vigorous shaking. The organic layer was separated by centrifugation and evaporated to dryness using a water-bath at 30° and a water pump vacuum. The residue was dissolved in 100 μl of methanol for analysis by HPLC.

RESULTS AND DISCUSSION

Fig. 1a shows a chromatogram of extract of human serum obtained from a patient administered HCFU. Water—acetonitrile (70:30) was used as mobile phase. An extract of tissue homogenate gave a similar chromatogram under the same conditions. The metabolites were not separated with this solvent system. No interfering peak arose from endogenous serum or tumor tissue components. This solvent system was shown to be suitable for the determination of HCFU. Recovery of HCFU added to serum was 97.2 ± 1.0%.

HCFU, CPEFU and CPRFU were separated with a solvent system composed of water—tetrahydrofuran—acetonitrile (50:35:15), their retention times being 9, 4.5 and 3.5 min, respectively. The retention times of CPEFU and CPRFU were so close that their simultaneous determination in dilute biological samples would meet with difficulties. A better separation was achieved with the solvent system tetrahydrofuran—water (35:65). Fig. 1b is a chromatogram of human serum with this solvent system. The retention times of CPRFU and CPEFU were 6.4 and 10.0 min, respectively. This solvent system

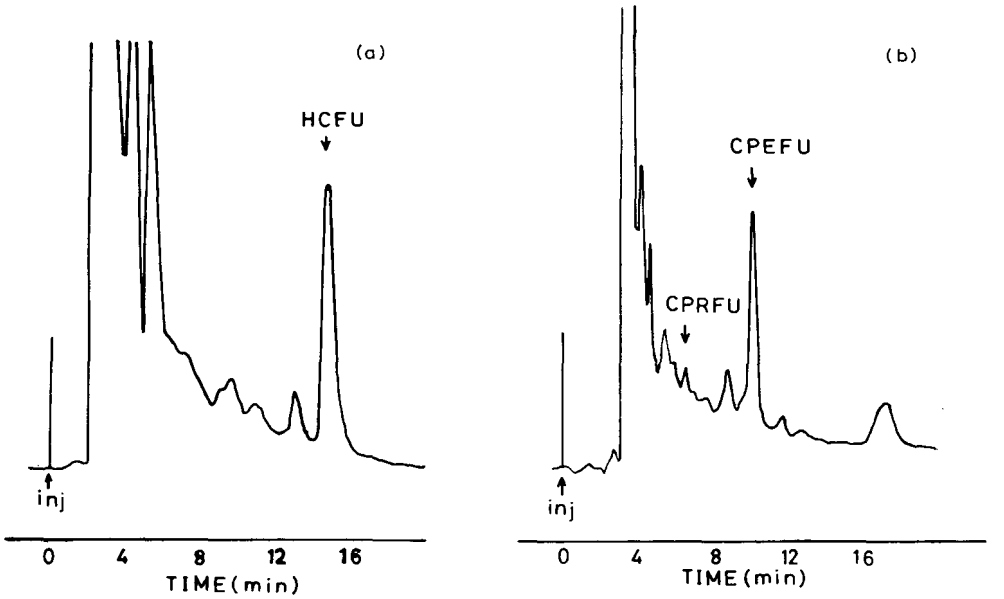

Fig. 1. Chromatograms of an extract of serum collected 3 h after the oral administration of 200 mg HCFU to an adult woman with cancer. The mobile phases are (a) water—acetonitrile (70:30) and (b) tetrahydrofuran—water (35:65). For HPLC conditions, see text.

proved suitable for the determination of CPRFU and CPEFU, but not of HCFU, since the latter compound has a long retention time. Recoveries of CPRFU and CPEFU added to serum were 79.4 ± 7.1 and 85.0 ± 6.2%, respectively.

Since FU is more hydrophilic than the other three compounds, it was not separated with the solvent system mentioned above. Use of a water—organic gradient method was not successful. FU was well separated from its derivatives and from the serum and tissue components with distilled water. Chromatograms of human serum and tissue homogenates of patients administered FU and HCFU are shown in Figs. 2 and 3. With water, a linear relationship was obtained between the peak height and the amount of FU injected into the chromatograph. Recoveries of FU added to serum and homogenate were 68.3 ± 4.9 and 54.2 ± 3.0%, respectively. The low recoveries come from the low lipophilicity of FU.

