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CHROMBIO. 107

CHROMATOGRAPHIC PROFILE OF HIGH BOILING POINT ORGANIC ACIDS IN HUMAN URINE

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(Received August 24th, 1977)

SUMMARY

The profile of high boiling point organic acids in urine samples from both normal subjects and patients suspected of having some form of metabolic disorder has been determined by combined gas chromatography—mass spectrometry. Fifteen different compounds eluting after hippuric acid have been identified, including two, cinnamoylglycine and acetyltributylcitrate, which have not been recognised previously. Relative retention times and abbreviated mass spectra of the identified compounds are presented.

INTRODUCTION

The diagnosis of many inborn errors of metabolism is based on the determination of organic acids in urine. In recent years the separation and identification of these acids has become greatly facilitated by the development of methods based on combined gas chromatography—mass spectrometry (GC—MS) [1—5]. With these methods, screening of samples from patients suspected of having such disorders is now possible.

Together with the development of the methodology, there has been a gradual accumulation of knowledge of the organic acid constituents of urine under a wide range of conditions [6—9]. The compilation of such data is

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necessary for the rapid assessment of chromatograms of urinary organic acids in screening situations.

Most gas chromatograms of urinary organic acids from adult subjects are characterised by a large peak of hippuric acid which forms a readily recognisable landmark. Most compounds of interest in the diagnosis of metabolic disorders elute between the solvent front and this peak. In general, the commonly occurring compounds in this region of the chromatogram have been well characterised in both normal and abnormal states.

For compounds eluting after hippuric acid, the data are much less extensive for a number of reasons. Firstly, few peaks are found in this region of the chromatogram in most samples and, so far, none has been associated with a particular metabolic disorder. Secondly, many of the compounds are eluted at temperatures close to the maximum thermal stability of the commonly used chromatographic columns (such as OV-17). This often results in considerable masking of the urinary constituents by bleeding of the stationary phase.

To extend the data on urinary organic acids excreted under a variety of conditions, we undertook a study of the compounds eluting after hippuric acid in acid diethyl ether extracts of urine samples from both normal subjects and patients suspected of having some form of metabolic disorder. The results were obtained using the type of procedure commonly employed in screening situations rather than the more time-consuming quantitative methods which are preferable when studying particular disorders. The resulting profile contains those compounds which are likely to be observed during normal screening for organic acids and should aid in the interpretation of chromatograms obtained under such conditions.

MATERIALS AND METHODS

Chemicals

Reference compounds were obtained commercially or synthesised if unavailable. Cinnamic acid, 3- and 4-hydroxybenzoic acids were obtained from Fluka (Buchs, Switzerland) and 3-hydroxybenzaldehyde from Sigma (St. Louis, Mo., U.S.A.).

Glycine conjugates of cinnamic acid and the hydroxybenzoic acids were prepared by reaction of the acid with glycine ethyl ester using N,N′-dicyclohexylcarbodiimide with subsequent transesterification to the methyl ester as previously described [10].

3-(3-Hydroxyphenyl)hydracrylic acid was prepared by a Reformatsky reaction of 3-hydroxybenzaldehyde with ethyl bromoacetate and zinc.

Acetyltributylcitrate was prepared from citric acid by acetylation with acetic acid—acetic anhydride followed by butylation with HCl-saturated 1-butanol.

Preparation of samples

Early morning urine samples were obtained from 10 normal subjects and 50 patients suspected of having some form of metabolic disorder. Three methods were used to obtain an organic acid extract of these samples. In most cases the

compounds were extracted from acidified urine into diethyl ether, either manually or with a continuous extraction apparatus [2]. These procedures were used for most samples as the aim was to determine those compounds which would be detected under normal screening conditions where a rapid answer is often required. Under these conditions, speed and ease of extraction are more important than quantitative recovery.

For quantitative recovery of the organic acids and extraction of highly polar compounds, the ion-exchange chromatography method of Horning and Horning [1] was used. Analysis of samples using this method ensured that no commonly occurring compounds had been overlooked by using the less-quantitative extraction procedures.

The organic acids were converted to their methyl esters with diazomethane prior to GC, as previously described [2]. *n*-Eicosane was added to all samples as an internal standard.

Gas chromatography

A Varian Model 2100 gas chromatograph was fitted with a 2-m glass column (4 mm I.D.) packed with 6% Dexsil 300 on Chromosorb W HP, 80—100 mesh, obtained from Analabs (North Haven, Conn., U.S.A.). The injector and detector temperatures were set at 280° and the column temperature was programmed from 150° to 350° at a rate of 6°/min. The carrier gas was nitrogen at a flow-rate of 20 ml/min. The gas chromatograph was fitted with a hydrogen flame ionisation detector.

Dexsil 300 was chosen as the stationary phase in this study as it has a particular high thermal stability. This allows analysis of compounds with a high boiling point without interference from bleeding of the column material. In addition, this material is slightly less polar than the stationary phases which are commonly used in the analysis of organic acids so that retention times are generally shorter [11].

Gas chromatography—mass spectrometry

A 2-m glass column was packed with Dexsil 300 as described above and used in a Varian Model 1440 gas chromatograph connected to a Varian CH7 mass spectrometer (Varian MAT, Bremen, G.F.R.) by means of a glass-frit type molecular separator. The GC conditions were as described above except that the carrier gas was helium at a flow-rate of 30 ml/min. The mass spectrometer was operated at an ionisation energy of 70eV.

For detection of known compounds by MS the mass spectrometer was connected on-line to a computer system (SpectroSystem 100 MS, Varian MAT, Bremen, G.F.R.). In addition, an off-line computer system was used to match peaks of unrecognised mass spectra with a library file of recorded spectra.

Compounds from the various urine samples were identified by comparison of GC retention times and mass spectra with those of reference compounds. In general, quantitation of the amounts of the different compounds was not attempted.

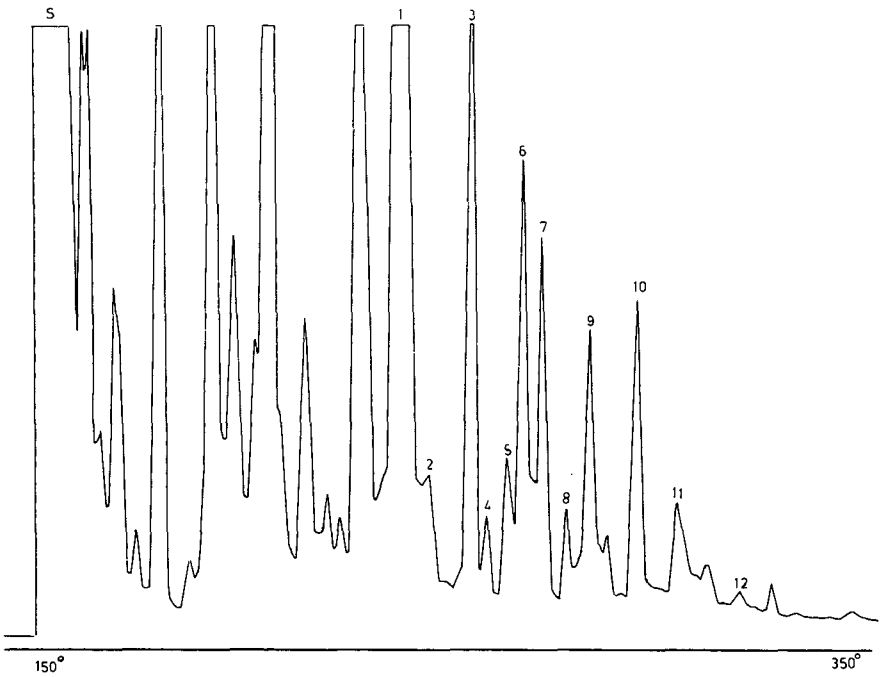

Fig. 1. Profile of high boiling point organic acids in a continuous ether extract of a normal urine sample. The extract was analysed on a column of Dexsil 300 as described in the text. The compounds identified are: 1, hippuric acid; 2, 3-(3-hydroxyphenyl)hydracrylic acid; 3, indoleacetic acid; 4, palmitic acid; 5, caffeine; 6, eicosane; 7, unknown compound of molecular weight 296; 8, 3-hydroxyhippuric acid methyl ester, methyl ether; 9, 4-hydroxyhippuric acid methyl ester, methyl ether; 10, cinnamoylglycine; 11, 4-hydroxyhippuric acid methyl ester; 12, phenylacetyl glutamate. The solvent front is indicated by S.

RESULTS

A gas chromatogram on Dexsil 300 of a continuous diethyl ether extract of a normal urine sample is shown in Fig. 1. In this particular sample, ten compounds eluting after hippuric acid have been identified. Under normal screening conditions, only three or four of these compounds would be detected in an average urine sample as the efficiency of the extraction process is much lower. Even when quantitative extraction methods are used, few urine samples contain more than five or six peaks in this region of the chromatogram. Fig. 1 shows clearly that the compounds under consideration can be readily separated under the GC conditions used in this study.

Fig. 2 shows compounds which were commonly found in both normal urine and in samples from patients with undiagnosed metabolic disease. This list of compounds was prepared from the results obtained by studying a large number of urine samples each of which contained only a few of the compounds. The compunds are located according to their relative retention times on Dexsil 300 with reference to hippuric acid.

To aid the identification of these compounds, the five major peaks in their mass spectra and their molecular weights are presented in Table I.

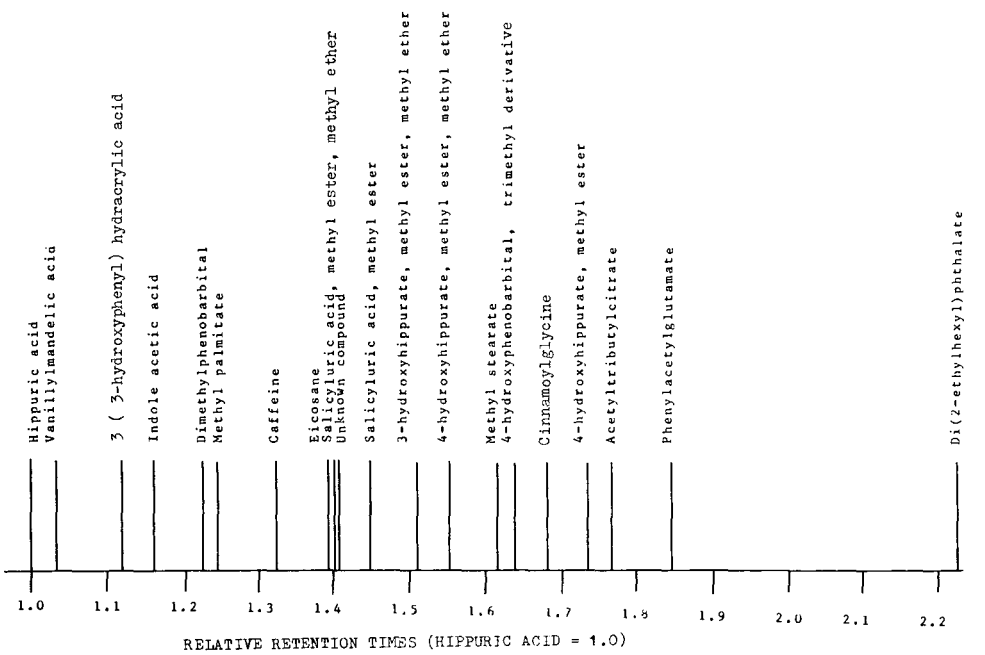

Fig. 2. Compounds commonly found in organic acid extracts of human urine both in normal subjects and patients suspected of having some metabolic disorder. The compounds are presented according to their relative retention times on Dexsil 300 with respect to hippuric acid.

DISCUSSION

The compounds shown in Fig. 2 were commonly found in urine samples and merit consideration when examining chromatograms of urinary organic acids. Of the compounds, only five are of mainly endogenous origin and these rarely occur as major peaks. These compounds are vanillylmandelic acid, indoleacetic acid, palmitic and stearic acids and phenylacetylglutamate (from phenylacetylglutamine).

Three drug metabolites are included as they are commonly found in urine samples submitted for organic acid analysis and may be present as major peaks. The presence of these drug metabolites, salicyluric acid, phenobarbital and 4-hydroxyphenobarbital, may cause some confusion if methyl esters are prepared for GC, as several derivatives can be formed from each compound. In particular, there may be up to six derivatives of phenobarbital and 4-hydroxyphenobarbital depending on the degree of methylation.

The major group of compounds eluting after hippuric acid comprises the phenolic acids and related compounds. Many of these have a high boiling point as they are excreted as glycine conjugates, for example the hydroxyhippuric acids and cinnamoylglycine. Under normal circumstances, these compounds are considered to arise from dietary sources, particularly the flavonoid constituents of plants [12]. However, in some forms of gastrointestinal disease,

TABLE I

THE FIVE MAJOR PEAKS IN THE MASS SPECTRA OF THE COMPOUNDS IDENTIFIED IN THE HIGH BOILING POINT FRACTION OF HUMAN URINE ORGANIC ACID EXTRACTS AS IN FIG. 2

Compound	*m/e* ratios	Molecular weight
Hippuric acid	105 77 134 51 193	193
Vanillylmandelic acid	167 139 226 114 108	226
3-(3-Hydroxyphenyl)hydracrylic acid	123 95 122 196 121	196
Indoleacetic acid	130 189 77 105 103	189
Dimethylphenobarbital	232 117 146 118 175	260
Methyl palmitate	74 87 43 55 270	270
Caffeine	194 109 55 67 82	194
Eicosane	43 57 71 41 85	282
Salicyluric acid methyl ester, ether	135 77 90 92 136	223
Unknown compound	179 236 222 147 207	296
Salicyluric acid methyl ester	121 209 120 65 92	209
3-Hydroxyhippuric acid methyl ester, ether	135 77 223 107 92	223
4-Hydroxyhippuric acid methyl ester, ether	135 223 77 92 107	223
Methyl stearate	74 87 43 56 75	298
4-Hydroxyphenobarbital(trimethyl derivative)	261 290 234 148 262	290
Cinnamoylglycine	131 103 77 102 218	219
4-Hydroxyhippuric acid methyl ester	121 209 65 93 150	209
Acetyltributyl citrate	185 259 129 43 57	402
Phenylacetyl glutamate	91 116 142 174 84	292
Di-(2-ethylhexyl)phthalate	149 57 167 71 70	279

they can be formed from the metabolism of phenylalanine and tyrosine by gastrointestinal microorganisms [13].

The 3- and 4-hydroxyhippuric acids were found in almost all samples and have been listed in several previous tables of urinary organic acids [6—9]. They are readily detected during organic acid screening. Cinnamoylglycine was also found in almost all samples although the amount present was usually quite small. This compound does not appear to have been reported in earlier lists of urinary organic acids.

In samples from adult subjects, caffeine was a common constituent. The excretion of this compound has been extensively studied in man [14].

Most urine samples also contained 3-(3-hydroxyphenyl)hydracrylic acid, although the amount varied from a major peak to just detectable levels, in agreement with the findings of Duncan et al. [15]. This compound was first characterised by Armstrong and Shaw [16] and is considered to be synthesised from phenylalanine by gastrointestinal microorganisms.

Two other compounds were commonly found in samples from patients which were submitted for organic acid analysis. These are the plasticisers di(2-ethylhexyl) phthalate and acetyltributyl citrate. The first of these is a common contaminant of biological materials [17] and usually arises from plastic storage containers. Acetyltributyl citrate is present in PVC transfusion tubing [18] and many other plastic materials but has not previously been described in

human urine. Whether it is excreted following exposure or accumulates in the sample during storage in plastic containers is unknown.

In addition to the compounds identified above, one other compound was found in almost all samples but has so far not been identified. This compound has a relative retention time of 1.41 with respect to hippuric acid and a molecular weight of 296 in the form of the methyl ester. The parent compound is resistant to acid hydrolysis. The major peaks in the mass spectrum of this compound are presented in Table I.

Whereas the method described above allows the identification of the most commonly occurring organic acids eluting after hippuric acid, it is not particularly suitable for the metabolites of catecholamines which are formed endogenously and which are mainly present in this region of the chromatogram. This group of compounds contains a number of hydroxy- and methoxyphenolic acids. The formation of methyl esters of these compounds for GC often also results in the formation of methyl ethers with the free phenolic hydroxyl groups and the extent of this process is quite variable. This alters the relative number and position of the hydroxyl and methoxyl groups, making identification difficult. Analysis of catecholamine metabolites is therefore best carried out using some other type of derivative (such as trimethylsilyl esters and ethers) which does not affect the number of methoxy substituents.

The GC profile of compounds with a high boiling point and their characteristic mass spectra presented in this paper include those compounds which are most likely to be found in organic acid extracts of human urine. The information should be useful in the rapid evaluation of organic acid chromatograms under normal screening conditions.

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CHROMBIO. 120

O-TRIMETHYLSILYLQUINOXALINOL DERIVATIVES OF AROMATIC α-KETO ACIDS

MASS SPECTRA AND QUANTITATIVE GAS CHROMATOGRAPHY

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SUMMARY

As an extension of earlier work on aliphatic α-keto acids, a method is described for the quantitative gas chromatographic determination of urinary aromatic α-keto acids. The keto acids are derivatized with *o*-phenylenediamine to yield the quinoxalinols. These compounds are chromatographed after trimethylsilylation.

The aromatic keto acids are stabilized by sodium dithionite (4 mg/ml urine) and storage below 0°. The final derivatives are stable for weeks at room temperature.

Low resolution mass spectra are reported. The fragmentation mechanisms are elucidated by analysis of O-trimethylsilyl-(TMS)-quinoxalinols, O-(TMS-d_9)-quinoxalinols and O-TMS-6(7)-chloroquinoxalinols.

INTRODUCTION

α-Keto acids are of considerable biomedical interest, as a number of hereditary metabolic defects are known to disturb the metabolism of these compounds [1] . They are also a challenge to the analytical chemist because of dimerization, enolization and decarboxylation.

In a series of papers [2—4] we showed that aliphatic α-keto acids are conveniently determined by gas chromatography (GC) of the O-trimethylsilyl-(TMS)quinoxalinols, either with conventional flame ionization detection, or the most sensitive selective ion monitoring [5] , in a gas chromatograph—mass spectrometer combination. In the present paper we discuss our experience with the quantitative GC determination of urinary β-phenylpyruvic acid (PPA) and *p*-hydroxy-β-phenylpyruvic acid (PHPPA) using the same analytical principle. In addition, the mass spectra of the O-TMS-quinoxalinols are presented.

Some of the results we have reported at two recent conferences [6, 7].

*Some of the results described here were obtained during medical thesis work by K.-P.D., H.-U.M. and M.P.

While our own work was in progress a report appeared [8] which documented the value of another bifunctional reagent, naphthalene-2,3-diamine, in the analysis of PPA by high-speed liquid chromatography.

MATERIALS AND METHODS

The gas chromatograph and other equipment and the sources of most chemicals have been described in a previous publication [3]. The sodium salt of PPA was obtained from Fluka (Buchs, Switzerland). Free PHPPA and 4-chlorophenylene-1,2-diamine [9] were from EGA-Chemie (Steinheim, G.F.R.). The PPA sodium salt was stored at 5° and PHPPA at −20° . Both compounds were used without further purification. Sodium dithionite ($Na_2S_2O_4$) was from Merck (Darmstadt, G.F.R.) and L-phenylalanine (pharmaceutical grade) from Serva (Heidelberg, G.F.R.).

Our GC method, as well as the determination of molar response factors (using peak heights), has also been described in detail in the same publication [3]. We have standardized two more steps in the procedure since then. Extraction of the quinoxalinols is performed after the addition of exactly 1.5 g ammonium sulphate to the reaction mixture. After evaporation of the chloroform extract the quinoxalinols are taken up with exactly 1 ml ether after shaking for 1 min. (This modification somewhat improves the precision of the ketoglutaric acid assay.)

We have meanwhile abandoned the use of free α-ketocaprylic acid (Sigma) as a second internal standard because of problems encountered with the long-term stability of this hygroscopic acid. For good results the sodium salt must be prepared also for this internal standard.

In our standard procedure for aromatic α-keto acids, $Na_2S_2O_4$ (4 mg/ml) is added to the urine, immediately after voiding, instead of toluene and acetic acid. All other steps, as well as the preparation of O-TMS-6(7)-chloroquinoxalinols [9], were the same as described before [3]. The deuterated derivatives were prepared as described previously [2].

Urinary creatinine was measured using the picric acid—NaOH kit supplied by Boehringer (Mannheim, G.F.R.). Quality control of these measurements was carried out with Monitrol II (Merz & Dade, München, G.F.R.).

Low resolution mass spectra of O-TMS-benzylquinoxalinol, O-(TMS-d_9)-benzylquinoxalinol and O-$(TMS)_2$-*p*-hydroxybenzylquinoxalinol were obtained on a Finnigan Model 3000 quadrupole mass spectrometer. Samples were introduced via a Varian Model 1400 gas chromatograph equipped with a 3% Dexsil 300 GC column (180 cm) and a glass jet separator kept at 250°. The injection port temperature was 200°. GC was performed isothermally at 230° . The electron energy was 70 eV and the ion energy between 4 and 6 V.

Low resolution mass spectra of the other derivatives were obtained on a Varian Model CH 7 magnetic mass spectrometer. Samples were introduced via a Varian Model 1700 gas chromatograph equipped with a 3% SE-30 column (150 cm) and a Biemann-Watson separator at 250°. The injection port was kept at 275°. GC was performed isothermally at 240°. The electron energy was 70 eV, and the accelerating voltage was 3 kV. All mass spectra were recorded on an oscillograph and evaluated manually.

RESULTS

Mass spectra

The quadrupole mass spectra of the O-TMS-quinoxalinols derived from PPA and PHPPA are presented in Fig. 1. For the sake of brevity the spectra of per-deutero and chloro derivatives are not shown. They may be obtained on request. In the quadrupole mass spectrometer *m/e* 73 (TMS) is the base peak. In contrast, the spectra obtained with the magnetic instrument have the molecular ion as the base peak. These spectra of O-TMS-quinoxalinols with an aromatic substituent in position 3 have few details only (cf. refs. 4 and 9).

The PPA derivative loses a methyl radical from the TMS group. There is also a distinctive (M—17) ion in the normal as well as in the chloro derivative. This ion has 19% relative abundance (R.A.) and is composed of two even electron species. One has lost two hydrogens, most probably from the phenyl ring (M—20 ion in the deuterated derivative, with 14% R.A.). The other has lost one hydrogen from the phenyl ring and one from the TMS group (M−21 ion in the deuterated derivative, with 7% R.A.). Migration of a TMS-related methylene group to the phenyl ring may have occurred in the latter ion.

The ion *m/e* 217 carries nine TMS-derived hydrogens as do all TMS-quinoxalinols which are not able to perform a McLafferty type rearrangement [4]. This ion can be used for single ion monitoring [5] of PPA using α-ketovaleric acid or α-ketocaprylic acid as internal standard [3, 4].

The ion *m/e* 219 carries the quinoxalinol moiety but no TMS-derived hydrogen. It is formed by the loss of $(CH_3)_2SiO$ from the M—15 ion. Most interesting is the ion *m/e* 146 (isotope peak at *m/e* 146.5). In the deutero and chloro derivatives it shows up as *m/e* 149 (isotope peak at *m/e* 149.5) and

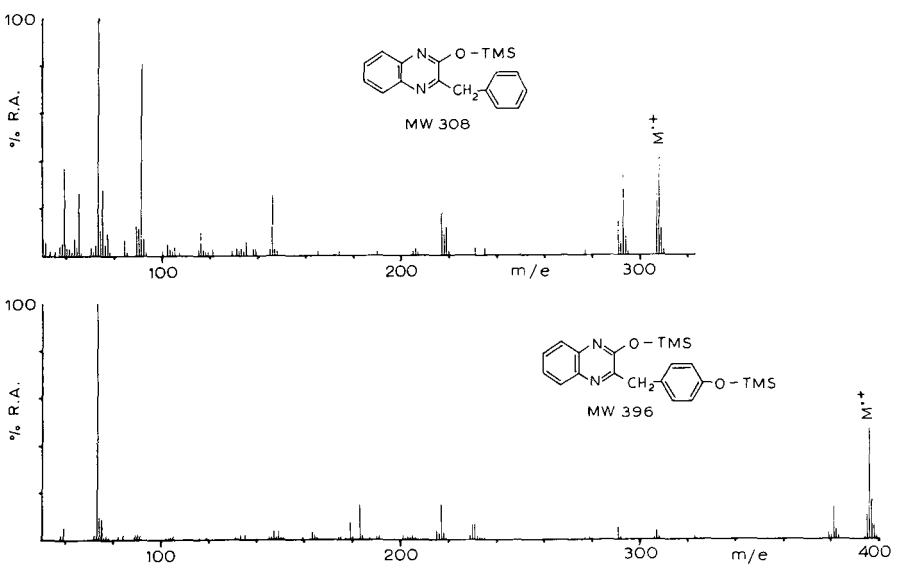

Fig. 1. Quadrupole mass spectra (70 eV) of O-TMS-quinoxalinols derived from β-phenylpyruvic acid (top) and from *p*-hydroxy-β-phenylpyruvic acid (bottom). The spectra of the deutero and chloro derivatives may be obtained on request.

m/e 163 (isotope peak at *m/e* 163.5 and 164), respectively. It thus represents an $(M-15-1)^{2+}$ ion where the single hydrogen most probably is abstracted from the phenyl ring. The ion *m/e* 135 carries six TMS-related hydrogens but no chlorine in the respective derivative. The structure $[C_6H_5-Si(CH_3)_2]^+$ is suggested. The ion *m/e* 116 carries neither a TMS-related hydrogen nor a chlorine in the respective derivative. Its origin is uncertain.

The most prominent ion in the lower mass range, expectedly, is the tropylium ion *m/e* 91. The trivial TMS-related ions in the lowest mass range need not be discussed here [4].

In the mass spectrum of the PHPPA derivative (Fig. 1, lower mass spectrum) M—15 and M—17 are found as described above for the PPA derivative. Three major fragments in the upper mass range of the PHPPA derivative contain six TMS-related hydrogens and the chlorine from the quinoaxaline moiety, respectively (*m/e* 291, 307, 323). The ion *m/e* 291 is therefore formed by loss of trimethylsilanol from the $(M-15)^+$ ion, a loss similarly observed in sterol-TMS ethers [10]. The ion *m/e* 217 is observed also in the PHPPA derivative (see above).