From the results described above the following solvent systems were used for the determinations; water—acetonitrile (70:30) for HCFU, water—tetrahydrofuran (65:35) for CPRFU and CPEFU, and water for FU. It was not too inconvenient to change the mobile phase during the HPLC study.

HCFU, CPEFU, and CPRFU were somewhat unstable in neutral and alkaline conditions, and gradually gave rise to FU. They were quite stable in 1 *N* HCl.

HCFU and its metabolites were dissolved in methanol at a concentration of 25 μg/ml. Appropriate dilution of these solutions with methanol was made to give the desired concentration. A 1-μl aliquot of the methanol solution was injected into the liquid chromatograph. Standard curves obtained by plotting the peak height against the amount of the substances injected were linear in

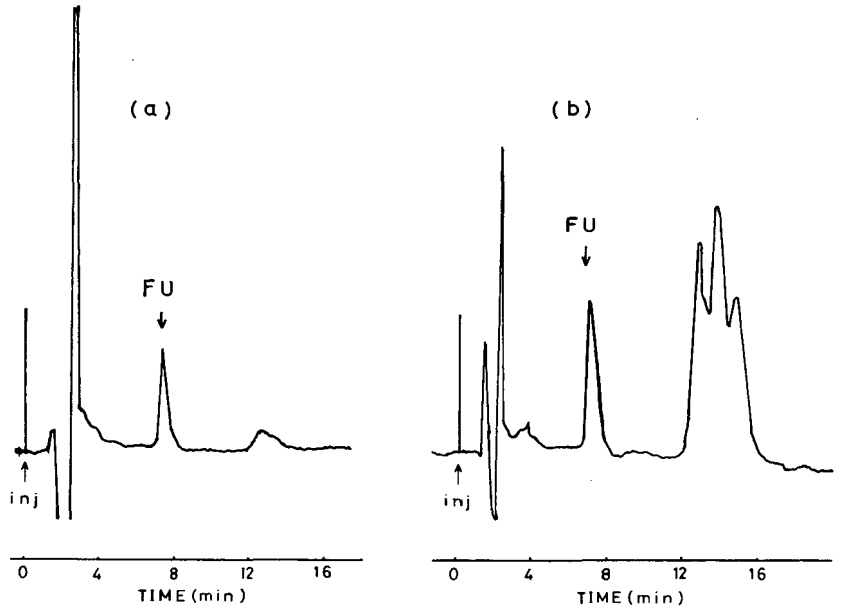

Fig. 2. Chromatograms of an extract of (a) serum and (b) tumor tissue homogenate collected 15 min after the selective arterial injection of 100 mg FU to an adult man with cancer. The mobile phase is distilled water. For HPLC conditions, see text.

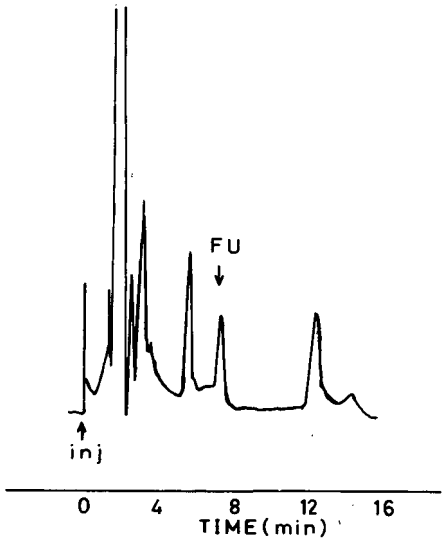

Fig. 3. Chromatogram of an extract of serum collected 3 h after the oral administration of 200 mg HCFU to an adult woman with cancer.

the range 0.5—25 ng. In the determination of the compounds in solutions containing 15 ng, standard deviations were 0.30, 0.66, 0.33, and 1.0 ng for FU, HCFU, CPRFU, and CPEFU, respectively.

Appropriate amounts of the substances were added to blank serum or homogenate. These spiked standards were carried through the procedure de-

scribed above and the peak heights plotted against the amounts of the spiked substances. The curves thus obtained were linear and comparison of the slopes with those obtained with the standard methanol solution gave recovery values for each substance described above. With this method, as little as 100 ng/ml serum and 250 ng/g tissue of HCFU and its metabolites were determined.

Along with the clinical studies, pharmacokinetic studies of the anticancer agent are in progress in this laboratory using the analytical method described. The present method is suited for drug monitoring studies in the therapeutic dose range (600—1800 mg/day). The results will be reported in the near future.

ACKNOWLEDGEMENTS

This work was supported by the Grant-in-Aid for the Cancer Research from the Ministry of Health and Welfare, for which we are grateful. We are also grateful to Nihon Waters Co., Osaka, for their technical advice on HPLC.

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Biomedical Applications

CHROMBIO. 307

Note

Analysis of trimethoprim and sulphamethoxazole in human plasma by high-pressure liquid chromatography

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Co-trimoxazole, a broad-spectrum antimicrobial combination of trimethoprim and the sulphonamide sulphamethoxazole, is used in the treatment of many common infections such as urinary and respiratory tract infections [1].

Several analytical procedures for the two drugs in plasma and other biological fluids have been reported. For sulphamethoxazole, spectrophotometric methods [2, 3] are available and are adequate in many instances. For trimethoprim, a microbiological assay [4], spectrofluorometric [5—7] and differential pulse polarographic [8] procedures have been used but most of these are laborious and require considerable attention to detail in order to obtain enough sensitivity for measurements in plasma. A sensitive and specific assay of trimethoprim in plasma or urine has recently been achieved using gas chromatography [9].

The use of high-pressure liquid chromatography (HPLC) on C-8 alkyl reversed-phase columns and variable-wavelength ultraviolet detection for analyses of trimethoprim and sulphamethoxazole in pharmaceuticals has been reported [10] and, more recently, has been applied to measurements in body fluids [11]. Using simpler HPLC apparatus and an octadecylsilane reversed-phase column in the present study, plasma concentrations of trimethoprim and sulphamethoxazole are determined separately. Sample preparation is identical for both drugs and merely involves precipitation of plasma protein and injection of supernatant directly into the chromatograph.

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MATERIALS AND METHODS

Plasma samples (1 ml) were pipetted into tubes containing 50 μl of 4 *M* trichloroacetic acid. After mixing for 20 sec on a vortex mixer, these were centrifuged for 15 min at 0° to separate the precipitated protein. Aliquots of 100 μl supernatant were injected into the chromatograph.

Analyses for both trimethoprim and sulphamethoxazole were performed on a reversed-phase column (Spherisorb, 10 μm ODS; 250 × 4.6 mm; Phase Separations, Queensferry, Great Britain) at ambient temperature. A single-piston high-pressure pump (Altex Model 110), which delivered solvent at constant flow-rates, a sample injection valve containing a 100-μl loop (Chromatronix) and a fixed-wavelength ultraviolet detector with a 20-μl flow cell (Altex Model 150) formed the basis of the chromatograph.

For trimethoprim, the eluting solvent was acetonitrile—aqueous 0.1 *M* KH_2PO_4 (pH 2.5) (30:70) containing 1% acetic acid and 1% ethyl acetate. The flow-rate was 1 ml/min. The mobile phase for sulphamethoxazole analysis was methanol—water—acetic acid (40:60:1) (pH 3.2) at a flow-rate of 2 ml/min.

Absorbance of the effluent from the column at 254 nm was monitored at a sensitivity of 0.02 a.u.f.s. for trimethoprim and at 0.32 a.u.f.s. for sulphamethoxazole. Peak heights were used for quantitation of both assays. Quality control was achieved by external standardization .

To prevent contamination of the reversed-phase column by plasma constituents remaining after the protein removal step, a pre-column (50 mm × 4.6 mm) containing 10 μm reversed-phase packing was incorporated into the system. Significant increases in perfusion pressure necessitating a change of pre-column (at 3000 p.s.i.) did not occur until several hundred samples had been assayed.

All chemicals used were analytical grade and water was double distilled. Sulphamethoxazole was B.P. grade. Trimethoprim lactate was donated by Wellcome, Australasia. All concentrations of drugs are in reference to the base.