The ion *m/e* 183 (15% R.A.) is an $(M-15-15)^{2+}$ ion as evidenced by an isotope peak at *m/e* 183.5, a shift of 6 a.m.u. in the deuterated derivative and a shift of 17 a.m.u. in the chlorinated derivative. Most probably each silicon atom carries one of each charge. The ion $(M-30)^+$ has only a very low intensity (less than 0.3% R.A.). Intense $(M-30)^{2+}$ ions have also been observed by VandenHeuvel et al. [11] in di-TMS derivatives of dihydroxydiphenyls and related compounds. The situation with the PHPPA derivative is evidently analogous.

All ions below *m/e* 180 are free of chlorine in the respective derivative. Most prominent in this range is ion *m/e* 179 with nine TMS-related hydrogens. Its composition is therefore $[CH_2{=}C_6H_4{=}O{=}TMS]^+$.

Gas chromatographic properties

In Table I we present the methylene units (MU) [12] of the PPA and PHPPA derivatives for three commonly used phases. The O-TMS-6(7)-chloroquinoxalinols yield symmetrical peaks on OV-17 only. It was not possible to deduce which structural isomer (6-chloro or 7-chloro) it is that elutes first on the less-polar phases.

Interestingly, O-TMS-3-(*p*-hydroxybenzyl)quinoxalin-2-ol eluted with two peaks on OV-101 (not shown). On OV-1, as well as on OV-17, this derivative yields perfectly symmetrical peaks, as does the respective derivative of PPA. Due to the presence of a benzyl moiety all derivatives are highly polar, as evidenced by the large ΔMU values [2].

Quantitative parameters

One of us has introduced $Na_2S_2O_4$ as a protective substance in TLC and GC assays of PPA [13]. Our experience with this reducing substance in the GC assay of aromatic α-keto acids is shown in Table II. At biomedically relevant concentrations these acids are stable for at least four days when urine is kept (and mailed) frozen in the presence of $Na_2S_2O_4$. Clearly, PHPPA is more labile than PPA.

The molar response factors on OV-1 with α-ketovaleric acid as internal

TABLE I

MU VALUES OF O-TMS-QUINOXALIN-2-OLS SUBSTITUTED IN POSITION 3 AND 6 OR 7

Temperature program was run at 2°/min starting at 50°.

Substituent in position 3	Substituent in position 6 or 7	Parent α-keto acid	MU values			ΔMU (OV-17) − (OV-1)
			OV-1	OV-17	Dexsil 300	
Benzyl	H	PPA	20.63*	23.31	21.32	2.68
Benzyl	Cl	PPA	22.49**	25.01	23.31, 23.41	2.52
p-Hydroxybenzyl	H	PHPPA	24.06	26.52	24.67	2.46
p-Hydroxybenzyl	Cl	PHPPA	26.0**	28.26	n.d.	2.3

*Taken from ref. 2.
**Peak broadened.
n.d. = Not determined.

TABLE II

STABILITY OF AROMATIC KETO ACIDS IN URINE

4 mg sodium dithionite were added per ml. Numbers denote % of initial concentration.

Days storage	Room temperature		Refrigerator		Freezer (−20°)	
	PPA	PHPPA	PPA	PHPPA	PPA	PHPPA
0	100	100	100	100	100	100
1	102	86	107	113	119	134
2	92	79	124	114	119	100
3	68	7	111	121	112	121
4	71	7	103	86	111	105
Conc.	1.7 m*M*		1.5 m*M*		1.5 m*M*	

standard [3] were 0.769 ± 0.057 (n = 11) for PPA and 0.769 ± 0.062 (n = 5) for PHPPA. The data obtained for estimation of these response factors yielded mean coefficients of correlation [3] of r = 0.997 (0.989—0.999) for PPA and r = 0.997 (0.996—0.999) for PHPPA.

In Table III it is demonstrated that the final O-TMS-quinoxalinols are stable at room temperature for at least four weeks. It is expected that the stability would be found to be much greater if the vials are opened only once.

Finally, in Table IV parameters of quality control [14] for our assay are given. The specificity of the GC assay is determined by the size of contaminating peaks in the chromatographic position of PPA and PHPPA. In normal controls on OV-1 it never corresponds to more than 30 μM. Specificity for PPA is still better by a factor of 3 on OV-17 (not shown). As also shown in Table IV, there is no major influence of the initial concentration of the keto acid on the accuracy and precision of the assay.

Application of the procedure to the study of phenylketonuria (PKU)

We have applied our method to the analysis of urinary aromatic acids in

TABLE III

STABILITY OF O-TMS-QUINOXALINOLS FROM URINARY AROMATIC KETO ACIDS

Concentration of keto acids in urine was 1.5 m*M*. The sample was stored at room temperature. Until day 66 the sample vial was opened 11 times. Numbers denote % of initial peak height ratios of compound vs. internal standard.

Days	PPA	PHPPA
1	100	100
3	102	111
4	99	103
14	98	102
20	95	96
26	97	94
48	84	102
66	90	91

TABLE IV

RELIABILITY OF THE O-TMS-QUINOXALINOL METHOD FOR QUANTITATIVE DETERMINATION OF URINARY AROMATIC KETO ACIDS

Determinations were made on OV-1 under the chromatographic conditions described in ref. 3 and the legend to Fig. 2.

	PPA	PHPPA
Specificity	Very high at concentrations $> 30\ \mu M$	Very high at concentrations $> 30\ \mu M$
Accuracy	99.7% at 280 μM ($n = 7$) 98.8% at 710 μM ($n = 6$)	98.4% at 290 μM ($n = 7$) 92.6% at 760 μM ($n = 6$)
Precision (S.D./mean)	4.1% at 280 μM ($n = 7$) 2.8% at 710 μM ($n = 6$)	7.8% at 290 μM ($n = 7$) 8.8% at 760 μM ($n = 6$)

mentally retarded patients with PKU who had never been treated, and who were on a normal, unrestricted diet. The first urine in the morning was sampled for analysis. Fig. 2 shows an example. In 15 patients aged 13—40 years we found PPA in a concentration of 409.9 ± 149.8 μmoles/mmole creatinine (range 104—720). PHPPA was detected in all PKU urines. The mean concentration was 16.6 ± 6.5 μmoles/mmole creatinine (range 8—30). The detailed presentation of these and other relevant data will be the subject of a forthcoming paper.

Preliminary data on four controls and three obligate heterozygotes indicate the value of urinary PPA measurement in the detection of carriers by an oral load of L-phenylalanine (100 mg/kg body weight). In the first six hours after loading, controls excreted a total of 6.52, 8.13, 4.09 and 3.70 μmoles PPA. The three parents of PKU patients excreted 16.21, 47.19 and 19.18 μmoles PPA. In this small amount of material we have already found an overlap in

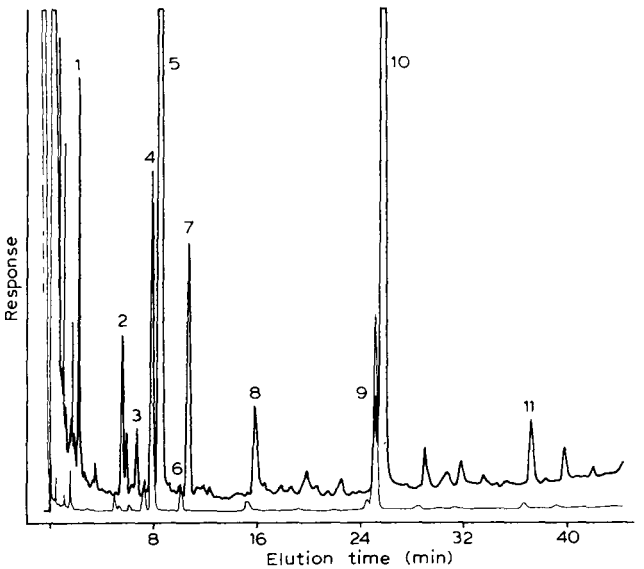

Fig. 2. Gas chromatogram of urinary acids from a 26-year-old mentally retarded male patient with untreated PKU (B. Th., Neuerkeröder Anstalten). Urine with $Na_2S_2O_4$ was received by post at ambient temperature and analysed 24 h after sampling. GC was carried out on 3% OV-1 in a glass column (180 cm long) with N_2 at 60 ml per min. Temperature program was from 70° to 160° at 2°/min. The lower trace is recorded at 10 mV recorder span, the upper one at 1 mV. The following acids are indicated in the figure (micromolar concentrations in brackets): 1 = phenylacetic acid; 2 = mandelic acid (319); 3 = pyruvic acid (91, determined on Dexsil 300 from urine treated with toluene—acetic acid [3]); 4 = *o*-hydroxyphenylacetic acid (855); 5 = phenyllactic acid (5.434); 6 = *p*-hydroxyphenylacetic acid; 7 = ketovaleric acid (internal standard); 8 = hippuric acid; 9 = ketoglutaric acid (177, determined on Dexsil 300 from urine treated with toluene—acetic acid); 10 = phenylpyruvic acid (4.912); 11 = *p*-hydroxyphenylpyruvic acid (166). Creatinine concentration was 10.5 m*M*. 500 µl urine were taken for derivatization. An equivalent of 5 µl urine was injected for analysis. *p*-Hydroxyphenyllactic acid remains in the aqueous phase on extraction with chloroform (unpublished results).

the excretion *o*-hydroxyphenylacetic acid (OHPAA). Further work on this topic is in progress [15].

We apply our method also to monitoring children with PKU who are dietetically treated at the Pediatric Department, University of Göttingen. Due to the sensitivity of the PPA assay it is possible to distinguish between acute and chronic derangements of dietary control [16].

DISCUSSION

Hemmerle [17] clearly demonstrated the great lability of PPA, which on contact with air is decomposed to benzaldehyde and oxalic acid. Also, spontaneous decarboxylation to phenylacetic acid is observed on extraction of free PPA [18].

The most successful procedure to prevent loss of PPA on derivatization for GC has been the addition of excess quantities of ascorbic acid to the urine, as

described by Wadman et al. [19]. This method could not be used here, as *o*-phenylenediamine reacts with ascorbic acid [20]. Because of smell and toxicity we did not try the sodium hydrosulphide procedure of Nielsen [21] but used sodium dithionite as a protective substance [13] instead. This procedure is fully evaluated in the present paper. While our work was in progress 2-mercaptoethanol was also tried successfully by Hayashi et al. [8] for stabilizing PPA.

Hinsberg [22], in 1887, suggested the use of *o*-phenylenediamine for the determination of α-keto acids. Wieland first reported such a procedure in 1949 [23]. We have shown in the present and three previous [2–4] papers that the GC determination of α-keto acids, both aliphatic and aromatic, can be performed very reliably after formation of the stable O-TMS-quinoxalinols with *o*-phenylenediamine and a silylating agent. In a carefully documented study Chalmers and Watts [24] found in 34 PKU patients almost the same urinary levels of PPA and PHPPA as we did. The concentrations (given as μmoles/mmole creatinine) they found were, for PPA 517.0 ± 182.7 (range 282–916), and for PHPPA 22.1 ± 26.7 (range 4–35). The correlation between the levels of both acids is +0.19 ($p>5\%$) in the study of Chalmers and Watts [24], and +0.88 ($p<0.1\%$) for our data. Evidently, due to stabilization of the urinary acids and to the very high stability of the final GC derivatives, our assay procedure for aromatic α-keto acids and especially for PHPPA is better controlled.

In the field of PKU research due credit can now be given to the importance of PPA as the major abnormal metabolite of phenylalanine. In the previous literature this view had been somewhat distorted because OHPPA was much more easily quantitated than PPA. Thus, without obvious biochemical reason, OHPPA was taken as the most important metabolite to diagnose heterozygotes [25–27; see also ref. 28] and to monitor dietary treatment [29]. Our own data [15, 16] indicate that a fresh look at these problems should be taken. Progress in human biochemical genetics is not only a consequence of the detection of new metabolites but also of careful quantitative analysis of known compounds [30].

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LIQUID CHROMATOGRAPHIC ANALYSIS OF SEBUM LIPIDS AND OTHER LIPIDS OF MEDICAL INTEREST*

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SUMMARY

A technique is described for the high-pressure liquid chromatographic (HPLC) analysis of sebum lipid classes. The lipid classes present in sebum are separated by gradient elution HPLC from a microparticulate silica column and detected using a moving-wire detector. The system described can be linked to a computer. Quantitation can be carried out by comparing peak areas obtained with those of an internal standard. Peak trapping for further investigations of the separated components, for example by gas chromatography—mass spectrometry, is very easy.

Sebum lipids are separated into the following lipid classes: hydrocarbons and squalene, cholesterol esters and wax esters, fatty acids as their methyl esters, triglycerides, 1,3-diglycerides, 1,2-diglycerides, free cholesterol, monoglycerides and other polar materials. Besides to sebum, the method has been successfully applied to other lipid mixtures, such as serum lipids. Examples of other applications are shown.

INTRODUCTION

High-performance liquid chromatography (HPLC) has not yet found widespread acceptance in the field of lipid analysis because of detector performance problems and gradient elution requirements. This situation has recently been reviewed [1]. For a number of analytical lipid class investigations, however, the replacement of thin-layer chromatography (TLC) by HPLC may have definite advantages.

Lipid class analyses of medical interest are still most often carried out by TLC and by enzymatic or colorimetric and photometric assay methods, although gas chromatography will remain the method of choice for the investigation of individual fatty acid or triglyceride compositions. In many cases these

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analytical methods include comparatively lengthy procedures, or they permit the determination of only one substance at a time, such as cholesterol or total glycerol in serum lipids by saponification with subsequent enzymatic analysis.

Adsorption TLC is fairly time-consuming if all the spots have to be scraped off the plate, eluted, and determined separately. Fast TLC procedures, involving densitometry of lipid spots after charring, may (in our experience) often lead to erroneous quantitative results if the various lipid classes present on the plate differ greatly in their contents of unsaturated compounds. Moreover, TLC plus charring is destructive and does not allow further investigation of the separated lipid classes.

For these reasons we have developed liquid chromatographic methods for lipid class analysis where detection is based on the so-called moving-wire detector in the methane conversion form described by Scott and Lawrence [2]. The wide applicability of such a gradient elution—transport—FID system for the analysis of lipids and mixtures of oleochemicals has been shown [3]. One of the first applications for this method was sebum analysis. The method described here is restricted to analyses of the so-called non-polar lipid classes, ranging in polarity from hydrocarbons to monoglycerides. HPLC analyses of polar lipid classes have also recently been described in the literature [4—6], and these may some day lead to new methods for the analysis of brain and membrane lipids of interest to the clinical chemist.

EXPERIMENTAL

The experiment set-up was very similar to those described in earlier reports [7,8]. Major differences are the use of chlorinated hydrocarbon solvents, slurry-packed microparticulate columns and the computer link.

The basic equipment which we use for lipid class analysis by HPLC consists of 3 solvent reservoirs, a solvent-gradient programming device with two solenoid valves (Ultrograd, LKB Produkter, Bromma, Sweden), a high-frequency reciprocating pump (Milton Roy 196-100, Dosapro, Neu Isenburg, G.F.R.) and pump-stroke dampening device, pre-column, pressure monitor, sample injection port (Precision Sampling, Baton Rouge, La., U.S.A.), the chromatographic column, a moving-wire detector (Pye Unicam LCM-2), a recorder (Servogor S) and an electronic integrator or connection to a calculating computer (Hewlett-Packard 3352) via an analog-to-digital converter [7,8].

Three mixed solvents (I, II and III) of increasing polarity are used to produce a sequence of two solvent gradients (from I to II and then from II to III), using the Ultrograd gradient programmer. The sequence of solvents used consisted of the mixtures carbon tetrachloride—isooctane (34:66) in solvent reservoir I, chloroform—dioxane—*n*-hexane (40:11:49) in reservoir II, and chloroform—methanol—diisopropylether (34:36:30) in reservoir III.

The columns used were of stainless steel slurry-packed with microparticulate silica gels such as LiChrosorb SI 60, of particle size 5 or 10 μm (E. Merck, Darmstadt, G.F.R.). In the previous reports [3, 7, 8] we had mostly relied on hydrocarbon—ether—alcohol sequences for generating the polarity gradients. However, the use of carbon tetrachloride and chloroform in the solvents resulted in better resolution between free cholesterol and the 1,2-diglycerides, due to

secondary solvent effects, and the microparticulate columns permitted electronic integration of the peak areas.

The Ultrograd program charts which we have developed for lipid class analysis are cut out of black paper and scanned by a photocell in the Ultrograd master unit, which switches the solenoid valves accordingly [7]. The charts are cut in such a way that column rinsing periods are carried out automatically (usually first with solvent II and finally with solvent I) before a new injection is made.

We have tried to keep analysis times as short as 30 min. This time includes both the sequence of two solvent gradients plus the column rinsing or regeneration periods. For a silica gel adsorption column, this regeneration period is very short [9] and, therefore, injection for analysis should be made only during continuous operation of the column. Whenever the column has been standing idle for some time, it is first started with a blank run.

The eluted lipid class peaks are detected and quantitated by a Pye-Unicam LCM-2 moving-wire detector, linked to an integrator or computer. For the HP 3352 computer we have developed a program for sebum analysis. Response factors are obtained from test chromatograms, and internal standards are used where applicable. In a few cases area percentage calculations were made.

In the LCM-2 detector, a small portion of the sample is transformed to methane, which in turn is detected by a flame ionization detector. It should be noted here, however, that the response of this detector depends not only on the carbon content of the sample, but also on a number of other factors. The most important of those is the solvent flow-rate, which in turn may depend on the column back-pressure if a reciprocating piston pump is used, and which may change during a gradient run. Rather frequent recalibration of the whole set-up is therefore a prerequisite for quantitative work. This is done by injecting a quantitative test mixture, followed by an RC ("recalibrate") order to the computer.

RESULTS AND DISCUSSION

Fig. 1 shows typical lipid class chromatograms as obtained from a mixture of test substances. Squalene appears first, followed by a wax ester, methyl oleate, triolein, two isomers of a monoglyceride diacetate, 1,3-diglyceride, 1,2-diglyceride, cholesterol, and finally monoglyceride.

A question that has often been raised in the recent literature [9] is that of the reproducibility of gradient elution. For a given equipment, this can be checked by looking at the relative standard deviations of peak retention times.

Table I shows that in our case the relative standard deviation (R.S.D.) of single values for retention times was generally around 1—3% and that the reproducibility of peak areas in the investigation was generally around 3—7%. It was found that evaporator oven temperatures play a significant role in this investigation. For example, an increase of that temperature will considerably increase the R.S.D. of both the squalene and fatty acid methyl esters peaks, while leaving the other peaks largely unaffected.

Fig. 2 shows chromatograms obtained for samples of human hair surface fat, or sebum. In this case the free fatty acids present in the sebum were meth-

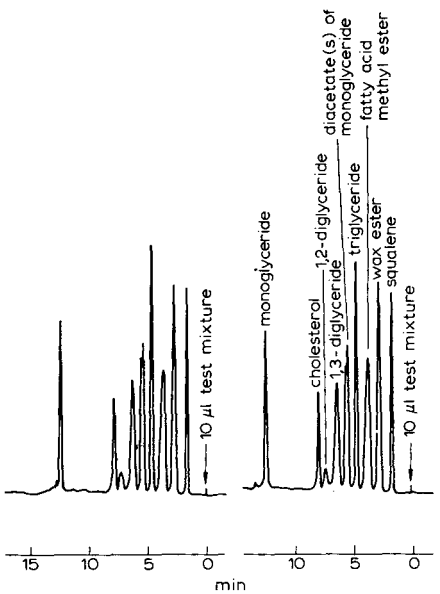

Fig. 1. Lipid class chromatograms of test mixture.

TABLE I

RELATIVE STANDARD DEVIATION OF RETENTION TIMES AND REPRODUCIBILITY OF PEAK AREAS

Ten chromatograms were checked. R.S.D., relative standard deviation. S, squalene (and hydrocarbons); CE, cholesterol esters and/or wax esters; ME, fatty acid methyl esters; TG, triglycerides; IS, internal standard; D, diglycerides; C, free cholesterol; M, monoglycerides (including diols or oxidation products of similar polarity).

	Retention		Peak areas	
	$\bar{x}$ (mm on chart)	R.S.D. (%)	$\bar{x}$ (area %)	R.S.D. (%)
S	13.55	± 1.2	6.23	± 3.4
CE	14.85	± 2.8	17.39	± 2.7
ME	20.00	± 1.7	22.29	± 2.9
TG	29.35	± 2.0	14.28	± 4.6
IS	46.35	± 1.0	12.14	± 3.6
D	52.75	± 1.1	11.60	± 5.3
C	62.80	± 1.1	5.46	± 7.5
M	73.90	± 0.9	8.53	± 6.8

ylated with diazomethane prior to the analysis. A number of individual differences can be detected in the sebum chromatograms. Monoolein diacetate was used as an internal standard for quantitation. The samples were obtained by diethylether extraction from hair of female test persons. The results of this investigation will be reported separately.

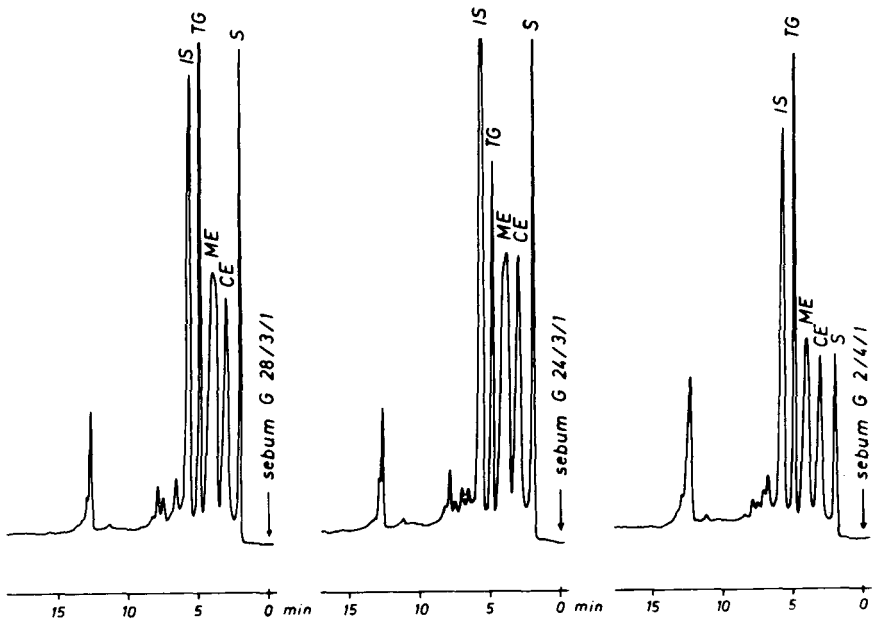

Fig. 2. Chromatograms obtained from various sebum samples. Samples were methylated prior to analysis. See Table I for abbreviations used.

The major differences that can be detected in the sebum chromatograms concern the ratios of the first four major peaks, plus a rather variable, but characteristic, region of small peaks in the vicinity of diglycerides and free cholesterol.

Fig. 3 shows a chromatogram of the unsaponifiable portion of human sebum. Major peaks for squalene, wax alcohols and cholesterol are clearly seen. In addition there is a peak in the polar region that may represent a mixture of dihydroxy compounds plus polar oxidation products, from squalene for example.

Fig. 4 shows a chromatogram of methylated atheroma lipids. In this case not all the peaks could be identified conclusively, but it is quite clear that the two major peaks represent free cholesterol and a very polar compound (with a retention time similar to monoglycerides). Some esterified cholesterol, fatty acid and triglyceride may also have been present.

Fig. 5 shows chromatograms of the chloroform-soluble portion of human cerumen, in the methylated and non-methylated form. It contains lipid classes similar to sebum.

Human blood plasma lipids have also been analysed and the result is shown in Fig. 6. Quantitation was achieved with the aid of monoolein diacetate as internal standard. The plasma lipid sample shown contained cholesterol esters, triglycerides and free cholesterol. Apart from the phospholipids, which usually remain on the column, chromatograms obtained from samples of erythrocyte lipids exhibit only one large peak for free cholesterol.

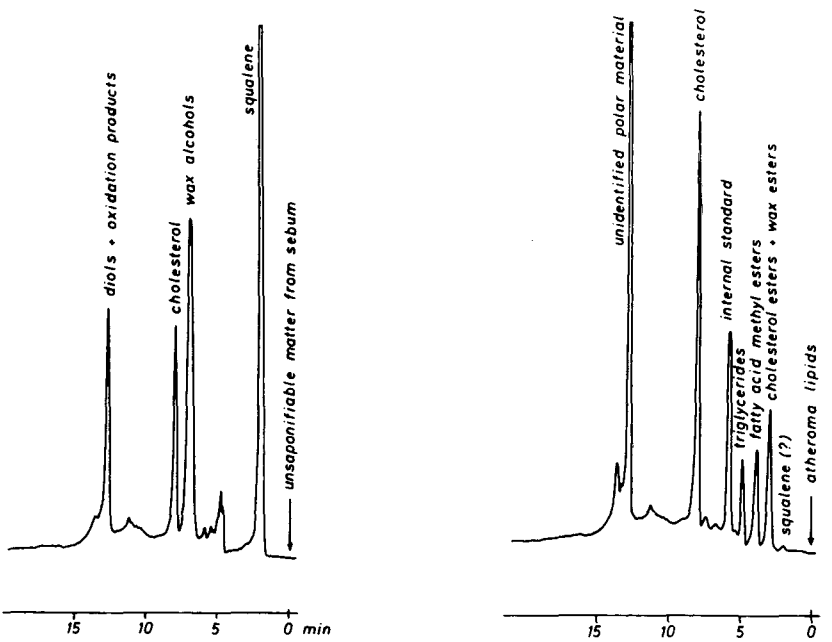

Fig. 3. Chromatogram of unsaponifiable portion of human sebum.

Fig. 4. Chromatogram of atheroma lipids. The extract was methylated by diazomethane prior to analysis.

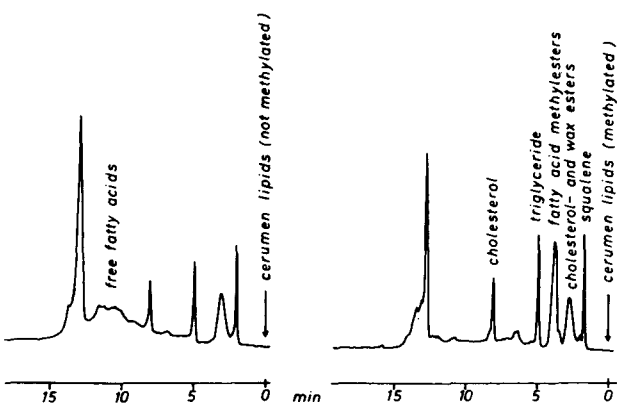

Fig. 5. Chromatograms of cerumen lipids. Methylated and nonmethylated chloroform extracts.