Calibration curves were derived from pooled blood bank plasma.

RESULTS

Trimethoprim

Typical chromatograms of plasma samples (Fig. 1) show that control samples are free from contaminating peaks. Trimethoprim was eluted in 6 min. Sulphamethoxazole was eluted immediately after trimethoprim. However, under conditions optimized for measurement of trimethoprim, sulphamethoxazole was incompletely resolved from a major metabolite (vide infra). Calibration curves for trimethoprim passed through the origin and were linear to the maximum concentration used (10 μg/ml). The detection limit was 0.1—0.2 μg/ml. Recovery of trimethoprim added to plasma was 95%. The coefficient of variation for the analysis was determined to be 3.2% (n = 10) at a concentration of 2 μg/ml.

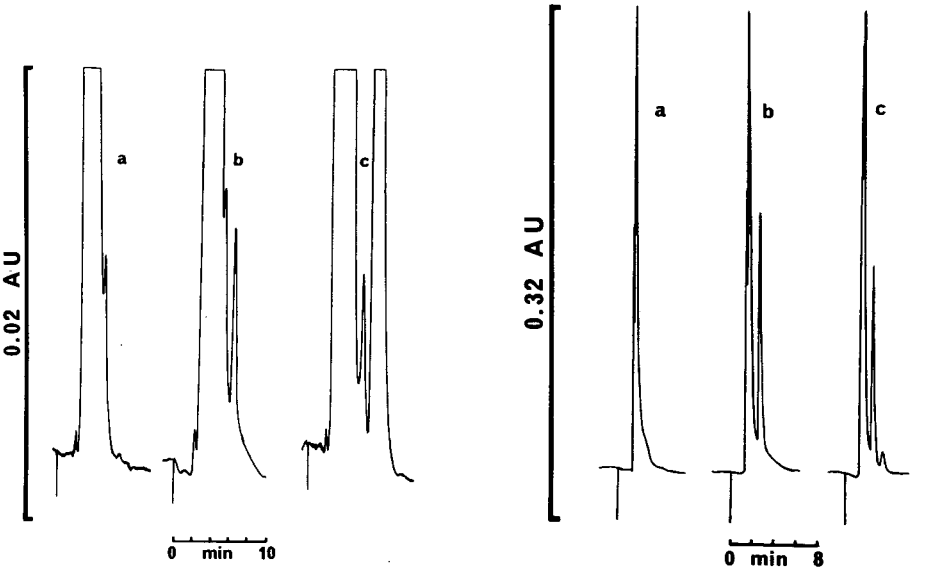

Fig. 1. Trimethoprim assay. Chromatograms of deproteinized plasma. (a) Blank plasma, (b) plasma containing 3.85 μg/ml trimethoprim, and (c) plasma from a patient receiving co-trimoxazole; the major component of the large peak following trimethoprim is sulphamethoxazole.

Fig. 2. Sulphamethoxazole assay. Chromatograms of deproteinised plasma: (a) blank plasma, (b) plasma containing 60 μg/ml sulphamethoxazole, and (c) plasma from a patient receiving co-trimoxazole; the small peak eluting after sulphamethoxazole is presumably due to the acetylated metabolite.

Sulphamethoxazole

Typical chromatograms of plasma samples (Fig. 2) show that control samples are free from interfering peaks. Sulphamethoxazole was eluted in 3 min and a peak, presumed to be its acetylated metabolite, appeared at 4 min (Fig. 2). The latter peak disappeared following hydrolysis by autoclaving acidified deproteinised plasma for 30 min, and there was a corresponding elevation of the sulphamethoxazole peak. Procurement of pure acetylated sulphamethoxazole standard should enable its quantitation in plasma. Trimethoprim could not be detected due to prolonged retention on the column. The calibration curves for sulphamethoxazole passed through the origin and were linear up to 200 μg/ml, the maximum concentration used. A sulphamethoxazole concentration of less than 1 μg/ml in plasma was easily detectable. Recovery of sulphamethoxazole added to plasma was 81%. At a concentration of 50 μg/ml, the coefficient of variation was 1.8% (n = 10).