Fig. 7 shows two chromatograms obtained from *vernix caseosa* lipids. The lipid class composition, calculated as area percentage (HPLC, uncorrected), compared well with published results [10]. Diol lipids, which are found only in the surface lipid of the human newborn, appear as a small peak just before the triglycerides.

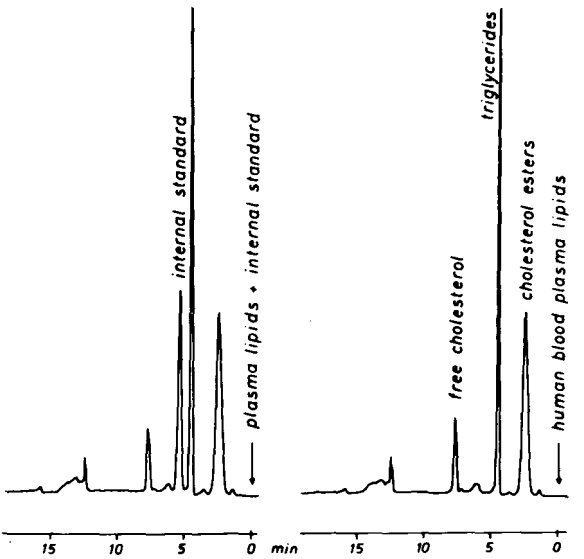

Fig. 6. Chromatograms of a sample of human blood plasma lipids, with and without addition of internal standard.

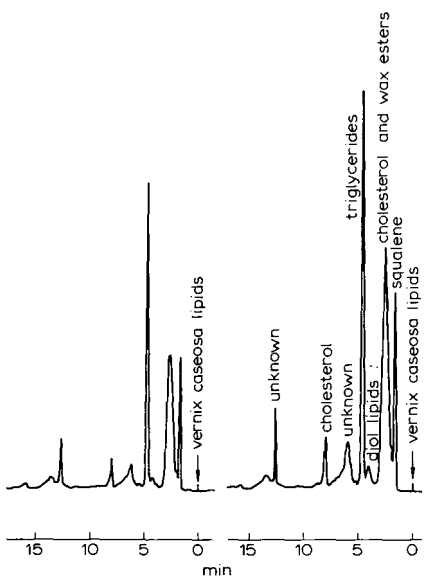

Fig. 7. Chromatograms of a sample of *Vernix caseosa* lipids. Diol lipids appear as a small peak just before the triglycerides.

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We are indebted to Mrs. G. Bittorf, Miss B. Kurth, Mrs. U. Siebert and Mrs. E. Arzberger for expert technical assistance.

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MICROCHROMATOGRAPHY OF HEMOGLOBINS

VIII. A GENERAL QUALITATIVE AND QUANTITATIVE METHOD IN PLASTIC DRINKING STRAWS AND THE QUANTITATIVE ANALYSIS OF Hb-F

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SUMMARY

This microchromatographic procedure for the quantitative analysis of the hemoglobin components in a hemolysate uses columns of DEAE-cellulose in a plastic drinking straw with a glycine—KCN—NaCl developer. Not only may the method be used for the quantitative analysis of Hb-F but also for the analysis of the varied components in mixtures of hemoglobins.

INTRODUCTION

Qualitative and quantitative microchromatographic procedures have been devised for the analysis of a variety of hemoglobin mixtures [1—7]. These methods have also been extended and used for the separation of complex mixtures of hemoglobins on more conventionally sized columns with gradient development [8—10]. This paper describes a miniaturization and simplification of one of these conventional procedures [9, 10]: the chromatographic column is packed in a plastic drinking straw and developed without a gradient

*Contribution No. 5626.

to the desired point of separation after which the column is sectioned with a sharp knife, each section is eluted separately, and the individual components are quantitatively estimated. Rather complex mixtures may be studied in this simple way, and the method is particularly suited for the quantitative determination of human hemoglobin F (Hb-F).

MATERIALS

Blood samples

Specimens were obtained through the Los Angeles Sickle Cell Center and the Childrens Hospital of Los Angeles. EDTA or heparin was the anti-coagulant.

For chromatograms in straws, whole blood was usually used to prepare the sample. When hemolysates were used, a previously described procedure [7] was applied. Only occasionally were samples dialyzed against a large volume of appropriate solution. For conventionally sized chromatograms [9, 10], the sample was prepared by hemolyzing saline-washed cells with four times their volume of water and 0.3 volume of carbon tetrachloride. After centrifuging to remove cell debris, an appropriate volume that contained about 40 mg of hemoglobin was applied directly to the column.

Equipment

Clear plastic drinking straws of the type that may be purchased from restaurant suppliers were used to contain the chromatographic column: their I.D. is about 0.6 cm and their length is 20 cm. It is convenient to have Lucite fittings* for the top and bottom of the straw (Fig. 1A). Only diameter A (0.572 cm; 0.225 in.) of the dimensions in Fig. 1A is critical and may have to be varied slightly, as our experience shows that about 20% of a given lot of straws have the wrong diameters for a given dimension of fitting. In place of such fittings, a 2—3 cm length of constricted glass tubing which is plugged with cotton and attached to the straw with rubber tubing is a somewhat less satisfactory bottom fitting; the other end of the straw is fixed with rubber tubing to a supply of solution. Any perforated object that fits the straw tightly can probably be used as a bottom fitting.

Developers

Two solutions, developers No. 1 and 2 of Ref. 7, are used. The ion exchanger is equilibrated with No. 1 which is 0.2 *M* glycine—0.01% KCN (15 g glycine and 0.1 g KCN per liter) and the chromatogram is developed with No. 2 which is 0.2 *M* glycine—0.015 *M* NaCl—0.01% KCN (15 g glycine, 0.88 g NaCl, and 0.1 g KCN per liter). The pH of these solutions is unadjusted.

Preparation of ion exchanger

DEAE cellulose (DE-52, microgranular and pre-swollen; Whatman, Clifton, N.J., U.S.A.) was equilibrated with developer No. 1 as previously described

*Such Lucite fittings may be obtained by sending US$ 3.00 per pair to W.A. Schroeder, 164-30, California Institute of Technology, Pasadena, Calif. 91125, U.S.A.

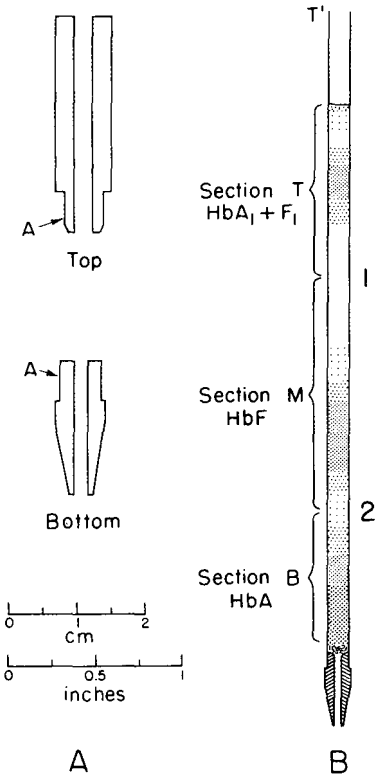

Fig. 1. (A) Dimensions of fittings. (B) Appearance of a typical completed chromatogram.

[3]. As will be discussed below, the chromatograms are equally satisfactory regardless of whether the pH of the ion exchanger is not adjusted or is adjusted as low as pH 7.1. Because of problems attendant to the adjustment of pH in stirred suspensions [3], measurement can also be made in the supernatant solution above the settled resin and adjustment made until the desired pH under these conditions is reached.

PROCEDURE

Chromatography

After a bottom fitting has been attached to a plastic straw and a small plug of cotton inserted, a 17.5-cm column is poured. The sample is approximately 10 mg of hemoglobin (5 mg if cord blood is used). This may be undialyzed hemolysate in 0.2–0.3 ml of water or a mixture of three drops of blood and 12 drops of water which is kept at room temperature for 5—10 min to allow for hemolysis. After the sample has been applied, the tube above the column is filled with developer No. 2, a top plastic fitting is inserted, and the assembly is attached by tubing to a funnel. A 60-ml portion of developer No. 2 is placed in the funnel and allowed to run through. If the initial liquid head above the bottom of the column is 20, 40, or 60 cm, the flow-rate is approximately 3, 6 or 9 ml/h. Because of the limiting amount of developer, the chromatogram may be started at any convenient time and will complete itself without attention when it runs dry.

*Elution of the zones**

The final appearance of the chromatogram will depend somewhat on the goal of the analysis. Fig. 1B depicts the typical final appearance of a chromatogram in which, for example, Hb-F is elevated in the sample. Hb-A_2 will have passed through as will part of the Hb-A. These two hemoglobins may be collected separately or together. The hemoglobins that remain on the column are eluted in the following way.

After the straw has been marked at 1 and 2 which are the midpoints of the interzones (Fig. 1B), the top fitting is removed and the straw is inserted into a 5-cm length of slightly oversize glass tubing as far as point 1. The end of the glass tubing that is positioned at point 1 should be constricted carefully so that the straw is relatively tightly held and will not flex when cut. The column is cut at point 1 and then at point 2.

After a 10-cm glass extension has been attached with rubber tubing to the top of the section B, the extension is filled with 2% KCN and the remaining Hb-A is eluted and combined with the portion that has passed through the column.

The middle section M is attached at one end with rubber tubing to a 2—3-cm piece of constricted glass tubing which has a cotton plug and at the other end to an extension which is filled then with 2% KCN for elution.

Finally, a small plug of cotton and a bottom fitting are inserted into the top section T at T'. By tapping at T', the column will slide against the cotton plug. Then 2% KCN is added for elution.

Absorbance of all fractions is read at 415 nm and percentages are calculated as usual.

Other methods

The alkali denaturation procedure was a modification [6] of that of Betke et al. [11]; results are listed as % F_{AD}. Microchromatographic determinations of Hb-F in the absence of Hb-A used a recently described method [6]; results are given as % F_{micro}. Chromatography on conventionally sized columns of DEAE cellulose followed the method of Abraham et al. [9, 10]; results are denoted by % F_{DE}.

RESULTS AND DISCUSSION

The initial goal of this study was the devising of a quantitative microchromatographic method for Hb-F in the presence of Hb-A to complement the method for Hb-F in the presence of Hb-S and/or Hb-C [6]. As the study progressed, however, it became apparent that the complete quantitative com-

*This description is more time-consuming and difficult than the actual procedure. After the cutting has been done, an alternative procedure may be used for elution. Each section is blown separately into a centrifuge tube, and the inside of the straw is rinsed with 2% KCN. After more KCN has been added, the suspension is shaken for a few minutes. After centrifuging, the supernatant solution is removed and the procedure is repeated. The combined supernatant solutions are used for spectrophotometry. For satisfactory quantitative work, the volume of each washing with KCN solution must be at least 10 times the volume of the centrifuged ion exchanger.

position of the hemoglobin in a sample could be obtained. We discuss first the general application and then the specific use of the method for the quantitative determination of Hb-F (expressed as F_{St}).

The general nature of the chromatogram

In a mixture that might contain Hb-A_2, Hb-C_0, Hb-C_1, Hb-S_0, Hb-S_1, Hb-A_0, Hb-A_1, Hb-F_0, and Hb-F_1 in various combinations, Hb-A_2 or a mixture of Hb-A_2 and Hb-C_0 will form the first zone to move down the column. Hb-A_2 and Hb-C_0 are the hemoglobins whose rate of movement is influenced by the pH to which the ion exchanger has been adjusted. If the DE-52 is equilibrated with developer No. 1 without pH adjustment, the pH of the supernatant solution is about 7.6. If the chromatogram is developed on this ion exchanger with developer No. 2, the Hb-A_2 (or Hb-C) forms a narrow zone and moves slowly. On the other hand, if the pH is adjusted to 7.1 in the supernatant solution, Hb-A_2 (or Hb-C) moves rapidly as a somewhat diffuse zone. Thus, quantitative estimation of Hb-A_2 is most conveniently done by using ion exchanger at the lower pH. At a flow-rate of 6 ml/h, collection of the Hb-A_2 can begin after about an hour and is complete at the end of two hours.

Hb-S_0 follows Hb-A_2 (or Hb-A_2 and Hb-C_0) through the column. If the sample contains no Hb-A, then Hb-S_1 is apparent in the position of Hb-A_0 above and well separated from Hb-S_0. In the presence of Hb-S_0 and Hb-A_0, Hb-A_0 and Hb-S_1 coincide, and analogously Hb-S_0 and Hb-C_1. Above Hb-A_0 (and/or Hb-S_1) is Hb-F_0 and finally near the top of the column will be Hb-A_1 + F_1. The identity of the zones has been confirmed by comparison with other chromatographic procedures and electrophoresis.

The goal of the analysis will determine the course of the procedure. Thus, if all components of an A-S sample are to be determined, the chromatogram would be started with ion exchanger at pH 7.1 two hours before the operator would leave so that Hb-A_2 may be collected separately. Collection in the second flask is then begun and the chromatogram is allowed to go to completion (and go dry) without further attention. If 55 ml rather than 60 ml of developer No. 2 is used, the Hb-S_0 will be almost completely in the second flask but some will be at the bottom of the column so that the midpoint between Hb-S and Hb-A_0 + Hb-S_1 is apparent. Finally, the midpoints between Hb-S_0 and Hb-A_0 + Hb-S_1, between the latter and Hb-F_0, and between Hb-F_0 and Hb-A_1 + Hb-F_1 are marked, the column is sectioned, the materials are eluted, and the quantities are determined.

If, on the other hand, the percentage of Hb-F_0 is the main interest, all components that precede it can be collected in one flask and, after sectioning and eluting, Hb-A_1 + Hb-F_1 can be combined with them while Hb-F_0 alone is eluted into a second flask. Actually, the determination of the complete composition requires so little additional effort that this procedure has rarely been used.

Complete determination of hemoglobin composition

Complete quantitative analysis of the components in the hemoglobin from a variety of hematological conditions was done both by conventional chromatography [9, 10] and by the straw method. Both procedures use DE-52

TABLE I

COMPARISON OF QUANTITATIVE DATA BY CONVENTIONAL CHROMATOGRAPHY [9, 10] AND BY THE STRAW METHOD

The first of each pair of numbers is the result from conventional chromatography [9, 10] and in brackets from the straw method.

Condition	Hemoglobin composition (%)
Normal adult	A_2 = 2.4(2.6); A = 92.5(89.0); F = 1.2(2.2); A_1 + F_1 = 3.9(6.2)
Normal adult	A_2 = 2.3(2.5); A = 88.7(84.8); F = 2.6(3.1); A_1 + F_1 = 6.3(9.6)
Normal adult	A_2 = 2.5(2.4); A = 90.5(86.1); F = 1.2(2.2); A_1 + F_1 = 5.8(9.4)
Normal adult	A_2 = 2.5(2.7); A = 91.7(89.8); F = 1.3(1.5); A_1 + F_1 = 4.5(6.0)
βthal trait (adult)	A_2 = 5.2(5.3); A = 87.4(84.8); F = 2.4(2.9); A_1 + F_1 = 5.1(7.1)
S trait (adult)	A_2 = 4.2(3.7); S = 35.9(35.0); A = 56.0(55.4); F = 1.2(1.6); A_1 + F_1 = 2.7(3.8)
D trait (adult)	A_2 = 2.2(2.2); D = 37.4(37.4); A = 47.5(45.1); F = 7.6(8.0); A_1 + F_1 = 5.5(7.3)
E trait (adult)	A_2 + E = 32.3(28.9); A = 61.5(62.9); F = 0.7(1.8); A_1 + F_1 = 5.3(6.4)
New York trait (adult)	A_2 = 2.8(3.4); A = 53.7(51.9); NY = 39.5(39.2); ? = 4.0(5.5)
SS disease (adult)	A_2 = 2.6(2.3); S = 67.3(73.0); F = 23.4(23.6); F_1 = 6.8(10.6)
S-HPFH (adult)	A_2 = 1.8(1.6); S = 64.0(65.2); F = 28.2(26.9); F_1 = 5.9(6.4)
SC disease (adult)	A_2 + C = 37.6(41.3); S = 42.5(37.0); F = 16.6(17.3); F_1 = 3.3(7.0)
SC disease (cord blood)	A_2 + C = 8.5(8.5); S = 7.9(7.5); F = 73.2(68.1); F_1 = 10.5(15.9)

as ion exchanger and glycine—KCN—NaCl solution as developer. Table I presents the results. In the data as given, the minor components (except for A_1 and F_1) are summed with the appropriate major component. As previously noted [10] and as will be discussed further below, F_{DE} (conventional chromatography) is not a true measure of Hb-F in the normal individual or if the Hb-F is less than 2—3%. The results of the straw method are in good agreement with those by conventional chromatography under almost identical conditions. The agreement compares favorably with what one might expect from duplicates by either method. The higher value for A_1 + F_1 or F_1 by the straw method is due to the complete removal of material from the top section in contrast to the tendency for trailing of the last zone and incomplete removal from the conventional chromatogram.

Quantitative analysis of Hb-F

In most chromatographic systems, Hb-F is mixed with minor components of Hb-A. The present procedure which employs a single developer provides the same excellent separation of Hb-F that is shown by conventionally sized columns with gradient elution [9, 10]. Above Hb-F_0 in the upper two centimeters of the straw chromatogram are Hb-A_1 and Hb-F_1 as two distinct but not well separated zones. In older samples, these increase. The procedure has now been applied to a wide variety of samples: normal adults, individuals with Hb-A and increased Hb-F, as well as patients with sickle cell anemia, SC disease, etc.

Reproducibility. In a series of 12 simultaneous determinations with the same sample of adult blood to which a small amount of cord blood had been added, the F_{St} averaged 5.1% with a range from 4.8 to 5.5%. In the same series, Hb-A_2 was 2.4% (range 2.3—2.5%), Hb-A_0 was 83.3% (range 82.5—83.8%), and Hb-A_1 + F_1 was 9.2% (range 8.9—9.4%). In a large series of duplicate determinations, the two values with few exceptions agreed within 10% of each other despite wide variation in the percentage of Hb-F.

Quantitative analysis in known mixtures. The straw method has been applied to varied mixtures of hemoglobin from a normal adult hemolysate and the hemolysate from the blood of an HPFH homozygote who has 100% Hb-F. These same mixtures had been analyzed by conventional chromatography and alkali denaturation, and provide the basis for Fig. 3 of ref. 10. The data are presented in Table II. In this normal adult, F_{St} was apparently not observed to be 1—2.5% (see below) and $A_1 + F_1$ was higher than the commonly determined 6—9% (see below). Although the % F_{St} will be higher than actual when the % F is low, the straw method can be considered to provide an accurate measure of the % F above about 10% F. This conclusion also obtains from the comparison of results by both the straw method and conventional chromatography (Fig. 2A); the latter is considered to be the most accurate procedure for the determination of Hb-F [10].

Hb-F in normal adults. So small a percentage of Hb-F is present in the normal adult that it is not apparent as a zone on the chromatogram. However, if as little as 1—2% of cord blood is added, a definite zone of Hb-F can be detected. When the amount of Hb-F in a sample is small, it is advisable to use a simultaneous control in which Hb-F is definitely elevated. In this way, the positions at which the cuts should be made can be more precisely delineated. When the method was applied to 219 adults with normal hematological indices, the results of Fig. 3 were obtained. A slight tailing of the Hb-A_0 is responsible for the values. The % F_{AD} (alkali denaturation) from 36 of 37 randomly selected samples of the series ranged from 0.4 to 1.6%; one sample had 3.0%

TABLE II

COMPARISON OF PERCENTAGES OF F_{St} WITH THEORETICAL PERCENTAGES IN KNOWN MIXTURES

Data provided by Dr. E.C. Abraham, Augusta, Ga, U.S.A.

$F_{Theor.}$	Determined values			Calculated values*		
	$A_0 + A_2$	F_0	$A_1 + F_1$	A_1	F_1	$F_0 + F_1$
2	82.4	4.6	13.1	12.0	1.0	5.6
5	79.3	6.3	14.4	11.6	2.8	9.1
10	75.0	9.5	15.5	11.0	4.5	14.0
15	73.5	11.0	15.5	10.7	4.8	15.8
20	68.5	13.8	17.7	10.0	7.7	21.4
25	62.8	19.6	17.6	9.2	8.4	28.0
30	59.9	22.0	18.1	8.7	9.4	31.4
40	53.4	26.0	20.6	8.0	12.6	38.6
50	48.2	31.0	20.8	7.0	13.8	44.8
60	34.5	40.1	25.4	5.0	20.4	60.5
70	28.6	45.1	26.3	4.2	22.1	67.2
80	19.8	53.3	26.9	2.9	24.0	77.3
AA	87.2		12.8	12.8	0	

*Approximate amounts of Hb-A_1 in the mixtures were calculated on the analysis of the hemolysate of the blood of the normal adult (AA). A constant proportionality 87.2 (% $A_0 + A_2$): 12.8 (% A_1) was assumed for all the mixtures.

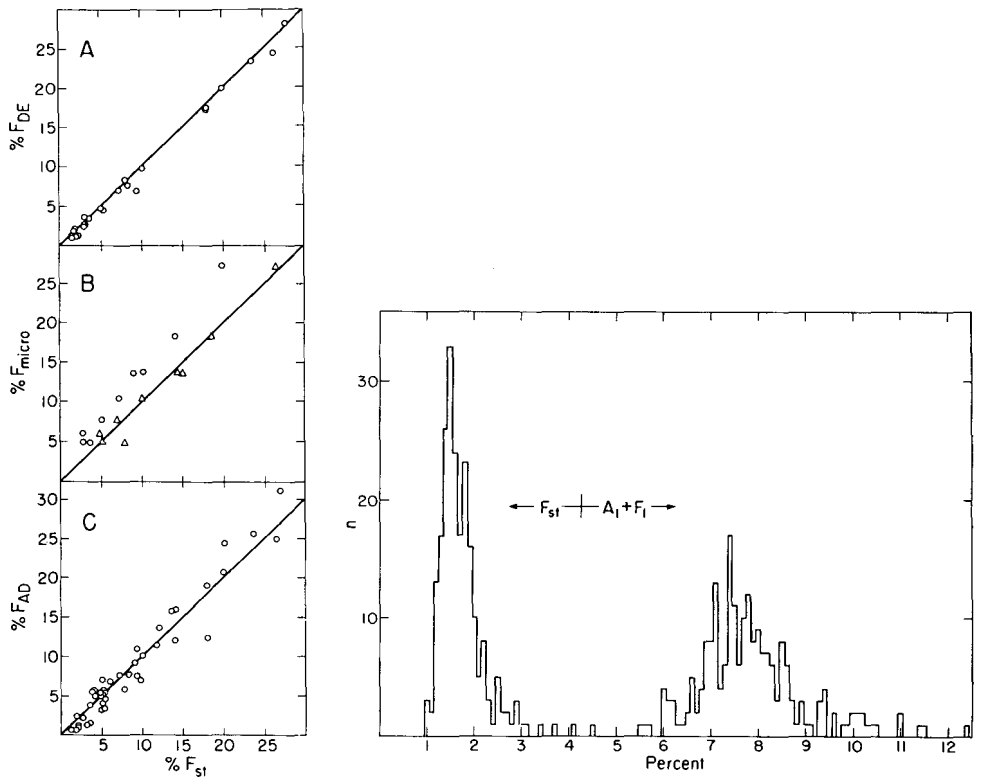

Fig. 2. Comparison of results by the straw method (F_{St}) with those from (A) conventionally sized chromatograms (F_{DE}), (B) from microchromatograms in the absence of Hb-A_0 (F_{micro}) (○= Hb-F_{St}; △ = Hb-F_{St} + Hb-F_1), and (C) from alkali denaturation (F_{AD}).

Fig. 3. Range of values for % F_{St} and % Hb-A_1 + F_1 in samples from normal adults.

F_{St} and 3.2% F_{AD} . Although the method provides an inexact measure of Hb-F in the normal adult, the normal range is about 1—2.5% F_{St}. Thus, when Hb-A_0 is the major hemoglobin, the % F_{St} will be 1—2% higher than the actual Hb-F because of the tailing of Hb-A_0. The Hb-A_1 + F_1 tends to fall between 6 and 9% (Fig. 3).

Comparison with other methods. The straw procedure has been compared with results from conventionally sized chromatograms (F_{DE}), from alkali denaturation (F_{AD}), and from another micro procedure (F_{micro}).

A comparison of F_{St} and F_{DE} data is given in Fig. 2A. There is an excellent correlation in the results. Because the F_{DE} method has excellent accuracy at values above 10% Hb-F [10], it may be concluded that the straw method provides a valid measure of Hb-F.

When Hb-A is absent, a previously described microchromatographic method [6] may be used for Hb-F. When F_{micro} and F_{St} data were compared for SS or SC samples, the results in Fig. 2B were obtained. In Fig. 2B, the data are plotted both as F_{St} and as F_{St} + F_1 against F_{micro}. The sum of F_{St} + F_1 agrees with F_{micro} because F_0 + F_1 do not separate in the latter method.

Fig. 2C depicts a comparison of F_{St} with F_{AD} results. The excellent agreement is somewhat misleading. F_{St} does not include Hb-F_1 yet both are alkali resistant. The alkali denaturation procedure appears to be measuring a proportion of $F_0 + F_1$ that perhaps fortuitously equals the fraction $F_0/(F_0 + F_1)$ (see also below).

Nature of Hb-A_1 + F_1. When Hb-A is absent, the top zone consists of Hb-F_1 only. Although this top zone contains mainly the acetylated version of Hb-F_0, heterogeneity is apparent in the straw chromatograms as also in more conventionally sized chromatograms [10]. When fresh cord blood samples are chromatographed on the straws, the ratio of F_1 + other components to F_{St} is 1:4 to 1:3. This is comparable to previously reported data [12]. Likewise, in 18 SS samples with F_{St} above 10%, the ratio of F_1 to F_{St} averaged 1:3. Because Hb-F_1 appears to be structurally identical with Hb-F_0 except for the acetylation of its γ chains, it must be considered as part of the total Hb-F of the sample. In Hb-A-containing samples, Hb-A_1 and Hb-F_1 are eluted together in the straw method. Because the quantity of Hb-F_1 is 20—25% that of the F_{St} in cord blood samples and SS patients, the total Hb-F in an Hb-A-containing sample may reasonably be calculated to be 1.25 F_{St}.

Some samples have a yellow zone that is strongly fixed at the top of the chromatogram. Although its spectrum is not that of hemoglobin, it does have absorbance at 415 nm. Consequently, if present, it should be removed from the top before cutting and discarded prior to elution of $A_1 + F_1$.

If the sample contains electrophoretically fast-moving hemoglobins such as Hb-J or Hb-N, they will be in the region of $A_1 + F_1$ [7]. Potentially, they could be confused with an old sample with an accumulation of altered products.

Technical considerations

Concentration of NaCl. The concentration of NaCl is critical for the separation of Hb-F_0 from Hb-A_0. If it is 0.013 or 0.017 *M* instead of 0.015 *M*, the separation is unsatisfactory.

Flow-rate. Most experiments have used a flow-rate of about 6 ml/h. At 9 ml/h, the zones are more diffuse and the separations worsen. Little seems to be gained by reducing the flow-rate to 3 ml/h.

Volume of developer. This variable determines the final appearance of the chromatogram. Whether more or less than the recommended 60 ml is used will depend upon the objectives of the analysis. For example, if a complete analysis of an AS sample is desired, it is advantageous to use a few milliliters less so that the interzone between Hb-A and Hb-S is still apparent in the lower column. On the other hand, if the determination of Hb-F is of main importance, a few milliliters more might be added so that, while most of the Hb-A in in the filtrate, the Hb-A—Hb-F interzone is still obvious.

Quantity of sample. For adult samples with normal or moderately reduced packed cell volumes, the hemoglobin in three drops (about 0.07—0.08 ml) or one microhematocrit tube is adequate. If the hematocrit is very low, it is desirable to centrifuge and remove some plasma. Only one or two drops of cord blood should be used because the high percentage of Hb-F will prevent adequate separation from Hb-A if the amount of Hb-F on the column is high.

Storage of samples. If samples are refrigerated, blood may be stored for 3–4 weeks as blood or hemolysate, and the % F_{St} will not change significantly. On the other hand, increasing amounts of material fall in the $A_1 + F_1$ region in older samples. The increase is observed within a few days in hemolysates but not for about 2 weeks with blood.

Time required. This microchromatographic procedure requires more time than other microchromatographic methods. However, little attention is required of the operator. We have routinely made 12 analyses per day. The chromatograms have been started about 2 h before the operator leaves for the night so that Hb-A_2 may be collected. The chromatogram completes itself (goes dry) during the night and cutting, elution, and spectrophotometric determination then are made in the morning. If an operator devoted his time exclusively to this method of analysis, 20–25 analyses per day seem a reasonable goal.

ACKNOWLEDGEMENTS

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CHROMBIO. 118

QUANTITATION OF DEXAMETHASONE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific method for the quantitation of dexamethasone in plasma and urine is described. The specificity of the method is obtained using adsorption chromatography on a high-performance liquid chromatograph. The dexamethasone is detected with a variable-wavelength UV detector. An internal standard technique is used for quantitation of dexamethasone with a minimum sensitivity of 15 ng. Preliminary results of the application of the method to pharmacokinetic studies of dexamethasone in humans are reported.

INTRODUCTION

Several recent studies [1—3] have shown that glucocorticoids administered prenatally can significantly reduce the incidence of respiratory distress syndrome in premature infants. Dexamethasone [3] and betamethasone [1, 2] have been used for this indication. The disposition of dexamethasone in the maternal—placental—foetal system is under investigation in this laboratory.

Limitations in selectivity and sensitivity of thin-layer [4] and paper chromatographic [5] techniques prevent their application in the analysis of dexamethasone in plasma at clinically relevant concentrations. Analysis of dexamethasone using gas chromatography (GC) is complicated because steroids with a C-17 hydroxyacetone side chain undergo thermal degradation at the

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temperatures employed [6]. The assay of dexamethasone by GC would also seem to require the prior formation of a stable derivative.

Previous studies of the disposition kinetics of dexamethasone in humans have been effected by administering radioactively labelled drug and monitoring levels of radioactivity in various biological fluids [7—9]. The success of radiotracer methods in providing information on the disposition of the unchanged drug relies on the use of an additional technique which provides adequate separation of the unchanged drug from its metabolites. More recently, three radioimmunoassay procedures [10—12] have been reported for the determination of dexamethasone in plasma and urine and these have been used to monitor its plasma concentration [13, 14] and urinary excretion [13] after oral administration to humans. Since earlier studies had suggested that the major part of the dose of dexamethasone was metabolized in humans, the development of a suitable radioimmunoassay procedure for disposition studies in humans necessitates the preparation of a specific antibody having no significant cross-reactivity with coexisting metabolites. Since the metabolism of dexamethasone in man has not been elucidated, the possibility of these antibodies exhibiting significant cross-reactivity with unidentified metabolites cannot be discounted.

A radio-receptor assay has been reported for the determination of plasma glucocorticoid activity in man [15] and has been applied in a study of the glucocorticoid levels of maternal and cord serum after prenatal betamethasone therapy [16]. This technique provides an estimate of the amount of betamethasone in terms of cortisol equivalents and hence this analytical approach appears unsuitable for most pharmacokinetic studies.

The recent emergence of commercial high-performance liquid chromatographic (HPLC) equipment coupled with sensitive ultraviolet (UV) detectors has made available a new analytical approach for the assay of steroids such as dexamethasone. HPLC offers the advantages that (a) no derivatization steps are required prior to quantitation and (b) the dexamethasone is detected directly. This paper reports a method using HPLC and UV detection for the assay of dexamethasone in plasma and urine.

EXPERIMENTAL

Reagents and materials

Dexamethasone and prednisolone were gifts from Merck, Sharp & Dohme (Sydney, Australia) and Upjohn (Sydney, Australia). Dichloromethane (Merck, Darmstadt, G.F.R.) and 1-butanol (Ajax, Sydney, Australia) were analytical grade solvents. The heptane was spectroscopic grade (Ajax). All other reagents were analytical grade and were used without further purification as were the solvents.

All the glassware was cleaned with a chromic acid mixture and washed with distilled water. The glass evaporation tubes were then silylated with Siliclad (Clay Adams, Parsippany, N.J., U.S.A.), washed with distilled water and dried.

Drug extraction from biological samples

Plasma or urine (3 ml) and a methanolic solution of internal standard (1 μg prednisolone per 50 μl) were washed with *n*-heptane (7 ml) in a 15-ml glass centrifuge tube with a PTFE-lined screw cap, by shaking on a reciprocal shaker for 15 min. After centrifugation for 10 min at 1500 *g*, the organic phase was discarded. Sodium chloride (300 mg) was added to the aqueous phase which was then extracted with dichloromethane (10 ml). To minimise emulsion formation, this extraction was done with gentle shaking on a reciprocal shaker for 30 min. After centrifugation at 1500 *g* for 30 min, the aqueous phase was discarded. The organic phase was then transferred to another centrifuge tube leaving the residual emulsion on the walls of the first tube. The extract was then washed successively with aqueous solutions of sodium carbonate (3 ml, 0.05 *M*), hydrochloric acid (3 ml, 0.1 *M*) and water (3 ml) by vortexing for 1 min and centrifuging at 1500 *g* for 10 min. The dichloromethane extract was then transferred to an evaporation tube, a 15-ml glass tube with a 100-μl capillary at the base. The extract was concentrated in a water bath at 48°. When no liquid dichloromethane remained, the tube was stoppered and immersed in an ice—water bath. This allowed the dichloro-

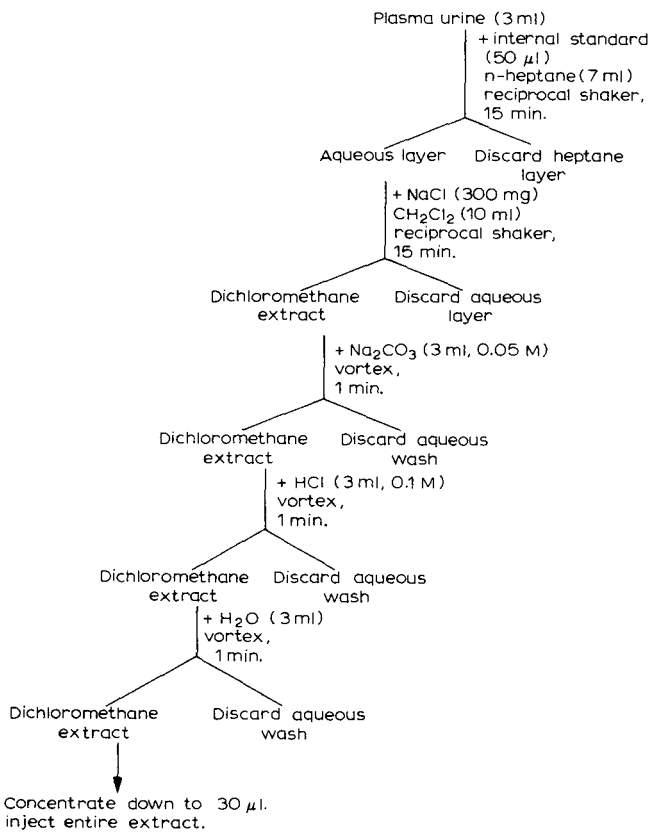

Fig. 1. Schematic diagram for the extraction of dexamethasone.

methane vapour to condense and wash down the internal walls of the evaporation tube. The evaporation and condensation procedures were repeated until approximately 30 μl of the dichloromethane extract remained. The entire extract was injected into the liquid chromatograph. The wall of the evaporation tube was again washed with 10 μl of the fresh dichloromethane. This was also injected into the liquid chromatograph. The use of a stop-flow injection system allows the dichloromethane rinse to be placed on top of the column with the original sample for each chromatographic run. Fig. 1 gives a schematic outline of the extraction procedure.

Chromatography

A Varian Aerograph Model 8500 high-performance liquid chromatograph equipped with a Varian Aerograph stop-flow, septum-less, high-pressure injection port and a Spectra-Physics Model 770 variable-wavelength UV detector operated at 240 nm was used. The column was 250 × 2 mm I.D. stainless-steel tubing packed with silica gel having an average particle diameter of 5 μm (Varian Aerograph Micropak Si-5). Analyses were performed using a mobile phase of dichloromethane containing 5% 1-butanol and 0.3% water at a flow-rate of 65 ml/h and an inlet pressure of 2000 p.s.i. Minor adjustments in flow-rate were made to maintain the retention times constant at 6.6 and 13.8 min for dexamethasone and prednisolone, respectively. The system was operated at an ambient temperature between 23° and 25°.

Calibration and reproducibility

Known quantities of dexamethasone (15—350 ng) were added to blank plasma and urine samples. The samples were then assayed for dexamethasone. Calibration curves were constructed by plotting the peak height ratios between dexamethasone and the internal standard versus the amount of dexamethasone added. To check the reproducibility of the analytical procedure, three different plasma calibration curves were constructed on three different days using human plasma samples from four different sources: a healthy male, a healthy female and two healthy pregnant women near term. A calibration curve using human urine was similarly prepared.

Drug disposition study

Dexamethasone phosphate (8 mg, Decadron®; Merck, Sharp & Dohme) corresponding to 6.66 mg of dexamethasone was administered intravenously over 5 sec to a healthy female volunteer (age 25 years, weight 60 kg). Blood (10 ml) was withdrawn via a cannula from the antecubital vein and placed in tubes containing 100 units of ammonium heparin and separation granules at 12, 19, 34, 65, 123, 153, 190, 247, 298, 357, 417 and 468 min. The plasma was separated immediately and stored frozen until analysis. All urine passed in the 24 h after dosing was collected as a bulk sample and stored frozen prior to analysis.

RESULTS AND DISCUSSION

The major problem encountered in the development of this method was

devising a combination of an extraction procedure and a chromatographic system that consistently resolved dexamethasone from endogenous compounds which absorbed significantly at the $\lambda_{max.}$ of dexamethasone (240 nm). The system reported here has overcome this problem reliably and has been applied in the analysis of over 300 biological samples.

The extraction of dexamethasone from biological fluids using dichloromethane, chloroform and ethyl acetate was investigated, but dichloromethane was found to be the most suitable solvent since (a) it extracted the least number of endogenous compounds, (b) the recovery of dexamethasone was approximately 75% and (c) this solvent was easily evaporated on a water bath. It was necessary, however, to wash the dichloromethane extract with solutions of sodium carbonate and hydrochloric acid to remove the endogenous acids and bases which were unresolved from either dexamethasone or prednisolone on all the chromatographic systems that were examined. The instability of the C-17 side chain in the presence of base [17] necessitated the use of a mild alkaline solution (Na_2CO_3) in this washing step.

The activity of the silica column is dependent on the water content of the mobile phase [18]. It was found that the incorporation of 0.3% water into the mobile phase stabilized the activity of the column and improved the peak shape of both dexamethasone and prednisolone.

A significant loss of activity occurred when the recycled mobile phase became polluted and the resolution of dexamethasone from the endogenous compound eluting before it, decreased significantly. The column was then re-

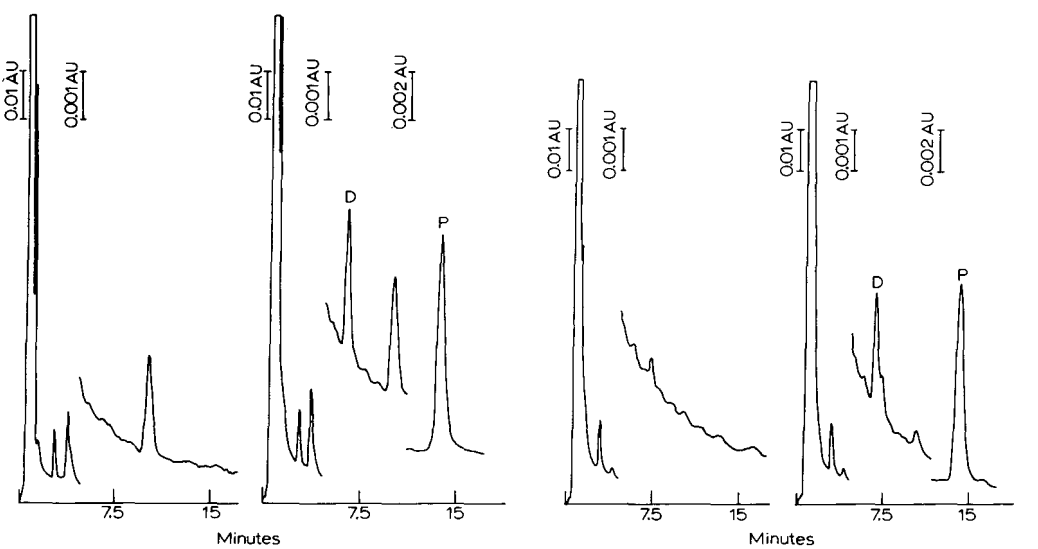

Fig. 2. Chromatograms of blank human plasma and human plasma to which were added 150 ng of dexamethasone (D) and 1 μg of prednisolone (P). Chromatographic conditions are given in the text.

Fig. 3. Chromatograms of blank human urine, and blank human urine to which were added 150 ng of dexamethasone (D) and 1 μg of prednisolone (P). Chromatographic conditions are given in text.

equilibrated with fresh mobile phase and resolution was restored within a few hours.

A similar loss in resolution also occurred when the same grade of dichloromethane from another manufacturer was used to prepare the mobile phase. The identity of the impurity in the solvents that caused this difference in resolution is unknown.

The selectivity of the extraction procedure and chromatographic system is demonstrated by the chromatograms from human plasma and urine extracts in Figs. 2 and 3, respectively. The analysis of blank plasma and urine indicated that there was no significant interference from endogenous compounds. The possibility that metabolites of different polarity [7] from dexamethasone, if extracted, would co-chromatograph with the unchanged drug in this chromatography system is remote. Attempts to confirm the homogenity of the quantitated dexamethasone by mass spectral analysis was frustrated by the thermal instability of the C-17 hydroxyacetone side chain.

Reproducible quantitation of dexamethasone was achieved by using prednisolone as the internal standard. This compound's physical and chemical properties were similar to those of dexamethasone and resulted in a similar recovery for prednisolone (72%) and dexamethasone (75%), and hence contributed to the acceptable linearity of the calibration data (Fig. 4). The calibration plots derived during the application of this method were superimposable within the methodological variation whether the biological fluid being analyzed was plasma or urine. The method enabled the reliable quantitation of 15 ng of dexamethasone and thus provided a lower sensitivity limit of 5 ng/ml for a 3-ml plasma sample. This sensitivity has permitted studies of the pharmacokinetics of dexamethasone in healthy volunteers and patients receiving parenteral doses of 8 mg of dexamethasone phosphate. Fig. 5 presents a typical plasma concentration—time profile of dexamethasone after intravenous administration to a healthy female adult. The terminal phase half-life was 154 min and 2.4% of the dose was excreted unchanged in urine in 24 h. These plasma concentration—time data for dexamethasone were amenable

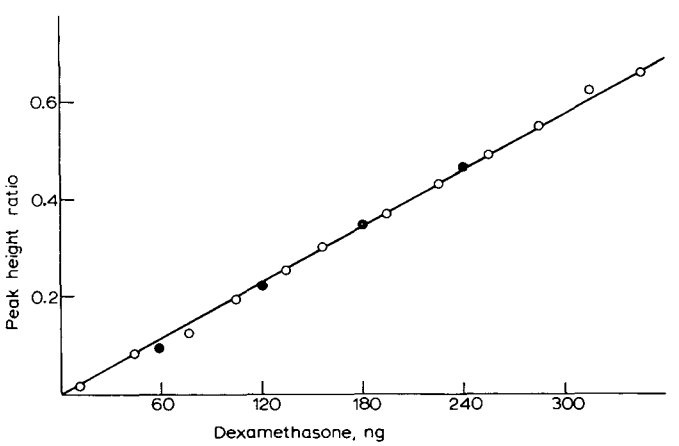

Fig. 4. Calibration curve for dexamethasone in (0) plasma and (•) urine.

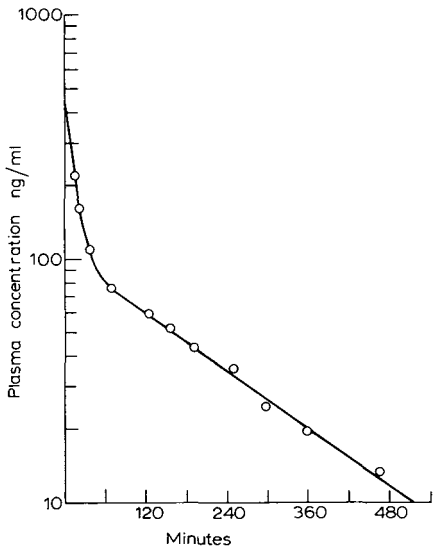

Fig. 5. Plasma concentration—time profile of an 8-mg intravenous dose of dexamethasone phosphate (equivalent to 6.66 mg of dexamethasone) to a healthy female volunteer. ○ Experimental data; —, computerized non-linear regression fitting to a biexponential equation.

to pharmacokinetic analysis and this and urinary excretion studies will be reported elsewhere.

The results obtained with this HPLC technique suggest that, in humans, there may be unidentified metabolite(s) which are cross-reactive with the antibodies used in the radioimmunoassay of dexamethasone. The most pronounced discrepancy is in the estimates of the fraction of the dose excreted in urine. Duggan et al. [14] report the urinary excretion of 10.6% of the intravenous dose unchanged after 48 h while English et al. [13] found 31% of an oral dose unchanged after 24 h. It also appears that Duggan et al. [14] may be correct in their suspicion that the shoulder appearing in their plasma concentration—time curve after intravenous dosage may be caused by the appearance of metabolite(s) to which the antibody is cross-reactive.

This HPLC method is simple, direct, sensitive and accurate for the quantitation of dexamethasone concentrations in biological fluids and has been found to be adequate for clinical applications.

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CHROMBIO. 104

SENSITIVE FLUORIMETRIC METHOD FOR THE DETERMINATION OF PUTRESCINE, SPERMIDINE AND SPERMINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO HUMAN BLOOD

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SUMMARY

A fast and sensitive method for the determination of putrescine, spermidine and spermine by high-performance liquid chromatography is described. These compounds are converted to their fluorescent dansyl derivatives and are separated by a reversed-phase chromatographic system (Micropak CH-10) with water and acetonitrile as mobile phase. The sensitivity of the method is 30 pmoles.

The application of the method to the determination of polyamines in blood is described. It was found that most of the polyamines circulating in blood are localized in the erythrocytes, their content in normal human blood being spermidine 14.1 ± 3.1, and spermine 8.4 ± 2.8 nmoles/ml packed erythrocytes. Putrescine is not present in normal human erythrocytes. The polyamine level in serum is less than 0.1 nmole/ml.

The polyamine content of the erythrocytes from patients with malignant neoplasm was significantly elevated.

INTRODUCTION

Interest in the determination of the polyamines spermidine and spermine and their precursor putrescine in physiological material has been stimulated by the reports of Russell et al. [1,2], who described elevated levels of these compounds in the urine of patients with metastatic cancer. Following this finding, a number of papers for the estimation of polyamines in various biological samples such as urine [3—7], cerebrospinal fluid [8] and bone marrow cells [9] from cancer patients have appeared.

Since it was to be expected that polyamine levels in physiological fluids could be used as a clinical test in the diagnosis and follow-up of patients with

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cancer, it was desirable to develop a fast, simple and efficient method of analysing polyamines. A number of improved techniques have recently been developed, which are based on automated ion-exchange chromatography [10—14], gas chromatography [15—17], combined gas chromatography—mass spectrometry [18], thin-layer chromatography [19—21], or high-performance liquid chromatography (HPLC) [22—25] of polyamines and their derivatives.

In the course of our studies on haematopoietic malignancies we were interested in analysing polyamine levels in the blood of patients. Among the methods for polyamine analysis, the fluorescence method is the most sensitive one. However, hitherto reported methods are somewhat complicated and time-consuming. At the beginning of our studies we had to develop a less complicated, highly sensitive and reproducible method for polyamine analysis, especially suitable for analysing polyamines in blood.

This present work describes a quick and highly sensitive method for the determination of polyamines in blood as their dansyl derivatives by HPLC. The use of the method for the determination of polyamines in blood from normal subjects and patients with malignant neoplasm will be demonstrated.

EXPERIMENTAL

Materials

Dansyl chloride was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and recrystallized from *n*-hexane. Putrescine, spermidine and spermine as their hydrochlorides were purchased from Sigma (St. Louis, Mo., U.S.A.). Precoated silica-gel 60 plates for TLC were products of Merck (Darmstadt, G.F.R.) Acetonitrile, acetone, benzene, dioxane, chloroform, morpholine, triethylamine, perchloric acid, and L-proline were of special grade reagent from Wako Junyaku (Osaka, Japan). The ^{14}C-labelled polyamines, putrescine dihydrochloride (60 mCi/mmole), spermidine trihydrochlorine (122 mCi/mmole) and spermine tetrahydrochloride (115 mCi/mmole), were obtained from New England Nuclear (Boston, Mass., U.S.A.).

High-performance liquid chromatography

The high-performance liquid chromatograph was constructed in our laboratory. A high-pressure piston pump (Hitachi, Model 634) supplied the mobile phase at a rate of 1 ml/min to the column. The mobile phase for elution was a linear gradient between 20% acetonitrile in water (10 ml) and acetonitrile (10 ml), which was performed by using a pair of cylindrical glass vessels of the same size (1 × 20 cm). The column (25 cm × 2.2 mm I.D.) was Micropak CH-10 octadecylsilanized silica particles of 10 μm average diameter (Varian, Palo Alto Calif., U.S.A.). The eluted fluorophores were detected with a Union Giken ultraviolet—fluorospectrophotomotor Model SM 303A, equipped with a Xenon lamp (Jobin Yvon), using wavelengths at 342 and 512 nm for activation and emission, respectively.

Procedure for determination of polyamines in human blood

Human blood, which was collected from the cubital vein with a heparinized syringe, was centrifuged at 550 *g* for 5 min. After the plasma and buffy coat

had been carefully removed by suction, the erythrocytes were suspended in 4 volumes of 0.9% NaCl, and the suspension was centrifuged at 2000 rpm for 5 min. After the sediment had been washed five times with 0.9% NaCl, packed erythrocytes were obtained. In this procedure, the packed erythrocytes had a haematocrit value of 90 ± 2%, and were contaminated with less than 5% of leucocytes.

To 0.25 ml of packed erythrocytes was added 0.75 ml of water and the cells hemolysed by mixing on a vortex mixer for 20 sec. To the resulting hemolysate was added 1 ml of 10% perchloric acid and extracted by mixing on the vortex mixer for 1 min; the precipitate was removed by centrifugation at 1200 *g* for 10 min. The, 0.5 ml of 5 *N* sodium carbonate and 2 ml of dansyl chloride (10 mg dansyl chloride/ml acetone) were added to the supernatant solution. Dansylation was allowed to proceed in the dark at room temperature for 16 h. Excess reagent was consumed by reaction with 0.1 ml of 1 *M* L-proline for 30 min in the dark at room temperature. Acetone was removed in vacuo on a rotary evaporator at 50°. The dansylated derivatives were extracted twice using 1.5 ml of benzene each time. The benzene was evaporated in vacuo at 50° on a rotary evaporator, and the residue was redissolved in 50 μl of benzene; 5 μl of the solution were subjected to HPLC.

Determination of recovery

The ^{14}C-labelled putrescine, spermidine and spermine were added to the haemolysate and processed as described in the determination procedure. Separated dansyl putrescine, dansyl spermidine and dansyl spermine were collected in counting vials from the outlet of the flow-cell of the high-pressure liquid chromatograph, monitoring with the fluorescence monitor, and the radioactivity was measured with a Beckman liquid scintillation spectrometer, Model DPM 100, using Bray's scintillator.

Preparation of dansyl polyamines

A 100-μl portion of 5 *N* sodium carbonate and 0.5 ml of dansyl chloride (10 mg dansyl chloride/ml acetone) were added to 0.4 ml of 0.25 m*M* spermine, and the reaction mixture was left to stand for 2 h at room temperature in the dark. After the excess dansyl chloride had been converted to dansyl proline by the addition of 50 μl of 1 *M* L-proline, acetone was evaporated in vacuo at 50°. Dansyl spermine was extracted twice with 1 ml of benzene each time. The benzene extract was evaporated to dryness in vacuo at 50° to give dansyl spermine.

Dansyl spermidine and dansyl putrescine were prepared in the same way as described above.

RESULTS

Resolution and quantitation

The best separation was accomplished by using a reversed-phase column, Micropak CH-10, with linear gradient elution from 20% acetonitrile in water to acetonitrile as a mobile phase. As shown in Fig. 1, the mixture of dansyl deriv-

TABLE I

CONCENTRATION OF SPERMIDINE AND SPERMINE IN BLOOD

The procedure for whole blood was the same as for erythrocytes as described in Experimental but using 0.25 ml of whole blood instead of packed erythrocytes. Leucocytes were isolated by the method using 6% dextran. Isolated leucosytes (10^6 cells) in water (0.4 ml) were subjected three times to freezing and thawing, and the resulting supernatant was dansylated. Spd, = spermidine; Spm,= spermine; n.d., not determined.