To demonstrate the effectiveness of the assays in a clinical situation, blood samples from ten hospitalised patients dosed orally with co-trimoxazole (two tablets*, twice daily) were taken at least three days after starting treatment. In each patient, blood was taken 2 h before the evening dose on the day of sampling. Plasma concentrations of trimethoprim ranged from 0.39 to 3.16 μg/ml (mean 1.83 μg/ml). Sulphamethoxazole concentrations ranged from 28.2 to 128.1 μg/ml (mean 66.6 μg/ml).

*Each tablet contained 400 mg sulphamethoxazole and 80 mg trimethoprim.

No interference with the measurement of trimethoprim or sulphamethoxazole was observed despite concurrent medication with a wide range of drugs. These included amiloride, ampicillin, aspirin, chloral hydrate, chlorothiazide, chlorpromazine, cloxicillin, codeine, cortisone, dextropropoxyphene, digoxin, dioctyl sodium sulphosuccinate, frusemide, glyceryl trinitrate, hyoscine-N-butylbromide, indomethacin, mebendazole, metoclopramide, morphine, neomycin, nitrazepam, nystatin, oxycodone pectinate, paracetamol, pethidine, phenytoin, prednisolone, pindolol, quinine, salbutamol, sodium valproate and triamterene.

DISCUSSION

Assay of sulphonamide levels has been available for decades but it is only recently that such data have made a contribution to clinical deliberations. Most assays of trimethoprim have been rather laborious and unsuitable for routine use. The HPLC method described in this paper permits specific determination of the level of both drugs to be accomplished rapidly and therefore to influence decisions on dosing. Its value for routine use is enhanced by lack of interference from a large number of other drugs and their circulating metabolites. Furthermore, only relatively inexpensive HPLC equipment is required.

Co-trimoxazole is now one of the most commonly used antimicrobial agents and is given by both oral and parenteral routes. It is increasingly being employed in severely ill patients and monitoring of plasma levels is likely to make a substantial contribution to ensuring that maximum benefit is derived from this valuable treatment. This is emphasised by the eight fold variation in trimethoprim levels found among ten patients in this study.

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Note

Comparison of thin-layer and gas chromatographic assays for caffeine in plasma

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Caffeine is one of the most widely ingested natural alkaloids and in some countries the per capita annual consumption may be as high as 100 g. Recently there has been an upsurge of interest in the clinical, toxicological and teratogenic effects of chronic caffeine consumption. Until recently the usual technique for the estimation of caffeine in biological fluids has been ultraviolet spectrophotometry [1]. This method is unsatisfactory and lacks the specificity and sensitivity required for pharmacokinetic studies. A radioimmunoassay procedure has been described for the measurement of caffeine in plasma and saliva but requires expertise for the preparation of the caffeine antibody. High-pressure liquid chromatographic (HPLC) methods for caffeine have also been described [2, 3] and one technique has been compared with radioimmunoassay and been found to give comparable results [2]. Thin-layer chromatographic (TLC) methods for caffeine have been developed for plant extracts [4, 5] and recently this method has been extended to measurements of caffeine in plasma [6, 7]. Gas—liquid chromatographic (GLC) procedures for estimation of plasma caffeine levels have also been described [8, 9]. One of these utilised an external standard (hexobarbital) added following the extraction step [8]. A GLC technique has also been developed for caffeine determination in combined analgesic formulations [10]. During the course of a study of caffeine kinetics in man we have had the opportunity to develop and compare TLC and GLC assays for caffeine in plasma.

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EXPERIMENTAL

Thin-layer chromatography

Materials and methods

Caffeine base was obtained from Sigma (London) Chemicals (Poole, Great Britain). All other chemicals were of analytical grade. Thin-layer plates were 10 cm × 20 cm high-performance TLC Fertigplatten (Merck Kieselgel 60 F 254, supplied by BDH, Poole, Great Britain).

Apparatus

Developed chromatograms were scanned on a Vitatron TLD 100 flying spot densitometer (MSE Instruments, Crawley, Great Britain). The instrument settings were excitation filter, UVB (mercury lamp); emission filter 523 nm; scanning speed, 3 cm/min; log-mode; level f; span 95; c = 4, and damping 1.