Subject	Polyamine	Polyamine concentration in:			Hematocrit (%) (C)	BxC 100 (D)	Dx100/A (%)
		Whole blood (nmoles/ml) (A)	Erythrocytes (nmoles/ml) (B)	Leucocytes (nmoles/10^9 cells)			
Normal	Spd	7.30	14.0	135	47	6.58	90
	Spm	4.50	8.62	338		4.05	90
Normal	Spd	6.84	12.43	77	44	5.47	80
	Spm	4.85	8.27	305		3.64	75
Gastric cancer	Spd	14.5	33.6	n.d.	38	12.8	88
	Spm	14.6	33.8			12.8	88
Gastric cancer	Spd	8.70	24.2	n.d.	31	7.50	86
	Spm	15.3	36.8			11.4	75

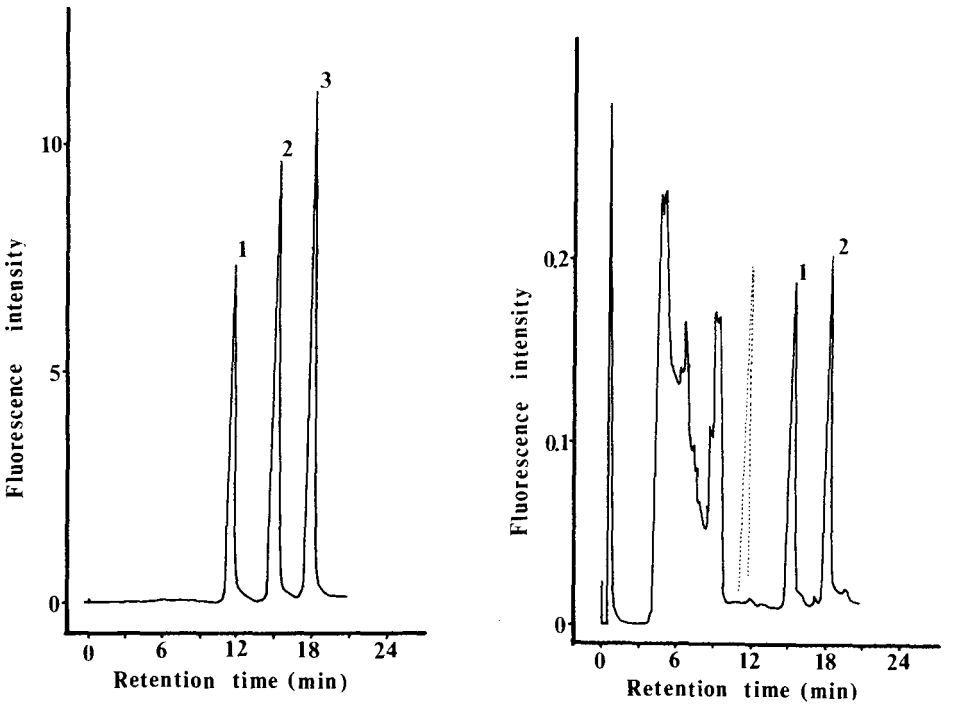

Fig. 1. Chromatogram of dansyl derivatives of putrescine, spermidine, and spermine. Standard samples were prepared as described under Experimental. Dansylated polyamines in benzene were injected into the high-performance liquid chromatograph. Conditions: column, Micropak CH-10 (25 cm × 2.2 mm I.D.); mobile phase, linear gradient between 10 ml of 20% acetonitrile in water and 10 ml of acetonitrile; flow-rate, 1 ml/min, 1, Putrescine; 2, spermidine; 3, spermine.

Fig. 2. Chromatogram of dansylated polyamines from human erythrocytes. Running conditions were the same as described in the legend of Fig. 1. All procedures are described in the text. Injected amount (5 μl) is equivalent to 25 μl of packed erythrocytes. 1, Spermidine; 2, spermine. The dotted line indicates the elution position of putrescine.

atives of putrescine, spermidine and spermine were well separated in a total analysis time of 20 min. The limit of detection was 30 pmoles. Peak heights of the fluorescence intensity trace on the recorder were plotted against the amount of each polyamine added, and a satisfactory linearity was obtained.

Determination of polyamines in human blood

Fig. 2 represents a chromatogram of dansylated derivatives obtained from erythrocytes. The injected amount of dansylated derivatives in benzene (5 μl) was equivalent to 25 μl of packed erythrocytes. As can be seen from the figure, determination of the polyamines can be achieved without any purification step such as cation-exchange column chromatography. This is achieved by using ^{14}C-labelled polyamines. As shown in this figure, erythrocytes of normal human blood contain both spermidine and spermine, but the content of putrescine is negligible. In this method, putrescine is eluted in the position indicated in the figure by the dotted line.

To determine the recovery of putrescine, spermidine and spermine throughout the procedure, the ^{14}C-labelled compounds were added to the haemolysate, and the effluent from the flow-cell of the high-pressure liquid chromatograph was collected and the radioactivity counted. Recoveries of putrescine, spermidine and spermine were 95%, 94% and 91%, respectively.

Polyamine concentration in healthy human blood

We examined the concentrations of polyamines in whole blood, erythrocytes, leucocytes and plasma. Several cases are presented in Table I. Putrescine was not detected in whole blood nor in blood fractions. Concentrations of polyamines in plasma were less than 0.1 nmole/ml of plasma. As indicated in the last column of Table I, more than about 80% of the spermidine and spermine in circulating blood was present in the erythrocytes. Most of the remaining amount of polyamines, corresponding to less than about 20% of spermidine and spermine in circulating blood, was localized in the leucocytes. The same situation was also found in the blood from a patient with malignant neoplasm, as shown in Table I.

As shown in Table II, the average concentrations of spermidine and spermine in erythrocytes were, respectively, 14.1 ± 3.1 and 8.4 ± 2.8 (mean ± S.D.) nmoles/ml packed erythrocytes. It is of interest that erythrocytes contain more spermidine than spermine and, on the other hand, that leucocytes contain more spermine than spermidine. The ratio of spermidine to spermine in erythrocytes was found to be 1.7 and that in leucocytes 0.25.

TABLE II

POLYAMINE CONTENT OF ERYTHROCYTES AND LEUCOCYTES

	Spermidine (mean ± S.D.)	Spermine (mean ± S.D.)	Spermidine/Spermine
Erythrocytes (nmoles/ml; n = 27)	14.1±3.1	8.4±2.8	1.7
Leucocytes (nmoles/10^9 cells, n = 7)	95±26	387±61	0.25

Polyamine content of erythrocytes from patients with malignant neoplasm

In order to know whether it is possible to use the polyamine level in erythrocytes as a marker of malignant neoplasm, the concentrations of polyamines in erythrocytes from patients with malignant neoplasm were determined.

The concentrations of polyamines were found to be significantly elevated in the erythrocytes as shown in Table III. As shown in the last column of Table III, the values of the spermidine/spermine ratio were widely distributed, and the relation between these values and the activity of malignant neoplasm is uncertain at the present. From these findings we consider it useful to determine the polyamine level in erythrocytes as a clinical test in the diagnosis and follow-up of patients with malignant neoplasm.

TABLE III

POLYAMINE CONTENT OF ERYTHROCYTES FROM PATIENTS WITH MALIGNANT NEOPLASM

F, female; M, male; IBL, immunoblastic lymphadenopathy.

Case	Age (Sex)	Diagnosis	Spermidine (nmoles/ml packed erythrocytes)	Spermine (nmoles/ml packed erythrocytes)	Spermidine/ Spermine
S.Y.	81 (F)	Gastric cancer	49.2	23.6	2.08
E.S.	51 (F)	Gastric cancer	164.0	101.0	1.62
T.T.	37 (F)	Duodenal cancer	42.4	111.0	0.38
H.N.	78 (M)	Rectal cancer	68.4	27.6	2.08
T.H.	74 (F)	Hepatoma	62.4	19.2	3.25
H.S.	64 (M)	Pancreatic cancer	48.8	16.7	2.92
F.F.	72 (M)	Pancreatic cancer	36.0	10.4	3.46
A.K.	37 (F)	Ovarian cancer	18.9	35.4	0.53
F.O.	72 (F)	Lymphosarcoma	34.8	11.1	3.14
M.T.	40 (M)	IBL	32.8	12.4	2.65

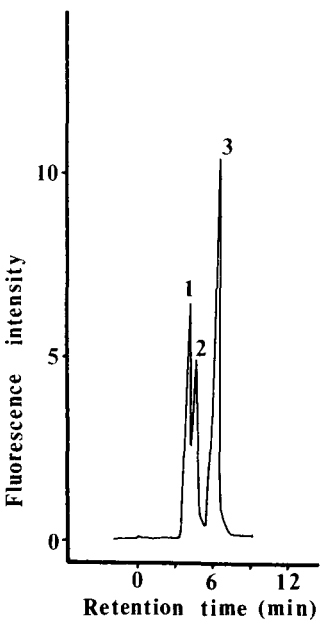

Fig. 3. Chromatogram of dansyl derivatives of putrescine, spermidine, and spermine. Running conditions: column, Micropak Si-10 (30 cm × 2.2 mm I.D.); mobile phase, chloroform—dioxane—triethylamine (100:10:1); flow-rate, 0.5 ml/min. 1, Spermine; 2, spermidine; 3, putrescine.

DISCUSSION

Several systems for the separation of dansylated polyamines were examined before a suitable one was selected. For example, at the beginning of the present work we tried TLC and found that dansyl derivatives of polyamines were well separated on a silica gel G 60 plate using chloroform—dioxane—N-ethyl morpholine (40:4:1) as a solvent system. On the basis on this finding a Micropak Si-10 (silica gel of 10 μm average diameter, Varian associates) column and a mobile phase consisting of chloroform—dioxane—triethylamine (100:10:1) was tested. As shown in Fig. 3 this system gave a fairly good resolution. We decided to abandon this system for the following reasons: (1) a good separation was not constantly obtained and depending on the lot number of the column overlap of the peaks of dansyl spermidine and dansyl spermine was observed: (2) irreversible contamination of the packing material occurred after about twenty runs.

Owing to the hydrophobic nature of the dansyl derivatives, it was apparent that a reversed-phase column would give satisfactory results. Finally, we found that Micropak CH-10 and a mobile phase consisting of a linear gradient between 20% acetonitrile in water and acetonitrile gave a good, reproducible resolution as shown in Fig. 1. The total analysis time is about 20 min. The limit of detection of this method is 30 pmoles. Only 0.25 ml of packed erythrocytes was needed for the analysis and the recoveries of putrescine, spermidine and spermine were 95%, 94% and 91%, respectively.

One of the most interesting findings of the present work is that most of the polyamines in circulating human blood are compartmentalized in the erythrocytes. It may be speculated that the erythrocytes function as polyamine carriers in the circulation.

The concentrations of spermidine and spermine in healthy human blood were, respectively, 14.1 ± 3.1 and 8.4 ± 2.8 (mean ± S.D.). nmoles/ml packed erythrocytes and the ratio of spermidine to spermine was about 1.7. Putrescine was not detected in the erythrocytes. In plasma the concentrations of polyamines was less than 0.1 nmole/ml. Leucocytes were found to contain more spermine than spermidine and the ratio of spermidine to spermine was about 0.25 (Table II). Very recently Cohen et al. [26] reported the same results in their extensive studies on the distribution of polyamines in blood from patients with cystic fibrosis and normal subjects.

Elevated levels of polyamines in urine, cerebrospinal fluid and bone narrow cells from cancer patients have been reported since 1971. In the present experiments we determined the concentrations of polyamines in the erythrocytes from patients with malignant neoplasms, and found that the polyamine levels in the erythrocytes from these patients were significantly elevated (Table III). Extensive work on the determination of polyamines in blood from patients with malignant neoplasm is in progress and will be published elsewhere.

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manuscript and valuable discussions. The skillful technical assistance of Miss Yasue Aomatsu is also appreciated.

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CHROMBIO. 110

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HIPPURIC ACID IN HUMAN URINE

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SUMMARY

A method is described for the determination of urinary hippuric acid by high-performance liquid chromatography. The method used ethyl acetate extraction for partial clean-up of the urine. The separation was carried out on a reversed-phase column using 20% methanol in 0.01 *M* aqueous potassium phosphate containing 0.5% acetic acid as a mobile phase. The column effluent was monitored with a UV detector at 254 nm. Hippuric acid was separated from other normal urine constituents in less than 10 min. Metabolites of xylene and styrene did not interfere with the assay. Analytical recoveries from urine were excellent and peak height and concentration were linearly related.

INTRODUCTION

Hippuric acid is normally present in human urine as a metabolite of dietary components. After exposure to toluene, large quantities of hippuric acid are excreted in the urine and quantities of it are correlated to toluene exposure. Therefore, the determination of hippuric acid in urine provides an exposure test [1—3]. Methods for the determination of hippuric acid in urine depending upon spectrophotometry [1, 4] and fluorometry [5] have been described. These methods, although simple and sensitive, suffer from the lack of specificity. Ogata et al. [6] have described a specific colorimetric method, which requires clean-up of the urine by paper chromatography or thin-layer chromatography before the determination step. A gas chromatographic method has been described by Buchet and Lauwerys [7]. However, this method requires derivatization. The present report describes a high-performance liquid chromatographic method for the accurate determination of hippuric acid in human urine. This method is simple and does not require the formation of volatile derivatives.

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EXPERIMENTAL

Materials

Hippuric acid, *o*-, *m*- and *p*-methylhippuric acids and mandelic acid were obtained from Tokyo Chemical Industry (Tokyo, Japan). Phenylglyoxylic acid and nicotinuric acid were from Aldrich (Milwaukee, Wisc., U.S.A.). Salicyluric acid was from Sigma (St. Louis, Mo., U.S.A.). The other reagents and solvents used were of reagent grade.

Standard solutions

A standard solution of hippuric acid in distilled water was prepared at a concentration of 1 g/100 ml. Hippuric acid was first dissolved in about 10 ml of 0.01 *M* NaOH before dilution with water. This solution was further diluted with water to produce solutions of the desired concentration.

Extraction procedures

Urinary hippuric acid was isolated as described by Ogata et al. [6]. To 1.0 ml of urine in a glass-stoppered tube were added 0.04 ml of concentrated HCl and 0.3 g of NaCl. This mixture was extracted with 4.0 ml of ethyl acetate by shaking vigorously for 2 min. The tube was centrifuged for 5 min at 1000 *g*, then 200 μl of the supernatant organic phase were transferred into a test tube. The ethyl acetate was evaporated to dryness in a water-bath at 70°. The residue was dissolved in 200 μl of water, and 4 μl was injected into the high-performance liquid chromatograph.

Apparatus and chromatographic conditions

A Waters Assoc. Model ALC/GPC 202 liquid chromatograph equipped with a μBondapak C_{18} prepacked column (30 cm × 4 mm I.D., Waters) at ambient temperature and a UV detector set at 254 nm, was employed for chromatographic analysis. The detector was operated at 0.05 absorbance units full scale for most samples.

The eluting solvent was 20% (v/v) methanol in 0.01 *M* potassium phosphate containing 0.5% (v/v) acetic acid. The flow-rate was 1.0 ml/min at a pressure of 2000 p.s.i. Sample injections were made on-column through a Waters U6K septumless injector with a 10-μl syringe (Hamilton 701 N).

RESULTS

Separation of hippuric acid in urine

Fig. 1 illustrates typical chromatograms obtained with samples prepared from (a) a urine control, (b) a urine sample to which synthetic hippuric acid has been added, and (c) a urine sample from a person exposed for two hours to 15—25 ppm of toluene in air. Samples prepared from the control urine gave a peak corresponding to endogenous hippuric acid. To illustrate the specificity of the method, the peak fractions were collected, extracted with ethyl acetate which was evaporated to dryness, and the residue obtained was examined by infrared spectroscopy. Infrared spectra were measured with a Hitachi Perkin-Elmer 225 grating infrared spectrophotometer. Fig. 2 shows the infrared

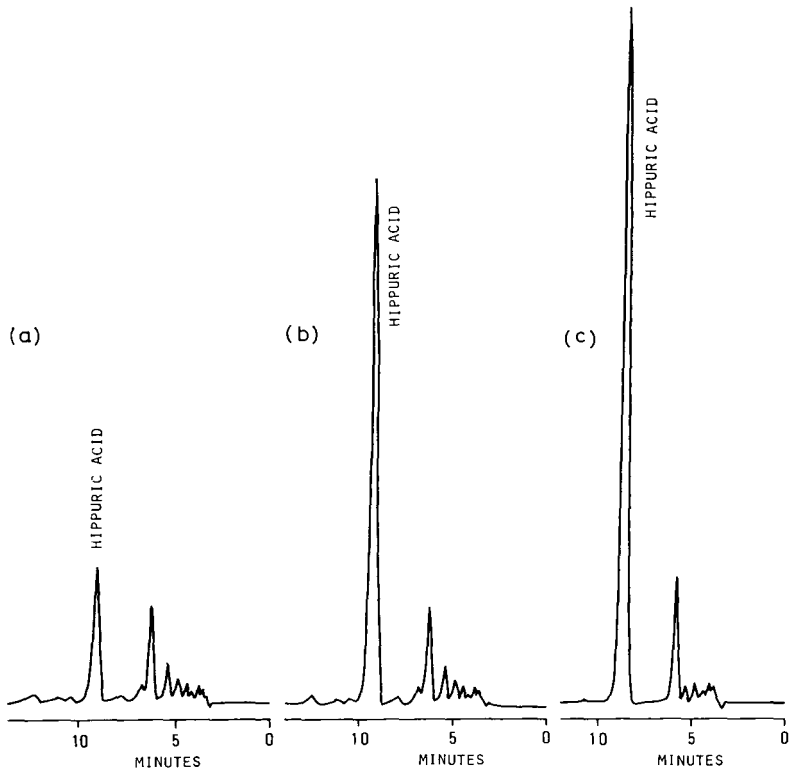

Fig. 1. Liquid chromatograms of extracts from (a) normal urine, (b) normal urine containing added hippuric acid, and (c) urine from a person exposed to toluene.

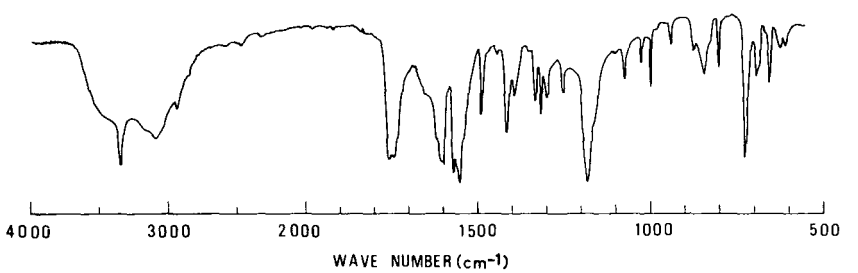

Fig. 2. Infrared spectrum of a peak fraction corresponding to hippuric acid in urine, which was separated by liquid chromatography. Spectrum was measured for KBr disc (200 mg of KBr and 0.8 mg of sample).

spectrum of the fraction. The spectrum was the same as that of synthetic hippuric acid. This result suggests that hippuric acid is separated well from other normal component(s) in urine.

Fig. 1c shows that an increased level of hippuric acid was found after exposure to toluene.

Calibration

Standard solutions were prepared containing various amounts of hippuric acid and analysed by the above extraction procedure which was the same as that for urine samples. The detector response at 254 nm is linearly related to the concentration of hippuric acid over a range of 0 to at least 5.0 mg/ml. By using the calibration curve the concentration of hippuric acid in a urine sample is easily determined after calculation of its peak height.

Recovery

The analytical recovery of hippuric acid from urine was determined by adding known quantities of hippuric acid to urine and analysing. Recoveries (Table I) ranged between 98 and 102%.

TABLE I

ANALYTICAL RECOVERY OF HIPPURIC ACID ADDED TO URINE

Hippuric acid (mg/l)		Mean recovery (%)
Added	Recovered*	
200	196	98
500	504	101
1000	1016	102

*Mean of 10 assays.

Precision

Within-run precision of the method was obtained by processing 10 aliquots of pooled urine. The concentration of hippuric acid was established at 868 ± 9.3 mg/l ± S.D. (coefficient of variation, 1.1%). Day-to-day precision was calculated from values for a single sample assayed on 10 consecutive days. The mean was 877 ± 11.9 mg/l ± S.D. (coefficient of variation, 1.4%).

Interfering substances

Other compounds for possible interference were studied by chromatographing aqueous solutions of them (Fig. 3). Mandelic acid and phenylglyoxylic acid, and methylhippuric acid, which are the known biotransformation products of the organic solvents styrene and xylene, respectively, were well separated from hippuric acid. Salicyluric acid and nicotinuric acid, which interfere with the determination of hippuric acid in Umberger's colorimetric method, did not interfere in this analysis.

Comparison with the paper chromatographic procedure

Thirty-two urine samples from persons not exposed to solvents were assayed by the liquid chromatographic method and the paper chromatographic method of Ogata et al. [6] in which hippuric acid is separated by paper chro-

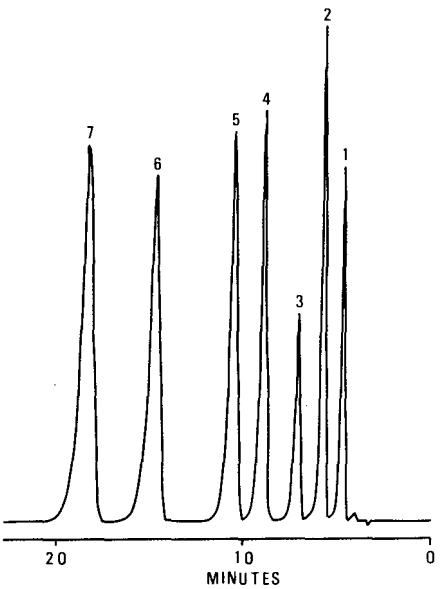

Fig. 3. Separation of a mixture of standards. 1 = Nicotinuric acid; 2 = phenylglyoxylic acid; 3 = mandelic acid; 4 = hippuric acid; 5 = *o*-methylhippuric acid; 6 = salicyluric acid; and 7 = *m*- (*p*-)methylhippuric acid.

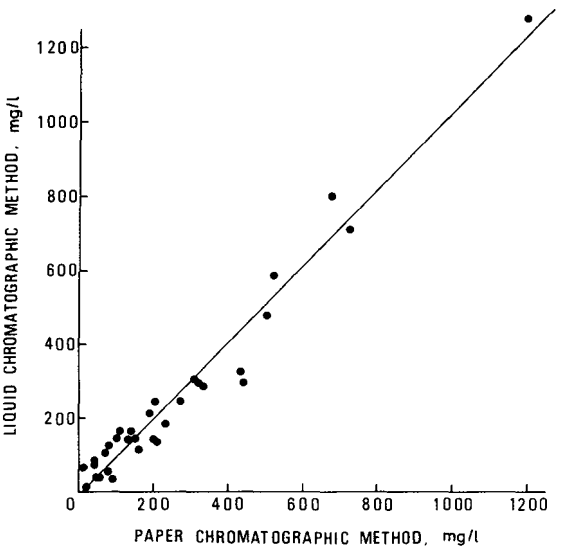

Fig. 4. Relation between hippuric acid values obtained by the paper chromatographic technique of Ogata et al. [6] and the present method.

matography, converted to azlactone and measured colorimetrically. Fig. 4 shows the results obtained with the two methods. The linear regression parameters for these results are: correlation coefficient, 0.959; y intercept, —0.0003; slope, 0.965.

DISCUSSION

High-performance liquid chromatography of hippuric acid in urine is more rapid and simpler than are spectrophotometric and gas chromatographic techniques, and does not necessitate derivatization of the acid as do gas chromatographic methods. Hippuric acid was separated from mandelic acid, phenylglyoxylic acid and methylhippuric acid, metabolites of styrene and xylene. This procedure can therefore be used for the estimation of exposure to toluene in cases of workers exposed to a mixture of solvents. Salicyluric and nicotinuric acids, interfering substances in Umberger's colorimetric method, did not interfere in the determination of hippuric acid. The specificity of this method is confirmed by infrared spectrophotometry of the eluted urinary hippuric acid. Results obtained by high-performance liquid chromatography and paper chromatography on the same urine sample correlated well for hippuric acid.

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SPECIFIC AND SENSITIVE COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC—FLOW FLUOROMETRIC ASSAY FOR INTRACELLULAR 6-THIOGUANINE NUCLEOTIDE METABOLITES OF 6-MERCAPTOPURINE AND 6-THIOGUANINE

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SUMMARY

A new non-radioisotopic technique is described for measuring rates of intracellular formation by human leukemic blasts of 6-thioguanine nucleotide metabolites, obligatory intermediates in the antineoplastic action of both 6-mercaptopurine and 6-thioguanine itself. The method is both specific and sensitive, and involves combined high-performance liquid chromatography and flow fluorometric detection of oxidized 6-thioguanine nucleotides in alkaline permanganate-treated cell extracts. Non-metabolized 6-thioguanine and 6-thioxanthine are also separated and quantitated in this system, permitting complementary in vivo pharmacokinetic analysis. The assay may be applied to detect resistant disease at an early stage in therapy, and thereby provides the opportunity for alternative treatments to be instituted.

INTRODUCTION

The 6-thiopurine antimetabolites 6-thioguanine (TG) and 6-mercaptopurine (MP) have found important application in the clinical chemotherapy of acute leukemia [1, 2, 3]. Both agents share the absolute requirement that they be metabolized to their ribosyl monophosphate, nucleotide, derivatives as the first step in their mechanism of action. This process, termed "lethal synthesis", necessarily occurs within the leukemic cell itself, and clinical resistance to the drugs has been associated with reduced nett rates of intracellular drug nucleotide accumulation [4]. MP nucleotide undergoes further metabolism

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to TG nucleotides, and, in terms of its mechanism of action, MP merely represents another way of administering TG to malignant cells [5, 6]. The antineoplastic activity of both thiopurines results from the ultimate incorporation of a TG deoxynucleotide metabolite into DNA in phosphodiester linkage [6, 7, 8]. Since intracellular TG nucleotide metabolites are central to the action of both drugs, it is apparent that through measuring rates of TG nucleotide formation from MP or TG by human leukemic leukocytes, either in vivo or in vitro, it may be possible to identify drug-resistant disease at the outset of therapy. In this way alternative treatment may be instituted before any significant amount of time has been wasted on ineffective therapy, and unnecessary systemic toxicity may be avoided. In the past, determinations of thiopurine disposition and nucleotide formation have utilized radioisotopically labeled drugs, and this has precluded routine measurement of drug pharmacokinetics and activation in vivo. Consequently, there have been only isolated cases where thiopurine bioavailability and cellular pharmacology have been investigated clinically.

In the present paper we describe a specific and sensitive high-performance liquid chromatographic (HPLC) technique utilizing flow fluorometric detection of oxidized TG derivatives for measuring rates of intracellular non-radioactive TG nucleotide formation from MP or TG by human leukemic cells. Development of this technique follows from the report of Finkel [9] who described a non-radioactive fluorescent assay for quantitating the free base in laboratory preparations of TG. However, the latter method cannot be applied to measurement of free TG in biological materials since the fluorescent oxidized drug is not separated from naturally occurring components which fluoresce upon oxidation. We have extended Finkel's observations by demonstrating that oxidized TG nucleotides also fluoresce strongly and may be separated and quantitated by combined anion-exchange chromatography and flow fluorometry. Unchanged TG and 6-thioxanthine are also separated and quantitated in this system, and therefore the pharmacokinetics of TG may be determined in conjunction with measurement of drug nucleotide accumulation by leukemic blasts. This approach obviates a major criticism of drug sensitivity tests based solely on in vitro methodology, where in vivo bioavailability is ignored.

Our techniques may be applied in selecting individual patients most likely to benefit from an extended course of thiopurine chemotherapy.

EXPERIMENTAL

Sample preparation

TG or MP treated leukemic cells were pelleted by centrifugation and extracted with 4% perchloric acid at 4° using [8-^{14}C] adenine as internal standard; extracts were neutralized with KOH. Serum samples from patients receiving TG chemotherapy were deproteinized by ultrafiltration through Centriflo membrane cones (Amicon, Lexington, Mass., U.S.A.). Neutralized cell extracts and deproteinized serum samples were oxidized with alkaline potassium permanganate [9]; 0.1 ml of sample was mixed with 0.1 ml 0.1 *M* sodium carbonate—sodium bicarbonate buffer pH 10.1, and 0.1 ml 0.24% potassium

permanganate solution, and oxidation was allowed to proceed for 5 min. Excess permanganate was reduced by addition of 10 μl 30% hydrogen peroxide solution, and the brown precipitate of manganese dioxide was removed by centrifugation.

Chromatography

Oxidized TG and its derivatives were separated by anion-exchange chromatography on a Model 7000B chromatograph (Micromeritics, Norcross, Ga., U.S.A.) using a Reeve Angel Partisil-10 SAX strong anion-exchange column, 25 cm × 4.6 mm I.D. × ¼ in. O.D. (Whatman, Clifton, N.J., U.S.A.), and a 15-min concave buffer gradient ranging from 5 m*M* potassium phosphate (pH 3.5) to 250 m*M* potassium phosphate—500 m*M* potassium chloride (pH 4.5), at a constant flow-rate of 5 ml/min and a pressure of approximately 2200 p.s.i. The separated TG derivatives in the column effluent were detected by their fluorescence, using an FS-970 flow spectrophotofluorometer (Schoeffel, Westwood, N.J., U.S.A.) set at an excitation wavelength of 330 nm, installed with an auxillary Corning 7-54 prefilter for excitation, and employing a 389-nm cut-off emission filter. In addition to recording the photomultiplier output on a 10-mV chart recorder, the 5-V full scale output at the rear of the instrument was interfaced to a PDP 11/34 minicomputer (Digital Equipment Corp., Maynard, Mass., U.S.A.) through the computer's LPS11 system, and the chromatographic data were acquired in real-time at 2-sec intervals using the 12-bit A/D converter in the LPS11 module. Sequential digitized detector output values were stored on IBM Diskettes and were subsequently analyzed by a user written Fortran program which performed peak area integration. Peak areas were converted to absolute quantities using predetermined calibration curves. Known amounts of [^{14}C]adenine were added to cell pellets as internal standard during acid extraction, and radioactivity determinations were used to correct for losses of material during extract processing.

The mono-, di-, and triphosphates of adenosine, guanosine, uridine and cytidine were also separated under the chromatographic conditions described above, and for cell extracts these constituents were detected simultaneously in the column effluent by their UV absorption at 254 nm, using a Chromonitor 785 flow spectrophotometric detector (Micromeritics) connected in series with the flow fluorescence detector.

Materials

TG, 6-thioxanthine, DNase I, purine ribonucleotides, pyrimidine ribonucleotides, crotalus adamanteus venom and venom phosphodiesterase were purchased from Sigma (St. Louis, Mo., U.S.A.). 6-Thioguanosine was supplied by Dr. H.B. Wood of Drug Research and Development (Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A.). Synthetic TG nucleotide standards were a gift from Dr. G.A. LePage of University of Alberta Cancer Research Unit (McEachern Laboratory, Edmonton, Canada). [8-^{14}C]-Adenine (50 mCi/mmol) was obtained from Amersham (Oakville, Canada).

RESULTS AND DISCUSSION

The bottom panel in Fig. 1 illustrates the HPLC separation of an oxidized synthetic standards mixture of TG, 6-thioxanthine, 6-thioguanosine mono-, di-, and triphosphate, using fluorescence detection of the column effluent. The top panel in Fig. 1 shows the separation of a standard mixture of the mono, di-, and triphosphates of adenosine, guanosine, cytidine, and uridine, visualized by monitoring the UV absorption of the column effluent at 254 nm, under identical chromatographic conditions to those employed in the separation of TG derivatives below. The alkaline potassium permanganate treatment did not affect the physiological ribonucleotides and the sole reaction with the thiopurines was at the sulfhydryl group, where oxidation resulted in the quantitative formation of fluorescent 6-sulfonate derivatives. By oxidizing

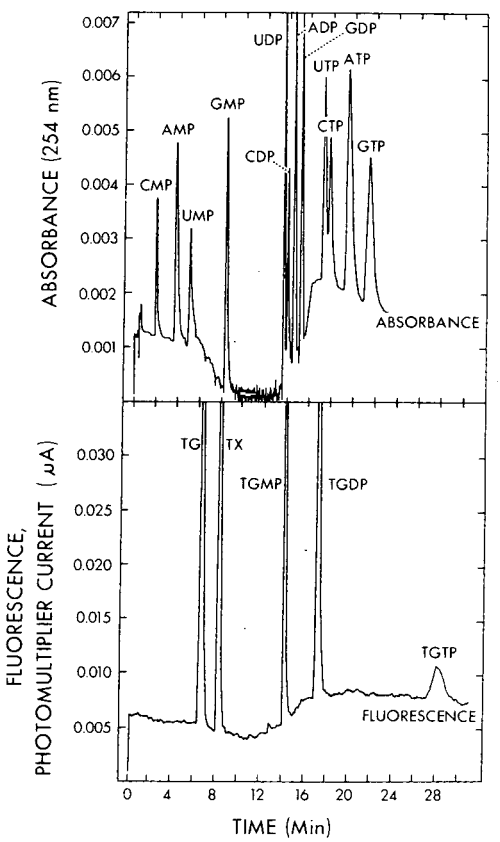

Fig.1. Separation of purine and pyrimidine ribonucleotides and oxidized 6-thiopurine derivatives by HPLC. Top: separation of a standards mixture of purine and pyrimidine ribonucleotides; absorbance profile at 254 nm. Bottom: separation of an oxidized standards mixture of 6-thiopurine derivatives; fluorescence emission profile. Abbreviations: TG = 6-thioguanine; TX = 6-thioxanthine; TGMP = 6-thioguanosine monophosphate; TGDP = 6-thioguanosine diphosphate; TGTP = 6-thioguanosine triphosphate. The actual species separated are the 6-sulfonate derivatives of the indicated thiopurines. Separation conditions as described in Experimental section.

standards mixtures it was demonstrated that this treatment did not affect the relative distribution of TG nucleotides amongst the three levels of phosphorylation. Prior to oxidation, samples of standard mixtures of TG nucleotides were separated at high concentration and quantitated by UV absorption at 340 nm (data not shown). Following oxidation the mixtures were diluted to permit more sensitive fluorescence measurements and the separation repeated. The relative distribution of TG nucleotides before and following oxidation were identical. No dephosphorylation occurred during oxidation. Oxidation did result in coordinated shifts of the thiopurine components to higher retention times, consistent with the generation of an additional negative charge on the molecules through formation of the sulfonate group. UV absorption measurements demonstrated that MP underwent a similar oxidation reaction, but the oxidized MP product did not exhibit detectable fluorescence under the conditions employed. Oxidation of 6-thioxanthine resulted in a fluorescent product, however, the intensity of the fluorescence was only approximately 10% of that exhibited by TG at the selected wavelengths for excitation and emission. Oxidation of pure synthetic TG deoxynucleoside monophosphate resulted in a fluorescent product with a retention time identical to that of the ribonucleoside monophosphate.

A calibration curve for oxidized TG is presented in Fig. 2 on a double logarithmic scale over the range of photomultiplier current settings. Peak area represents the product of the baseline corrected, computer-integrated A/D counts over the peak, and the value of the photomultiplier current producing full-scale output at the particular instrument setting corresponding to

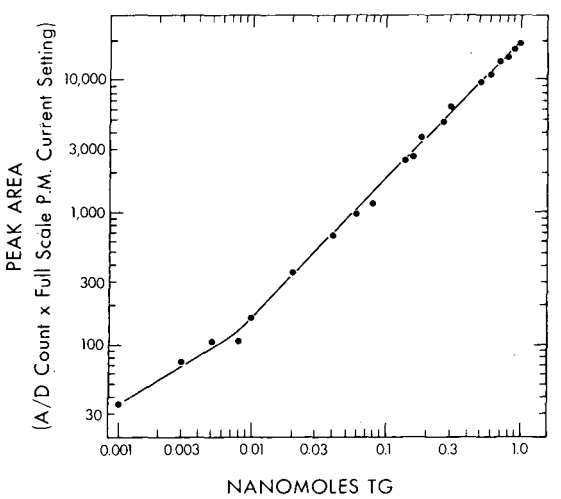

Fig.2. Chromatographic fluorescent peak areas of oxidized 6-thioguanine standards. Baseline corrected peak areas were determined by computer integration of the spectrophotofluorometer detector output signal acquired in real time. Areas are normalized as the product of the actual peak area and the detector photomultiplier current (μA) producing full-scale output at the signal amplification selected for each individual measurement. Chromatographic separation conditions were the same as those for Fig. 1 except that it was unnecessary to continue the gradients to completion.

each measurement. The abscissa represents the quantity of oxidized TG injected onto the column. The system is capable of measuring amounts of TG as low as 4 pmoles, or somewhat less than a nanogram. Quantities of TG nucleotides in cell extracts were derived from a TG calibration curve and are expressed as TG equivalents. Fig. 3 illustrates the simultaneous UV absorption and fluorescence emission chromatographic profiles for an oxidized acid extract of cultured mouse lymphoma L5178Y cells exposed to 3 μM TG. These cells are demonstrably sensitive to the cytotoxic effects of TG [5, 6]. The data correspond to an extract of 6.6×10^5 cells. TG, and TG nucleoside mono-, di-, and triphosphates are evident in the fluorescence emission profile (cf. Fig. 1). The peak immediately following TG represented a component of the tissue culture medium and was displaced from authentic 6-thioxanthine standard added to the cell extract (data not shown). In fact, 6-thioxanthine added during extract preparation may be used as internal standard in determinations of initial rates of TG nucleotide formation by human leukemic blasts (see Fig. 7). The triphosphates of uridine, cytidine, adenosine and guanosine are seen in the simultaneous UV absorption trace. Pretreatment of the acid extracts with crude crotalus adamanteus venom prior to oxidation resulted in complete nucleotide dephosphorylation and

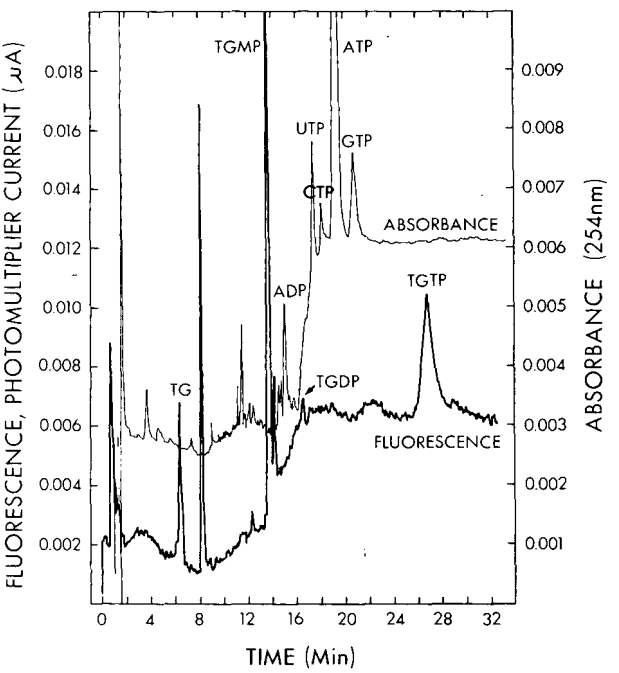

Fig.3. HPLC separation of an oxidized acid extract of cultured L5178Y cells exposed to TG. Simultaneous absorbance (254 nm) and fluorescence emission detection of the column effluent. The data represent 6.60×10^5 cells exposed to 3 μM TG for 13 h. Abbreviations: TGMP = 6-thioguanosine monophosphate; TGDP = 6-thioguanosine diphosphate; TGTP = 6-thioguanosine triphosphate. The actual thiopurine species separated are the 6-sulfonate derivatives of the indicated compounds. Separation conditions were the same as those for Fig. 1.

the disappearance of the nucleotide peaks in both profiles. In the case of the fluorescence emission profile, a new peak of equivalent area appeared with retention time slightly less than that of oxidized TG and identical to that of oxidized 6-thioguanosine. This result confirmed the nucleotide identity of the fluorescent peaks. Fig. 4 reproduces the fluorescence emission trace obtained on chromatographic separation of an oxidized extract of 1.65×10^6 cultured L5178Y cells exposed to 25 μM MP. The profile is very similar to that of Fig. 3 for cells exposed to TG, and demonstrates the formation of TG and TG nucleoside mono-, di- and triphosphate nucleotide metabolites from MP by L5178Y cells. Formation of TG nucleotide metabolites is essential to the cytotoxic activity of MP [6]. Oxidized MP did not exhibit any detectable fluorescence under the conditions employed to detect oxidized TG and its derivatives. Following acid extraction of TG-treated cells, the acid insoluble nucleic acid plus protein residues were subjected to alkaline hydrolysis at 37°. Acid soluble breakdown products of RNA released by this procedure were oxidized and chromatographed. Fig. 5 presents the simultaneous absorbance and fluorescence profiles of the column effluent obtained with one such hydrolysate. The absorbance trace shows a partial separation of the mixture of normal ribonucleoside 2′- and 3′-monophosphate derivatives produced by alkaline hydrolysis of RNA. The fluorescence trace demonstrates the incorporation of TG into RN— in phosphodiester linkage and its release

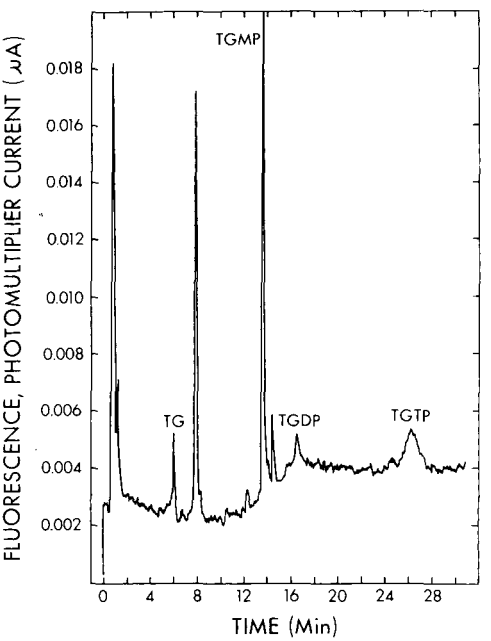

Fig.4. HPLC separation of an oxidized acid extract of cultured L5178Y cells exposed to MP. Fluorescence emission trace of the column effluent. Represented are data obtained with 1.65×10^6 cells exposed to 25 μM MP for 15.5 h. Abbreviations: TGMP = 6-thioguanosine monophosphate; TGDP = 6-thioguanosine diphosphate; TGTP = 6-thioguanosine triphosphate. The actual chemical species separated are the 6-sulfonate derivatives of the indicated compounds. Separation conditions were the same as those for Fig. 1.

by hydrolysis as a mixture of TG ribonucleoside 2'- and 3'-monophosphates. The acid-insoluble DNA plus protein residues of alkaline hydrolysis were suspended in 0.5 *M* Tris buffer (pH 7.0) and subjected to enzymatic hydrolysis of the DNA with pancreatic deoxyribonuclease (DNase I) and purified crotalus adamanteus venom phosphodiesterase. Ultrafiltrable hydrolysis products generated by this treatment were oxidized and separated on the chromatograph. Fig. 6 depicts the simultaneous absorbance and fluorescence profiles observed upon separation of a crude DNA hydrolysate prepared from TG treated L5178Y cells. The normal deoxyribonucleotide constituents of DNA are apparent in the UV absorbance trace and fluorescence detection of the column effluent demonstrated the presence of TG deoxyribonucleoside 5'-monophosphate, derived from TG which was incorporated into the DNA in internucleotide phosphodiester linkage. Incorporation of the TG deoxyribonucleotide metabolite into DNA is responsible for the cytotoxic action of both TG and MP [6, 7, 8].

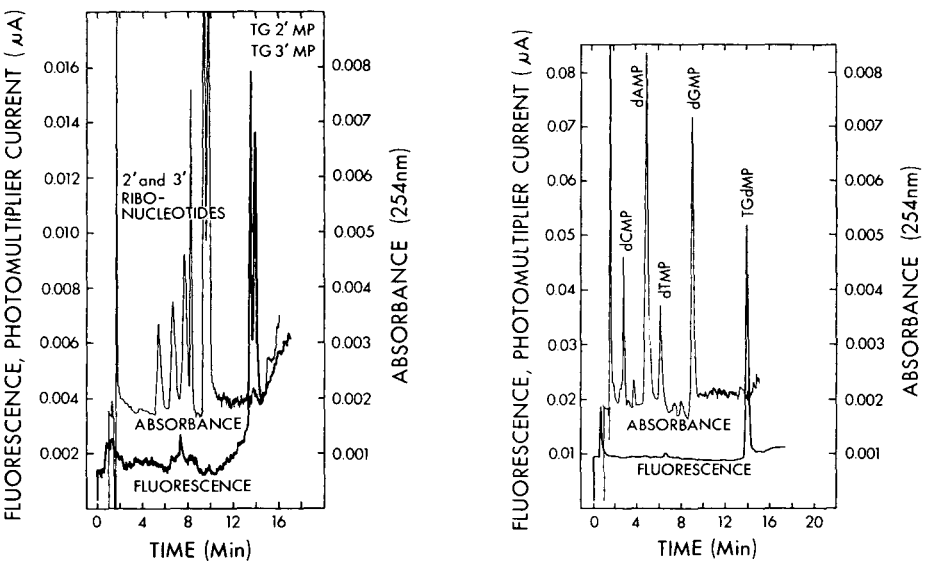

Fig.5. HPLC separation of an oxidized alkaline hydrolysate of the insoluble residue of acid extracted L5178Y cells exposed to TG. Simultaneous absorbance (254 nm) and fluorescence emission detection of the colum effluent. The data represent 1.20×10^5 cells exposed to 3 μM TG for 13 h. Abbreviations: TG2'MP, 6-thioguanosine 2'-monophosphate; TG3'MP, 6-thioguanosine 3'-monophosphate. The actual TG-related species separated are the 6-sulfonate derivatives of the indicated compounds. Separation conditions were the same as those for Fig. 1.

Fig.6. HPLC separation of an oxidized pancreatic deoxyribonuclease (DNase I) plus venom phosphodiesterase hydrolysate of DNA present in the acid insoluble residue following alkaline hydrolysis of L5178Y cells previously exposed to TG. Simultaneous absorbance (254 nm) and fluorescence emission detection of the column effluent. The data represent 2.14×10^5 cells exposed to 3 μM TG for 13 h. Abbreviations: TGdMP = 2'-deoxy-6-thioguanosine monophosphate. The actual TG-related compound separated is the 6-sulfonate of TGdMP. Separation conditions were the same as those for Fig. 1.

The procedures described in this paper were developed as a means of predicting thiopurine resistance in human leukemia, based upon the accumulation of TG nucleotides by blast cells either in vivo or in vitro. The data of Fig. 7 demonstrate the initial rates of TG nucleoside monophosphate formation from TG (50 μM) in vitro by freshly isolated leukemic cells of two patients with acute myelogenous leukemia. In these patients' blast cells, the extent of monophosphate accumulation from TG within 15 min was 25—50 times less than that observed with thiopurine sensitive cultured mouse leukemia L1210 and L5178Y cells (data not shown). In addition to possible differences in phosphoribosyltransferase activity, this lower nett rate of drug nucleotide synthesis was probably at least partially the result of a considerably higher dephosphorylation activity in the human vs. the mouse leukemic cells. When cells of patient 1 were washed free of drug at 15 min and incubated further at 37° in the absence of TG, intracellular TG nucleotide decayed rapidly and was essentially undetectable within 15 min. Therefore it is likely that during incubation with TG much drug nucleotide was degraded as soon as it was formed by the human cells, leading to the low overall rates of nucleotide accumulation. In this context, it is pertinent that a high activity of mem-

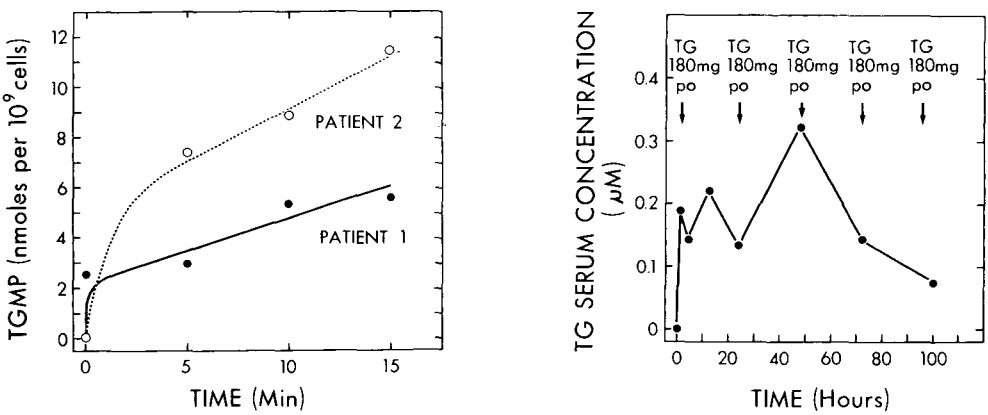

Fig.7. Initial rates of accumulation of TG nucleoside monophosphate, in vitro, by freshly isolated blast cells of human acute myelogenous leukemias. Cells (2×10^7 per ml, 2 ml per point) were incubated at 37° in MEM medium, supplemented with 15% fetal calf serum, and were exposed to TG (50 μM) for the indicated time intervals. Acid extracts of cell pellets were prepared, oxidized, and the 6-thioguanosine monophosphate (TGMP) separated and quantitated by HPLC with flow fluorescence detection. Separation conditions were the same as those for Fig. 1. Amounts of TGMP are expressed as nmoles TG equivalent, determined from a TG calibration curve using [8-^{14}C]adenine as internal standard for extract recovery.

Fig.8. Serum concentrations of TG in an acute myelogenous leukemic patient receiving oral TG. TG at the indicated dosage was administered at the times indicated by the arrows. Where the arrows and data points are coincident, the blood samples were taken immediately prior to administration of the drug. Each point represents the value determined from 30 μl of deproteinated serum which was oxidized and subjected directly to chromatographic separation without further manipulation. TG concentrations were determined from the area of the fluorescent oxidized TG peak detected in the column effluent, using a TG calibration curve. The chromatographic separation conditions were the same as those used to separate the nucleotides except that it was unnecessary to continue the gradients to completion.

brane bound alkaline phosphatase has been associated with thiopurine resistance in an animal model tumor system [10].

The HPLC—flow fluorometric methodology may also be used to measure concentrations of free TG in biological materials since TG is separated and quantitated in this system.

The serum levels of TG in an acute myelogenous leukemic patient receiving a course of oral TG are shown in Fig. 8. The data correspond to measurements on 30 μl of deproteinated serum, oxidized and applied directly to the chromatographic column without using any extraction or concentrative procedures. It can be seen that the serum concentration of TG fluctuated around 0.2 μM in this patient. Experiments with L5178Y cells in culture demonstrated that 0.2 μM TG was cytotoxic if cells were continuously exposed to this concentration for several days. The TG assay may be used routinely to evaluate a patient's capacity for maintaining an effective concentration of drug in the circulation during therapy. Such measurements, especially in conjunction with blast cellular drug nucleotide formation data, are of obvious value in determining the potential efficacy of an extended series of treatments with TG for individual patients. TG resistant disease might occur even in patients whose leukemic cells exhibited a high rate of TG nucleotide formation in vitro, if the drug was poorly absorbed or rapidly eliminated, with the result that cytotoxic plasma concentrations were not maintained throughout the period of treatment.

CONCLUSIONS

Through determination of TG nucleotide formation from MP or TG by leukemic blasts, our HPLC—flow fluorometric method may be used to predict resistance to MP or TG in human leukemia. In addition, predictions of both positive and negative responses to TG may possibly be made by combining cellular drug nucleotide determinations and TG serum concentration measurements during an initial course of therapy. We are currently evaluating the usefulness of these tests in the clinical management of human leukemia.

ACKNOWLEDGEMENT

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Biomedical Applications

CHROMBIO. 121

A RADIOIMMUNO—CHROMATOGRAPHIC SCANNING METHOD FOR THE ANALYSIS OF TESTOSTERONE CONJUGATES IN URINE AND SERUM

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SUMMARY

A method for the analysis of testosterone (and 5α-dihydrotestosterone) conjugates in human serum and urine samples is described. The samples were brought to pH 1 and extracted with a diethyl ether—methanol mixture. After evaporation the residues were run in a thin-layer chromatography system, individual samples' paths were cut into 1-cm long pieces and eluted with methanol. The methanol was evaporated and the residue subjected to acid hydrolysis. The released steroid was extracted by diethyl ether and measured by radioimmunoassay. The methodology described represents a new approach to the qualitative and quantitative study of steroid conjugates in serum and urine, and can easily be applied to the study of steroid conjugates in other biological material.