Extraction and development procedures

To 0.5 ml plasma in a ground-glass stoppered tube 5 ml chloroform, the extracting agent, were added and, after vortex mixing for 10 sec, the mixture was centrifuged for 5 min at 750 *g*. The chloroform layer was transferred to a conical tube and evaporated to dryness at 65° in a water-bath under a stream of nitrogen. The residue was dissolved in 100 μl acetone and 10 μl of this solution were applied to the thin-layer plate as a spot by hand using a micro-syringe. A series of extracts from plasma spiked with measured amounts of caffeine and a standard of caffeine in acetone were also applied to the same plate. The plate was developed in the solvent system [4] chloroform—carbon tetrachloride—methanol (8:5:1) in a saturated tank allowing the solvent to migrate about 8 cm.

Gas—liquid chromatography

Materials and methods

A Pye Unicam 104 gas chromatograph equipped with an alkaline flame ionisation detector (AFID) was used for this study. Two columns were found suitable: (1) 1.5 m × 4 mm I.D. borosilicate glass packed with 10% Apiezon M (Supelco, Bellefonte, Pa., U.S.A.) on Supasorb, 60—80 mesh (BDH, Poole, Great Britain); (2) 2.4 m × 4 mm I.D. borosilicate packed with 3% Poly S-179 (Supelco) on Gas-Chrom Q (DCMS-treated), 60—80 mesh (Supelco).

Extraction and chromatographic procedures

To 0.5 ml plasma in a ground-glass stoppered tube were added 20 μl of the internal standard solution of phenacetin (100 μg/ml in methanol), 0.1 ml 0.1 *N* NaOH and 3 ml ethyl acetate. The mixture was vortex mixed for 20 sec and centrifuged at 750 *g* for 5 min. The supernatant organic phase was transferred to a conical tube and evaporated to dryness under a stream of nitrogen in a water-bath at 75°. The residue was dissolved in 20 μl methanol and 1—3 μl were injected into the gas chromatograph. The chromatograph operating conditions for both columns were: column oven 225°; detector 330°; carrier

gas (nitrogen) flow-rate 75 ml/min; hydrogen flow-rate 37 ml/min; and air flow-rate 480 ml/min.

RESULTS AND DISCUSSION

A typical recording of the scanning of the thin-layer plate in the direction of solvent flow is shown in Fig. 1. In the system used to develop these plates the R_F of caffeine is 0.5. Calibration curves relating concentration of caffeine to peak height were linear over the range investigated (0—50 μg/ml). The minimum level of detection of the TLC assay was 0.1 μg/ml. Reproducibility was assessed by replicate analyses of spiked plasma samples. The coefficient of