INTRODUCTION

A large number and wide variety of steroid conjugates isolated from animal and human sources have been described [1]. The majority are in the form of glucuronides and sulphates though the presence of steroid phosphates has been reported in serum [2] and in urine [3]. Testosterone sulphate (TS) in human urine and serum has been reviewed by Dessypris [4]. According to this review only two papers reported on the concentration of TS in human serum [5, 6]. Testosterone glucuronide (TG) in urine and serum has been studied by various methods, including double isotope derivative [7, 8], gas chromatography [9] and more recently radioimmunoassay [10, 11]. Purvis et al. [12] in a recent study reported the presence of dihydrotestosterone sulphate in human seminal plasma.

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Thin-layer chromatography (TLC) has been used by workers in the past to separate steroid glucuronides and sulphates from the free steroids [13, 14]. In this paper a simple and sensitive method is described which provides means of studying profiles of testosterone (T) conjugates in biological material.

EXPERIMENTAL

Materials and instruments

The solvents used were of analar grade and obtained from BDH (Poole, Great Britain) or James Burrough (London, Great Britain). The TLC aluminium sheets pre-coated with silica gel (Art. No. 5554) were obtained from Merck (Darmstadt, G.F.R.). Sulphur trioxide—pyridine complex was obtained from Aldrich (Middlesex, Great Britain).

Unlabelled steroids: testosterone glucuronide, testosterone and 5α-dihydrotestosterone were supplied by Sigma (London, Great Britain). Tritium labelled steroids; 5α-dihydrotestosterone (TRK 395) and testosterone (TRK 402) were purchased from the Radiochemical Centre (Amersham, Great Britain). Tritiated testosterone glucuronide (NET-291) was purchased from New England Nuclear (Dreieichenhain, G.F.R.). Labelled and unlabelled testosterone sulphates were synthesized by a modification of the method of Sobel and Spoerri [15] and separated by TLC according to Klein and Giroud [16]. The testosterone sulphate produced was further identified as described elsewhere [17]. All labelled steroids including conjugated steroids were checked on TLC for purity before use. A Shandon Universal Chromatank (17.5 × 12.5 × 12 cm) was used for all TLC work. Scintillation counting was performed in an Intertechnique Model SL 36 liquid scintillation spectrometer. The scintillation fluid consisted of toluene—Triton (2:1, v/v) with 0.4% PPO (Intertechnique) and 0.05% α-NPO (Nuclear Enterprises). The counting sample (1.0—1.5 ml) was mixed with 10 ml scintillation fluid and this system had a counting efficiency for tritium of approximately 35%.

Samples

Serum and urine samples were from normal males and females aged 21—39 years and were either freshly obtained or had been stored at −20°.

Extraction of samples

The method used for extraction of T conjugates from urine and serum is modified from that of Kornel [18]. To 250 μl of serum or 500 μl of urine in a conical glass centrifuge tube a few drops of 6 *M* H_2SO_4 were added to bring the pH down to about 1 and the samples were then saturated with 50% (w/v) ammonium sulphate. The tube was shaken and allowed to stand for 5 min before extraction with 4 volumes of diethyl ether—methanol (3:1) followed by centrifugation at 2000 *g* for 5 min. The upper phase was transferred to disposable glass tubes containing 15—30 mg of sodium bicarbonate to neutralise any acid. The solvents were evaporated in a heating block at 40° under gentle air stream.

Chromatography of conjugates (System A)

The pre-coated TLC sheets were kept in an air-tight chamber containing silica gel granules before use. The chromatography tank was lined with filter papers at both ends and saturated at room temperature with chloroform—methanol—water (70:30:2), the system used to develop the sheets, for at least 30 min before use.

The residue from the ether—methanol extracts were dissolved in 100 μl methanol which was applied onto the TLC sheets 2 cm from the lower edge. Each tube was washed a second time with 100 μl methanol to secure satisfactory recovery. Samples were spaced 2 cm apart. Appropriate markers (10—20 μg) were applied on the margin of each sheet. The plates were developed by ascending chromatography and allowed to run until the solvent front had reached the upper edge (2.2 h). After drying, the zones for TG, TS and T were located by UV light. Each sample path was now cut into eighteen 1-cm long pieces, which were bent and put into conical glass centrifuge tubes where they were eluted with 1.5 ml of methanol. Centrifugation at about 2000 *g* for 3 min brought down suspended silica gel particles. The methanol was transferred into disposable glass tubes where it was evaporated in a heating block at 40° under gentle air stream.

Acid hydrolysis

The residues from the TLC pieces above were dissolved in 1.0 ml working phosphate buffer (0.1 *M*, pH 7.5 containing 0.2% bovine serum albumin and 0.01% thiomersal) or water and subjected to acid hydrolysis by 3 *M* H_2SO_4 for 20 h at 40° as described elsewhere. The working buffer was used to facilitate dissolution of non-polar compounds when the residue was transferred to other tubes for hydrolysis. The acid hydrolysate in each tube was extracted with 5 volumes of diethyl ether. The ether was evaporated at 40° in disposable glass tubes containing 15—30 mg sodium bicarbonate. The residue was dissolved in working phosphate buffer of which aliquots were taken for T assay or extracted for a run in system B.

Chromatography of androgenic steroids (System B)

The steroids liberated by the acid hydrolysis in the TG and TS peaks of System A were extracted by diethyl ether. After evaporation of the ether the residues were applied on the TLC sheets (Merck, Art. No. 5554) which were developed in a system of toluene—acetone (4:1). The mean R_F values for a few structurally related androgenic steroids were as follows: T 0.44, DHT 0.64; 5α-androstane-3β, 17β-diol 0.24; 5β-androstane-3α, 17β-diol 0.13; 5α-androstane-3α, 17β-diol 0.28. Further processing of the TLC sheets was similar to that of System A. The 1-cm pieces cut out of the sheets were eluted with diethyl ether and the residue, after evaporation, dissolved in working buffer and assayed in the immunoassay for T.

Assay of T and DHT

The radioimmunoassay has been described elsewhere [19] but was used here without prior chromatography to separate T and DHT. The antiserum

used was raised in rabbits against testosterone-3-carboxymethyloxime—bovine serum albumin and was specific for T (100%) and DHT (66%), other related steroids crossreacting 3% (5α-androstane-3β, 17β-diol), 2% (5α-androstane-3α, 17β-diol), 0.7% (androstenedione), 0.5% (epitestosterone), 0.3% (5β-androstane-3α, 17β-diol), <0.1% (androsterone, testosterone acetate, dehydroepiandrosterone) and <0.01% (testosterone sulphate, testosterone glucuronide).

RESULTS

Chromatography of conjugates

The TLC of the testosterone conjugates (System A) was highly reproducible. The R_F values (mean ± standard deviation) of the markers (TG, TS and T) in six consecutive runs were 0.13 ± 0.02, 0.48 ± 0.01 and 0.87 ± 0.02, respectively. The saturation of the tank before each run was found to be essential for securing good reproducibility.

Profiles in urine

The chromatographic (System A) profiles of T and T-containing conjugates in urine are shown in Figs. 1 and 2. Fig. 1 shows a representative profile in a male urine and Fig. 2 the same type of profile in two female urine samples. TG ran the shortest distance (4 cm) from the origin, TS ran about twice as fast (8 cm) and T itself ran fastest, close to the solvent front (16—17 cm).

In order to find out whether any urinary steroids other than T and DHT might be causing the readings in the peaks corresponding to the glucuronide and the sulphate, an aliquot of the hydrolysate from the respective peak frac-

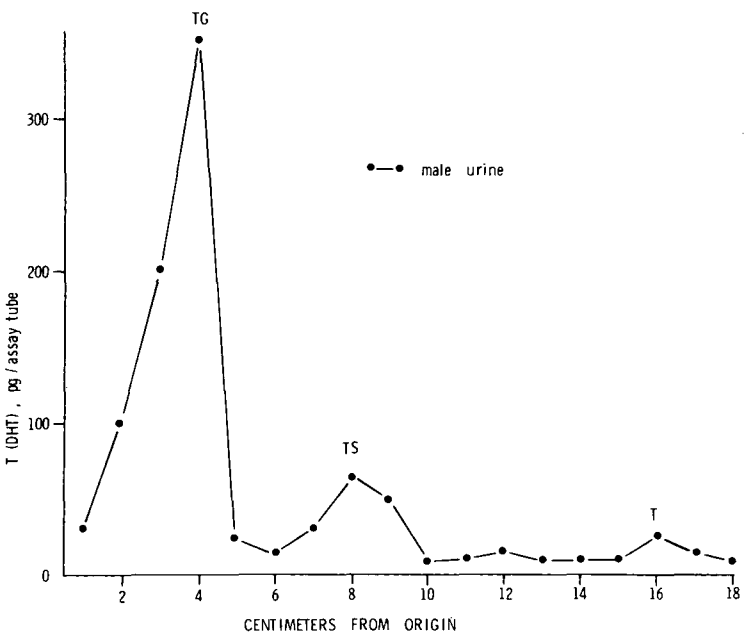

Fig. 1. TLC of a male urine using System A (Figs. 1—6 are all based on extractions of 1-cm sections of the chromatogram and radioimmunoassay of the extracts).

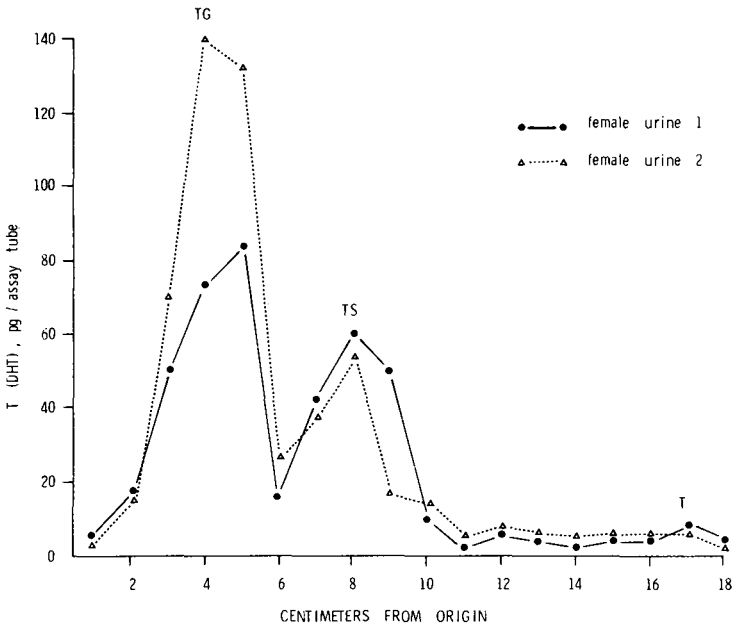

Fig. 2. TLC of female urine in System A.

tions (TG and TS) was run in System B. These results are shown in Fig. 3 (TG) and Fig. 4 (TS). It may be seen that practically all the readings are caused by T and DHT. The TG fraction is made up mostly by T (Fig. 3) whereas in the TS fraction DHT contributes about 30% to the peak.

Profiles in serum

Profiles of the T-containing compounds from two male serum samples are shown in Fig. 5. In male serum the proportional size of the individual peaks is the reverse of that in male urine. Unconjugated T has the highest concentration in male serum and TG the lowest. In female serum the TS peaks were slightly higher than the TG peaks and the unconjugated T peaks were the smallest.

Aliquots from serum TG and TS peaks are also run in System B. Again almost all of the conjugate peaks consisted of T and DHT. Also, as for the urine conjugates, the sulphate peak contained relatively more of DHT than did the glucuronide peak. Fig. 6 shows representative androgen profiles from the TS peak of a male and a female serum.

Recovery studies and quantitation

Unlabelled and labelled conjugates as well as unconjugated T were added to two urine and two serum samples and the recovery through the whole procedure assessed. The recovery ranged from 60 to 89% being similar for both the labelled and unlabelled compounds and no obvious difference was found between serum and urine samples. Based on an average recovery of

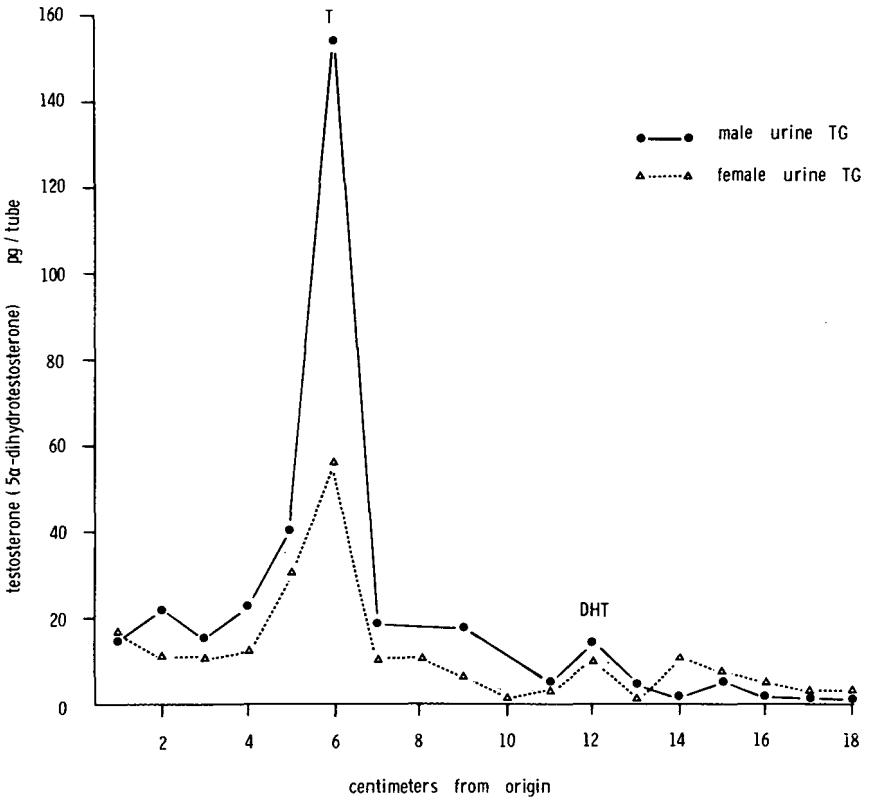

Fig. 3. TLC in System B of urinary TG hydrolysate from System A.

72% we estimated the amount of TG and TS in 4 urines and sera from both sexes. The ranges of TG values for males and females were 80—163 and 92—271 ng per 100 ml in serum and 17—54 and 12—60 μg per 24 h in urine, respectively. The ranges of TS values for males and females were 200—308 and 80—430 ng per 100 ml in serum and 0.3—13.6 and 0.1—2.9 μg per 24 h in urine, respectively. These values are expressed as the T-conjugates but include both the T and DHT conjugates.

DISCUSSION

The method presented for the study of T and DHT conjugates, which we have called a radioimmuno—chromatographic scanning (RICS) method, represents a new approach towards the study of steroid conjugates in biological material. It gives simultaneously the relative amounts and number of the individual steroid containing compounds. The correct detection hinges on the hydrolysis procedure used. We have studied the acid hydrolysis of the T con-

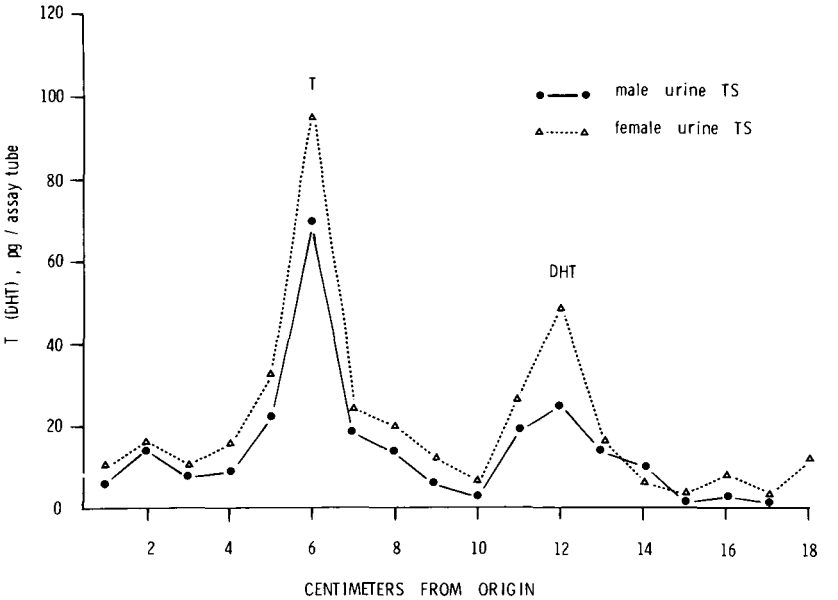

Fig. 4. TLC in System B of urinary TS hydrolysate from System A.

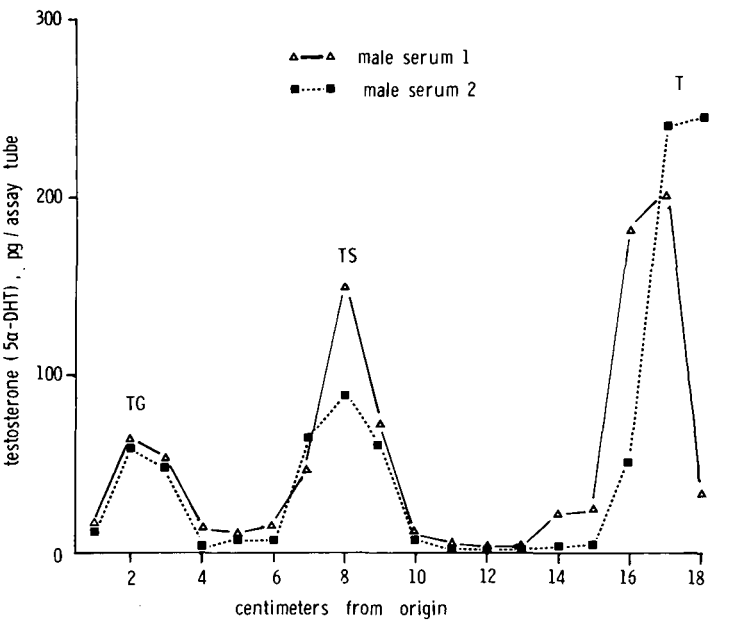

Fig. 5. TLC of male serum in System A.

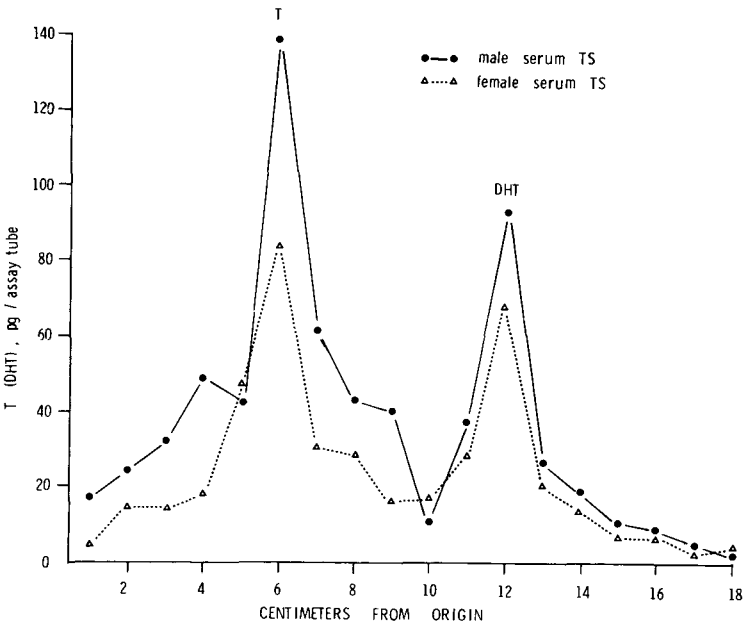

Fig. 6. TLC of serum TS hydrolysate in System B.

jugates specially [17] and did not find any evidence that it destroyed testosterone itself. Furthermore, besides a very acid-labile conjugate, these studies suggested that only the sulphate and glucuronide conjugates of T and DHT existed in normal human urine.

Our antiserum was sufficiently specific for the two androgens, T and DHT to allow us to use an ether extract of the acid hydrolysate directly for radioimmunoassay. This is clearly borne out of Figs. 3, 4 and 6 showing that the antiserum "sees" little if any steroids other than T and DHT.

The concentration of unconjugated (free) testosterone in urine is small [19, 20] and constitutes less than 1% of the total amount (conjugated and unconjugated). Because of the small volumes of urine samples used the peaks of unconjugated testosterone are barely visible. The average T—DHT ratio in the unconjugated fraction in urine from both sexes is 2.5 [19].

The acid extraction of the samples is a disadvantage as it might destroy acid-labile conjugates. We have in fact found evidence for the existence of such an acid-labile conjugate of testosterone in human urine [21]. If the pH-adjustment is omitted, however, the recovery of the glucuronide and sulphate conjugates is drastically reduced to less than 30%.

The method can easily be modified for quantitative measurements of the glucuronides and sulphates. Values published so far vary but our preliminary values in serum and urine are in reasonable agreement with those of other workers [4—7, 9—11]. Our values for TG in male urine are however somewhat lower and our TS values in serum higher than those reported. The later discrepancy can be explained to some extent by the relative amount of DHT

included in our measurements. It is conceivable that our conjugate peaks are not homogenous, consisting of more than one conjugate of similar polarity. Our studies with a different technique involving anion-exchange columns [22] do, however, agree with the present findings of only two conjugate peaks in human urine.

The higher T—DHT ratio in the sulphate peaks as compared to the glucuronide peaks is interesting and may prove to be of biological significance.

The method described here for T (and DHT) should be easily adopted for the study of conjugates of other steroids, provided relatively specific antisera are available and favourable hydrolysing properties of conjugates. Besides studying steroid conjugates in biological fluids it should be of interest to apply these techniques to the study of steroid conjugation in tissues.

ACKNOWLEDGEMENTS

We wish to thank Professor P. Shabasi, Teheran University, in co-operation with whom we raised the antiserum used. We also gratefully acknowledge support from the Kishinchand Chellaram Foundation and the Cancer Research Campaign to G.F. Joplin.

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Biomedical Applications

CHROMBIO. 103

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL CARRIED OUT WITH A SIMPLE DEVICE FOR POWER REGULATION

APPLICATION TO MAMMALIAN GROWTH HORMONES

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SUMMARY

A unit is described to regulate the electrical power delivered to an isoelectric focusing cell. The device consists in a resistance set, in series with a power supply, which can be adjusted to a suitable resistance value in order to permit only minor changes in wattage. Simultaneous monitoring of voltage and current is also carried out with a pen-recorder adaptor.

The improvements in operative conditions achieved by these means are tested by the fractionation of mammalian growth hormones.

INTRODUCTION

Notable improvements in resolution and operational time can be achieved in isoelectric focusing in polyacrylamide gel (IFPA) experiments by maintaining a constant wattage [1—5]. Different methods have already been described for this purpose. Many employ time-consuming manual adjustment of the voltage [1, 2] or expensive power supplies which utilize an electronic control [2] or a servo motor for continuous wattage regulation [4]. Alternatively, power can be regulated by inserting an electrical resistance in series with the IFPA system [5].

In this report we describe a simple and inexpensive apparatus that exploits this technical procedure. The apparatus also includes a device designed to record either voltage or current during the IFPA run. The applicability of this procedure is demonstrated with the fractionation of mammalian growth hormones (GH).

EXPERIMENTAL

Reagents

Acrylamide and N,N′-methylene-bis-acrylamide of commercial grade (Eastman Chemical, Rochester, U.S.A.) were recrystallized from chloroform and acetone, respectively. Coomassie Brillant Blue R 250 was purchased from Serva (Heidelberg, G.F.R.) and Ampholine carrier ampholytes from LKB (Bromma, Sweden). N,N,N′,N′-Tetra methylethylenediamine and other chemicals were of analytical grade. Human haemoglobin, supplied by Dr. L. Rossi-Bernardi, was obtained as previously described [6]. The highly purified growth hormone preparations, supplied by the N.I.H. Endocrine Study Section as a gift from Dr. A.E. Wilhelmi and Dr. A.F. Parlow, were: bovine growth hormone (BGH) B1003A; ovine growth hormone (OGH) 0743B; porcine growth hormone (PGH) P526B; rat growth hormone (RGH) Rat GH B 2; canine growth hormone (CGH) D887A; and human growth hormone (HGH) H1648E.

Preparation of gel slabs

Acrylamide gel slabs were prepared according to the procedure described in the LKB application note [7]. Gel plates were prepared by combining acrylamide with N,N′-methylene-bis-acrylamide as cross-linker with the composition T*=5%, C=2.8% [8]. After the riboflavin had been added, the mixture was poured into the gel mould and photopolymerized for 30 min under the light of a mercury lamp (Osram HQL, 80 W) situated at a distance of about 200 mm from the gel. The final concentration of Ampholine in the gel (2—4%, w/v) was obtained according to the method of Vesterberg [9,10]. The gel plate was used directly and maintained during the focusing period at 4° as monitored on the gel surface by a thermistor probe (sensitivity ± 0.5; LISI, Milan, Italy).

IFPA of pituitary GH preparations

In a typical run, the focusing was performed in gels (250 × 115 × 2 mm) containing 4% (w/v) Ampholine mixture (pH 3.5—9.5).

A 19.8/kΩ resistance was inserted in series and the voltage was set at 100 V/cm (LKB 3371D, d.c. power supply). After 90 min 50—100 μg of protein sample were applied by filter paper (10 × 10 mm Whatman 3 MM) placed on the gel near the cathode. The focusing time under these conditions was 3 h. The end voltage was 70 V/cm.

The pH gradient was measured by cutting out discs of gel (diameter 4 mm) and eluting carrier ampholytes for 1 h into closed tubes with 0.5 ml solution of 20 m*M* KCl free from carbon dioxide [11]. Measurements of pH were performed at 4° in a cold room using a pH meter (model D.P. 100, Gibertini, Milan, Italy) equipped with a micro glass electrode (LOT 205 M3; W. Ingold, Zurich, Switzerland) [12]. Replicate gels were stained for proteins with Coomassie Brilliant Blue R 250 [13] and the isoelectric points of the bands were calculated from the corresponding pH curve.

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*Symbols used: T=g. acrylamide + g.N,N′-methylene-bis-acrylamide per 100 ml; C=(g/100 ml)N,N′-methylene-bis-acrylamide/T.