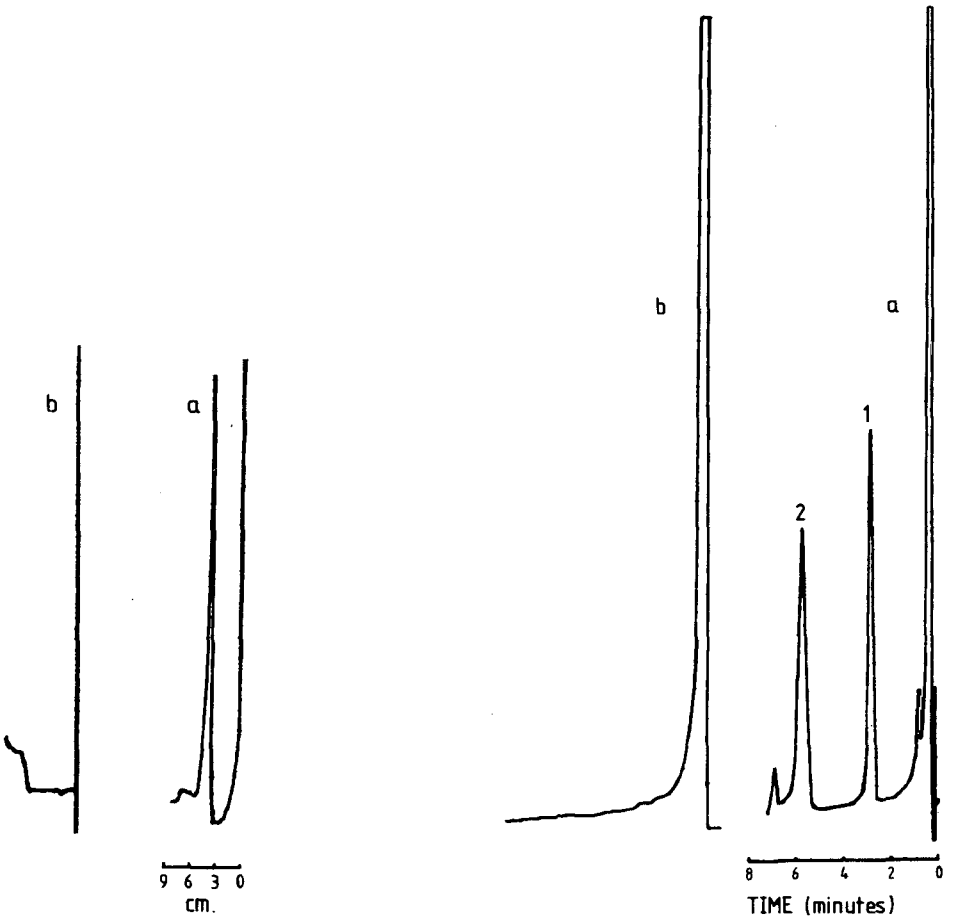

Fig. 1. Scan of thin-layer chromatogram along the plate in the direction of solvent flow. (a) Plasma containing caffeine (10 μg/ml); (b) control plasma.

Fig. 2. Gas chromatograms on Apiezon M system of human plasma. (a) At 5 h following oral administration of 300 mg caffeine base; peaks: 1, phenacetin (internal standard), 2, caffeine (3.2 μg/ml). (b) Control caffeine-free plasma.

variation between assays was 7.18 at 10 μg/ml (n = 15) and 7.25% at 2 μg/ml (n = 5). There were no interfering substances in blank plasma or urine.

Typical chromatograms for the GLC assay are shown in Fig. 2. The retention times on Apiezon M were phenacetin 2.5 min and caffeine 5.4 min, and on Poly S-179 were phenacetin 1.5 min and caffeine 3 min. The peak shape for both substances was symmetrical allowing quantitation by the peak-height ratio method. The minimum level of detection was 0.05 μg/ml, and the calibration curve was linear over the range 0—50 μg/ml. The coefficients of variation between assays were 1.67% at 10 μg/ml (n = 6) and 6.5% at 1 μg/ml (n = 6). There were no interfering endogenous substances in blank plasma, urine or saliva.

Duplicate samples of plasma were analysed by both TLC and GLC methods. The data were correlated by linear regression analysis which yielded a correlation coefficient of 0.91 for 76 observations. A paired Student's t-test of these values gave a value of 2.12, which is significant at the 5% level and indicates a systematic trend for the TLC assay to give a lower estimation than the GLC assay. This is demonstrated by the plot of the difference of the two assays vs. the mean of the assays (Fig. 3). Both assays yield data that are a measure of the caffeine level in plasma, and in a study of the pharmacokinetics of orally administered caffeine in man (to be published shortly) both gave identical estimates of the plasma $t_{½}$.

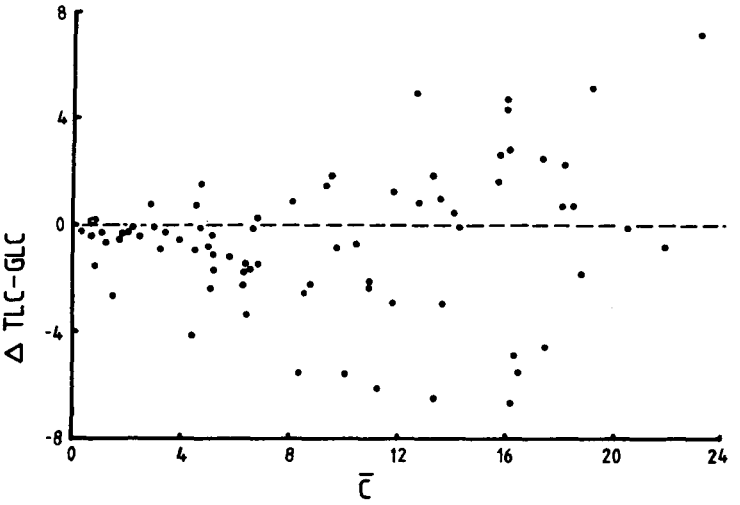

Fig. 3. Plot of difference between plasma levels estimated by TLC and GLC methods (ordinate, μg/ml) vs. the mean of the assays (C, = μg/ml) for 76 paired observations.

The TLC technique is simple, rapid and allows processing of a large number of samples. It is, however, less sensitive than GLC or HPLC methods. Although the use of the AFID allows a relatively simple extraction procedure, the GLC method requires more complex sample preparation but has greater sensitivity than the TLC method or that claimed for HPLC [2]. It is suitable for estimates of caffeine in saliva and can be used for very small volumes of blood. The

presently described TLC and GLC methods are considerably more sensitive than the previously described GLC assay [8] which required a 5-ml blood sample to achieve a reported sensitivity of 0.25 μg/ml. Both techniques are therefore suitable for use in studies of caffeine pharmacokinetics.

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Book Review

Analysis of steroid hormone drugs, by S. Görög and Gy. Szász, Elsevier, Amsterdam, Oxford, New York, 1978, 426 pp., price Dfl. 138.00, US$ 59.00, ISBN 0-444-99805-5.

This volume has been written primarily for analysts working in quality control laboratories, and for those concerned with the pharmaceutical analysis of steroid hormones. Analytical methods designed for clinical or biochemical samples have been largely excluded. The authors also express the hope that some chapters will be of value to chemists and biochemists working in the steroid field. The book does indeed fulfil its aims to a high degree.

The four principal sections of the book deal with basic information on steroids (Chapters 1—3), chromatography (Chapters 4 and 5), general analytical methods (Chapter 6), and details of quantitative analyses of steroids according to their functional groups (Chapter 7). Two further short chapters cover the analyses of formulations and of raw materials. Author and subject indexes conclude the volume, which is documented with over 1300 references, dating to about 1975.

The outline of steroid hormone chemistry in Chapter 1 is generally sound, but contains unfortunate errors in the diagrams on pp. 19—20 purporting to depict equatorial and axial substituents. Furthermore, cholic, desoxycholic and hyodesoxycholic acid are inexplicably misnamed cholanic, desoxycholanic and hyodesoxycholanic on p. 28 and in the Index.

Liquid chromatographic methods and their applications to steroid hormones are surveyed in Chapter 4 (by Gy. Szász). The growing importance of high-performance liquid chromatography is recognised by the authors, but many applications of this technique in steroid drug analysis have been published too recently to be included. The "classical" aspect of the chapter is exemplified by a table of hormone separations by partition column chromatography, in which the 19 references are dated between 1951 and 1965. Nevertheless, there remains much useful information, particularly in respect of practical details of thin-layer chromatographic mobilities and detection methods.

The account of the gas chromatography of steroid hormones (Chapter 5) seems to betray some lack of practical experience in this field. For example, the use of stationary phases in 2—3% concentrations (p. 148) is rarely necessary, 1% being quite satisfactory in properly prepared columns. No satisfactory illustration is given of the power and importance of retention data as indicators of structural features.

Chapter 6 contains a wide range of information on other physical methods

of analysis, principally spectrophotometry (UV, visible and IR), fluorimetry, and NMR spectroscopy. IR spectra (KBr discs) of 23 steroid hormones are illustrated and annotated.

The excellent review of functional group analysis of steroid hormones (Chapter 7) is the best feature of the book, and clearly reflects a wealth of experience on the part of its author. Not only steroid analysts, but also organic chemists and biochemists, will find instructive the elegant examples of selective reactions — including many devised by Dr. Görög — in this chapter. (It should be noted that an incorrect structure is shown on p. 281 for Girard P reagent.)

The quality of the book production is below the usual Elsevier standard: the typography is both imperfect and irregular.

In summary, this volume provides a valuable compilation of principles and methods of analysis of steroid hormones, and gives critical and detailed attention to methods used for pharmaceutical purposes. In addition to its usefulness for the specialised analyst, the book is recommended as a good source of practical information for anyone working with steroid hormones.

Glasgow (Great Britain) C.J.W. BROOKS

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