Apparatus

Gel isoelectric focusing was performed with an LKB Multiphor 2117 apparatus. Fiz. 1A shows the basic wiring diagram of the resistances (R_1—R_4). The resistance values were: $R_2 = 2 \times R_1$, $R_3 = 4 \times R_1$, $R_4 = 8 \times R_1$, so that the total resistance between the terminals A_1 and A_2 ranges from the R_1 value to $15 \times R_1$, according to the positions of the on—off switches (S_1—S_4). In order to obtain a wider range of total resistance values, the basic circuit has been modified by the use of three-positions switches (K_1—K_4) and eight (20 W) additional resistances (Fig. 1B). These switches, when all in the same position, can change the values of R_1, R_2, R_3 and R_4 but keep constant their original relationship (Table I). From the resistance values in Table I, it can easily be seen that when the switches K_1, K_2, K_3 and K_4 are in position I ($R_1' = 0.5$ kΩ) it is possible to obtain total resistance values ranging from 0.5 to 7.5 kΩ with 500-Ω steps, in position II ($R_1'' = 2$ kΩ) from 2 to 30 kΩ with 2-kΩ steps, and in position III ($R_1''' = 8$ kΩ) from 8 to 120 kΩ with 8-kΩ steps. Furthermore, it is possible to obtain a wider range of total resistance values by the different combinations of the switches K_1—K_4. This operation allows the resistance value to be set very close to the one required (e.g. a 28.5-kΩ value is obtained by setting switches K_2, K_3 and K_4 in position II and the switch K_1 in position I).

Fig. 2 shows the wiring diagram of the pen-recorder adaptor, which allows an alternative recording of two different signals (Y_1 and Y_2) varying very slowly with time. D_1 and D_2 are suitable adaptor units to attenuate Y_1 and Y_2 in

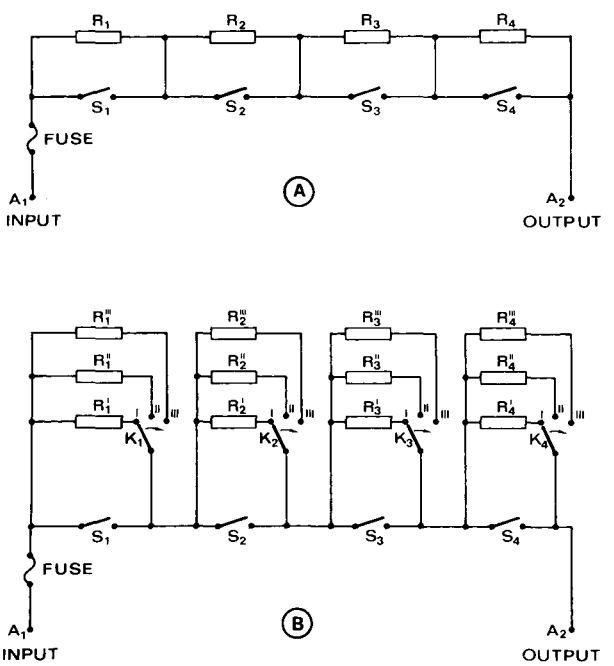

Fig. 1. Basic wiring diagram of the unit for power regulation. A, the resistance set with adjustable switches. B, detailed design of the resistance set of A.

TABLE I

RESISTANCE SET COVERING THE TOTAL RESISTANCE VALUES

Values in kΩ

Resistance	kΩ	Resistance	kΩ	Resistance	kΩ
R_1'	0.5	R_1''	2	R_1'''	8
R_2'	1	R_2''	4	R_2'''	16
R_3'	2	R_3''	8	R_3'''	32
R_4'	4	R_4''	16	R_4'''	64

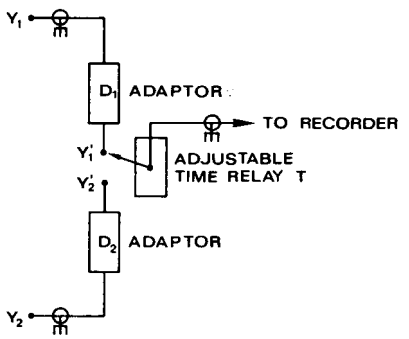

Fig. 2. Block diagram of the adaptor for one pen recorder.

order to maintain the pen of the recorder within the chart limits. An adjustable time relay provides that for a preset time, T, the unit is in position I and for a time T' (identical to T) the unit is in position 2. The time T must be long enough in comparison with the time required by the recording pen to pass from one position to the other. In such a way it is possible to have an alternate recording of two signals (i.e. voltage and current).

RESULTS

The developmental pattern of the voltage and the current during a typical experiment at pH 3.5—9.5, using a 19.8-kΩ series resistance, is shown in Fig. 3. At the start of the run, when a voltage of 100 V/cm is stabilized by the power supply, the effective voltage and current values, recorded between the electrodes, are 15 V/cm and 11 mA, respectively. The samples are applied 90 min after the start of the run when a condition of narrow power variation is achieved: voltage 60 V/cm, and current 5 mA. Four hours later the current is decreased to 4 mA and the voltage is raised to 70 V/cm. Therefore, the resistance inserted in series restrains the total power variation in the gel

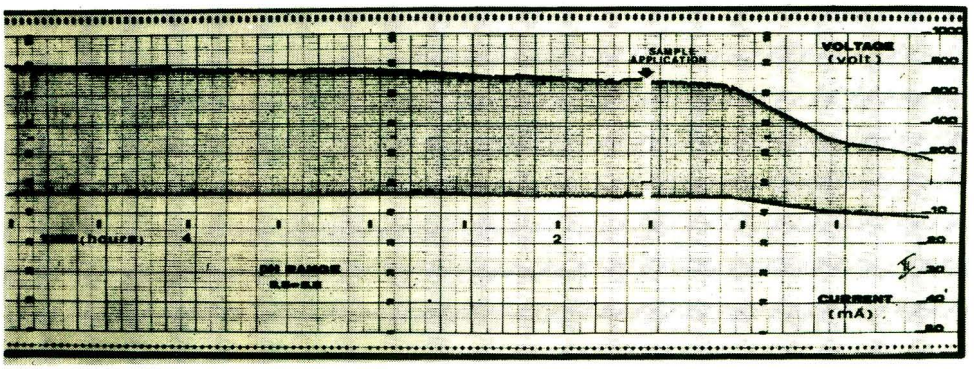

Fig. 3. Changes in the voltage (upper tracing) and current (lower tracing) during the IFPA experiment described in Fig. 4. The graph proceeds from right to left.

between 1.98 and 3.2 W. The IFPA patterns of six different GH preparations are shown in Fig. 4. Fig. 5 shows the band patterns of the same HGH preparation, refocused in a narrower pH gradient (Ampholine, pH 4.0—6.0 at 4% (w/v) concentration).

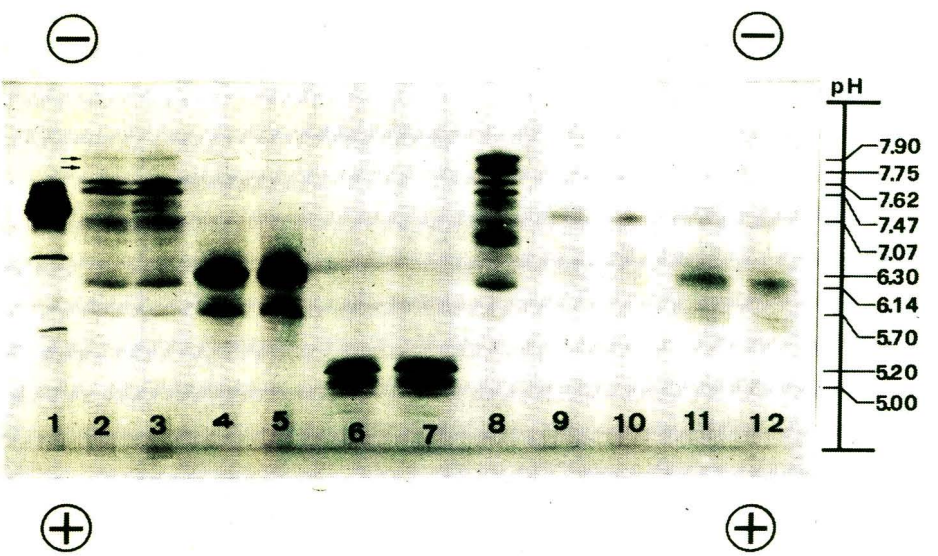

Fig. 4. IFPA of pituitary GH preparation of different animal species. Samples from left to right: (1) human haemoglobin; (2 and 3) ovine GH; (4 and 5) rat GH; (6 and 7) human GH; (8) bovine GH; (9 and 10) canine GH; (11 and 12) porcine GH. The arrows indicate the two more basic bands for OGH. The vertical scale shows the pH values along the gel, at 4°. Gel composition and electrolysis conditions are described in the text.

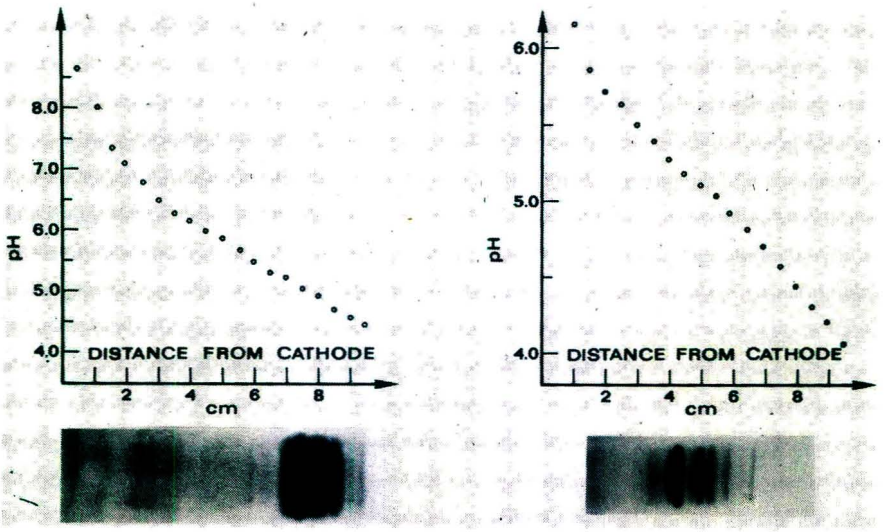

Fig. 5. Left: IFPA of HGH in the pH range 3.5—9.5 with a mixture of Ampholine in a final concentration of 3% (w/v) for a gel size of about 70× 115× 2 mm. A 35.4-kΩ resistance was inserted in series during the run. End voltage, 600 V; focusing time, 2 h. Right: IFPA of HGH in the pH range 4—6 with 4% (w/v) final concentration of Ampholine, for a gel size of about 120× 115× 2 mm, and with a 23.5-kΩ resistance inserted in series for power regulation during the run. End voltage, 500 V; focusing time, 3 h. The pH gradient was determined as described in the text, at 4°.

DISCUSSION

The circuit described is very simple and costs much less than a power supply built or adapted for constant power. It prevents heating and consequent sample denaturation by permitting only minor changes in power. The electrical resistances inserted in series with the electrophoretic cell can be quickly and simply adjusted according to the gel dimension, and the concentration and pH range of Ampholine.

By simultaneously monitoring voltage and current, a maximum power variation in the pH range 3.5—9.5 occurs within 90 min of the start-up operation. After this period it is possible to apply the sample and to control the development of the focusing conditions. By this means we have succeeded in considerably reducing the time of operation in medium-to-high voltage IFPA experiments. The focusing time can be estimated by determining the time required for the coalescence of samples applied at opposite ends of the slab.

In the GH fractionation experiments it is possible from the IFPA bands to distinguish clearly striking species differences. The isoelectric focusing of GH preparations has been previously reported using different procedures (IF in sucrose density gradient or IFPA in cylindrical tubes) [12—22].

We chose the flat gel as the analytical system because it allows comparison of very similar molecules side by side under the same experimental conditions.

The measured pI values of HGH and RGH reported are in good agreement with the data in the literature [14—19]. When HGH was refocused in a pH

gradient of 4.0—6.0 (Fig. 5) the isoelectric points of the major component and of the two successive bands were very similar to those obtained in the wider pH range. With regard to BGH and OGH the isoelectric point of banding patterns here reported are virtually identical to those obtained by Ellis et al. [14]. The resolution of our method appears to result in more distinct banding compared to other procedures. The higher pI values reported by Ellis for the two more basic components of either BGH or OGH are probably due to sucrose in the IF columns [21]. With regard to PGH, a pI value of 6.3 was first reported by Li and Liu [19], but a more recent paper [20] indicates that the apparent pI value for PGH is higher than 6.8. In our experiments PGH clearly shows a single major component at an isoelectric point of 7.08 as well as several minor components with acidic isoelectric points. On the other hand, as regards CGH, our isoelectric point for CGH is lower than that indicated by Hashimoto et al. [22]. However, the IFPA band pattern indicates that, in this case also, the native hormone consists of a single chain.

ACKNOWLEDGEMENT

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CHROMBIO. 095

MICROTECHNIQUES FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF BARBITURATES IN SMALL BLOOD SAMPLES

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SUMMARY

A method for the determination of therapeutic levels of barbituric acids in 25 μl of whole blood is described. After extraction and controlled concentration of the extract to a volume of 5 μl, the barbituric acids are N,N'-dimethylated using a microrefluxer. Of the total extract 20—100% is injected into the gas chromatograph. Low blanks, recoveries of 70—80% and peak ratios that are comparable to those in calibration experiments are obtained provided the detailed working instructions are followed strictly. In addition, barbiturates were determined (1 ng in 25 μl blood) using column-switching devices and nitrogen-sensitive detection.

INTRODUCTION

Determination of barbituric acids at therapeutic and toxic levels in blood is required in the following cases. (A) After suicide attempts — the type and quantity of the drug must be determined for adequate treatment; (B) if barbiturates have been misused in the drug sence — their determination is required after accidental overdosage and for forensic purposes; (C) due to their extensive use as sedatives and narcotics, pharmacokinetic studies with laboratory animals and voluntary test persons are important. (D) Some barbiturates, like phenobarbital, are widely used antiepileptic drugs. To suppress epileptic

fits a minimal concentration of the drug has to be maintained in the central nervous system. The blood level can be influenced by other drugs. It can drop to low and ineffective levels or rise to high and toxic values. Consequently the regular determination of blood levels is required as a basis for long-term therapy.

It would be of great advantage if the blood volume required for the test could be about 20—50 μl since these amounts of blood can be drawn from the fingertip or the lobe of the ear. This would be especially convenient in pediatrics and for experiments with small laboratory animals.

Therapeutic levels of barbituric acids are between 1 and 20 μg/ml of blood. Toxic levels reach up to about 100 μg/ml. With gas chromatography (GC) 5 ng of a barbiturate can be determined after methylation of the acidic —NH groups [1]. Therefore a sample of 20 μl of blood should be sufficient for a GC determination even if only 25% of its barbiturate content can be injected into the gas chromatograph.

Using microtechniques [1—4] and methods that have been described for other purposes [5, 6] it was possible to develop techniques which fulfill these criteria.

Foundations of the microlitre techniques

The method is based on the following principles:

(A). Extraction by stirring avoids formation of a colloidal solution of the aqueous phase in the organic phase [5]. Colloid formation occurs during extraction by shaking. It leads to the formation of interfering peaks in the gas chromatograms.

(B). Concentration of the extracts under partial reflux [6] avoids loss of solutes by sublimation.

(C). After difficult and time-consuming experiments prechromatographic microlitre techniques have been developed for the gas chromatography of very volatile compounds (e.g. anisoles) for the purpose of studying their metabolism in liver microsomes [2]. In order to solve this complicated problem many details had to be worked out which are important for trace analysis

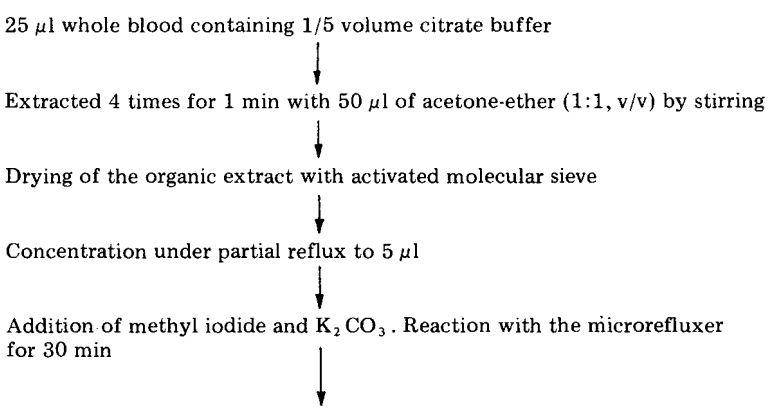

Fig. 1. Microlitre procedure.

in the μl range. These details concern, for instance, drying procedures, handling of small amounts of liquids, etc., and are to be published separately [7].

(D). Barbiturates can be determined below 100—200 ng per injection only after modification of the -NH groups. The reaction conditions of the derivatisation using methyl iodide in acetone lead to the quantitative conversion to N,N′-dimethyl barbiturates without any by-products [1, 4].

(E). The derivatisation mixture can be injected directly into the gas chromatograph [1]. Therefore it is desirable to carry out the reaction in as small a volume as can be injected almost totally. With the aid of a microrefluxer [3], derivatisation can be performed reproducibly in volumes of 5—7 μl. These principles lead to a method which is summarised in Fig. 1; but even after strictly adhering to these rules many interfering peaks did appear in the chromatograms of the first extraction experiments (see Fig. 4). In comprehensive test series the causes for these disturbances had to be liminated. Finally, detailed working instructions (see below) were obtained.

EXPERIMENTAL CONDITIONS AND STANDARD PROCEDURES

Chemicals

Acetone (pro analysi quality) from Merck (Darmstadt, G.F.R.) was used. If impurities were observed in control chromatograms, and for the experiments at 1 ppm, the acetone was purified over a column of aluminiumoxyd 90, aktiv, neutral (Aktivitätsstufe 1, Merck). In order to remove products of aldol condensation the eluate was distilled over a Vigreux-type column. Diethyl ether was purified over a similar alumina column. Small quantities of ether were purified every day. Methyl iodide and potassium carbonate (both pro analysi grade from Merck) were used directly. The molecular sieve, 3A, from Merck (2 mm pellets) was dried at 170° at 15 torr over P_2O_5 for 24 h.

Glassware

All glass vessels were specially made for the microlitre procedures. Glass of Duran or Solidex quality was used throughout. It is important that the shapes and the inner and outer diameters of the vessels correspond the specifications (see Fig. 2). To ensure absolute water-tightness the glass joints (N S 5)* of the two-necked vessels were polished.

Cleaning of the glassware. All glassware was cleaned with a 10% solution of a suitable detergent (i.e. R.B.S. from C. Roth, Karlsruhe, G.F.R.). The two-necked flasks and the 5-μl vessels were filled with cleaning solution with the aid of capillary glass tubes. Air bubbles in the finer parts were carefully avoided. The filled vessels were transferred to a beaker which contained a similar cleaning solution so that the vessels were completely immersed. The solution was heated to about 95° (boiling should be avoided). Boiling or incomplete filling of the vessels leads to the deposition of solid detergent on the glass walls. These deposits are quite difficult to remove and cause erroneous values. The vessels were then rinsed carefully with running tap water and bidistilled water using capillaries which point into their farthest tips. After predrying at 60° all glass vessels were treated in a Bunsen-burner flame until the flame

*NS=Normschliff.

was lighty coloured from the glass. The vessels were heated slowly beginning from their lower tips and proceeding to their upper end, thus water and volatile impurities were removed in a kind of a steam distillation. The heating procedure was carried out under close observation. Only after the removal of the inner water layers did nonvolatile impurities become visible.

PTFE stoppers and caps

Stoppers and caps were prepared from a soft type of PTFE. All stoppers were fabricated so that they fitted tightly over the whole length of the ground-glass joints. Stoppers which showed the slightest irregularities were eliminated. When the reflux reaction was complete the 5 μl reaction vessels were closed with PTFE caps and stored at −20°. At this temperature the caps shrink and ensure absolutely tight closure. 5 μl of acetone could thus be stored for several months without noticeable losses.

The PTFE stirrers as shown in Fig. 2 were made from a 2-mm diameter steel wire which was partially coated with PTFE shrinking tubes.

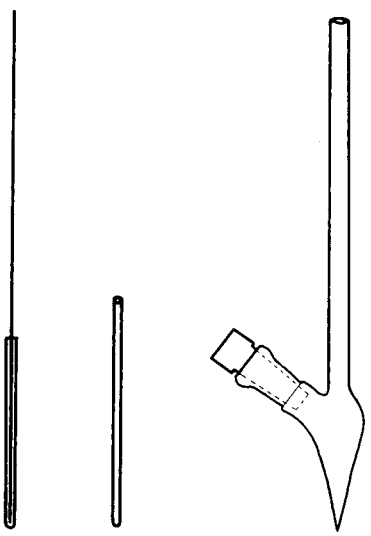

Fig.2. Glassware and microstirrer. Two-necked concentration vessel: length of reflux tube, 10 cm; I.D., 0.3 cm; ground-glass joint closed with PTFE stopper. Microvessel: length, 5.6 cm; I.D. 0.16—0.20 cm.

Extraction procedure★

20 μl of blood (either bovine or human blood) were measured into glass vessels (see Fig. 2) using Blaubrand micropipettes, (Brand, Wertheim, G.F.R.). The blood contained 1/5 of citrate buffer. Traces of blood should be avoided at the upper inner walls while adding the sample. The barbituric acids (or the internal standards) were added dissolved in citrate buffer; 5 μl of such a solution were mixed with blood. For extraction 50 μl of a mixture of ace-

★All glass vessels with or without liquid were kept in ice baths.

tone—ether (1:1, v/v) were added. A PTFE stirrer (see Fig. 2) was introduced into the tip of the vessel. It was driven by a lab motor at about 10 rpm. After 1 min the organic layer was removed with a pasteur pipette. Suction of small droplets from the aqueous layer should be avoided. The extraction procedure was repeated twice. The combined extracts were dried with activated molecular sieve for several minutes.

Concentration procedures★

The dried extract was transferred to a two-necked vessel (see Fig. 2). The side-arm of the vessel was closed with a PTFE stopper without use of grease. The glass vessels were immersed into water at 48°. The water should just cover the upper edge of the stoppers. After 10 min, ether was evaporated completely (under partial reflux). The vessels were then heated in a water-bath at 59° until the volume of the acetone solution was reduced to about 5μl (after 10—15 min). The vessels were then removed quickly and cooled in ice. The immersion in ice caused condensation of the vaporised acetone still present. The condensing liquid rinsed solutes from the walls of the vessel. The remaining acetone (about 20 μl) was transferred to the small flasks and reduced to a volume of about 3 μl in the water-bath of 59°. After immersion in an ice-bath the volume increased to about 6—7 μl.

Equipment for derivatisation

With the aid of the microrefluxer [3] reflux reactions are possible in the range below 50 μl. This device is available in Europe from Berghof GmbH (Tübingen, G.F.R.), and in U.S.A. from Regis Chemical Co. (Morton Grove, Ill.). With the millilitre attachment [4] reflux experiments in the range 0.2—5 ml can be performed in series of 6—9 experiments per run. This equipment has been useful for the preparation of small samples of internal standards, for example.

Derivatisation procedure

To the concentrated acetone extract (5 μl) the following additions were made: boiling chips (diameter 0.5—0.75 mm), about 1 mg freshly powdered potassium carbonate and about 2 μl of acetone containing methyl iodide in a 5—20 *M* excess to the barbiturates. For ultratrace analysis with N-sensitive detection the boiling chips were purified with acetone. The vessels were fixed in the appropriate holes in the cooler of the micro-refluxer. The lower parts of the vessels were immersed in the heated air-bath at about 120°. After a reaction time of 30 min the vessels were taken out of the micro-refluxer. Then they were closed with PTFE caps and cooled in ice.

Gas chromatography

All experiments in the ppm range were carried out with conventional flame ionisation detection (FID). A Perkin Elmer gas chromatograph Model 900 with FID was used. Conditions used were: nitrogen (as carrier gas) flow-rate, 30 ml/min; hydrogen flow-rate, 25 ml/min; air flow-rate, 250 ml/min; glass

★All glass vessels with or without liquid were kept in ice baths.

columns 65 cm × 0.2 cm I.D., filled with 3% OV 225 on Chromosorb W-HP, 120—140 mesh. Temperature programme: 100°, 2-min hold, then 8°/min to 240° (chromatograms in Figs. 3—5) or 100°, 4-min hold, then 4°/min to 240° (chromatograms in Figs. 6 and 7).

For the analysis of barbiturates in the ppb range an L 350 gas chromatograph from Siemens (Karlsruhe, G.F.R.) was used. It was equipped with an alkali flame-ionisation detector (A-FID, "N-FID, system R.E.K."*) [8] and with column switching modules as described by Deans [9, 10]. Column: 3% OV 17 on Chromosorb W-HP, 100—120 mesh; 0.2 cm I.D.; precolumn, 50 cm; separation column, 80 cm. Carrier gas: helium 25 ml/min; hydrogen 40 ml/min; air 110 ml/min. Temperature of detector: 270°. Temperature programme: 170 to 240°, 25°/min, then hold. Heart-cutting analyses were performed with a programme of type IV (see [11]) in the following way: (a) The solvent peak was cut up to 35 sec. During this time acetone and methyl iodide were vented before reaching the separation column. (b) Part of the main fraction was then transferred to the separation column for 15 sec. (c) The rest of the main fraction was then cut off and vented. The separation was performed as usual with this "heart-cut fraction" on the main column.

RESULTS AND DISCUSSION

Strict adherence to the experimental conditions (see above) avoided impurities which were otherwise detected in chromatograms such as shown in Fig. 3. Nearly perfect chromatograms from blanks were obtained reproducibly (see Fig. 4). It needs to be stressed that seemingly trivial deviations from the standard procedures can lead to unsatisfactory results. This implies

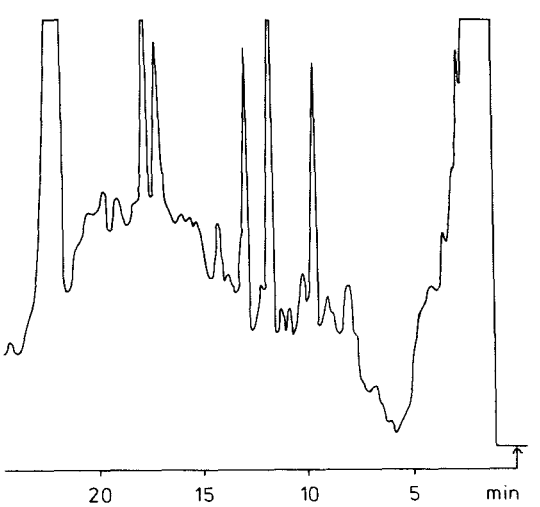

Fig.3. Gas chromatogram of an early blank experiment. 25 µl of whole blood submitted to prechromatographic techniques as described in Fig. 1 but without the use of more elaborate procedures. 1 µl injected into gas chromatograph from a total of 5 µl of the reaction mixture. Attenuation 2; range 10. For other GC details, see text.

*Manufactured by Siemens AG.

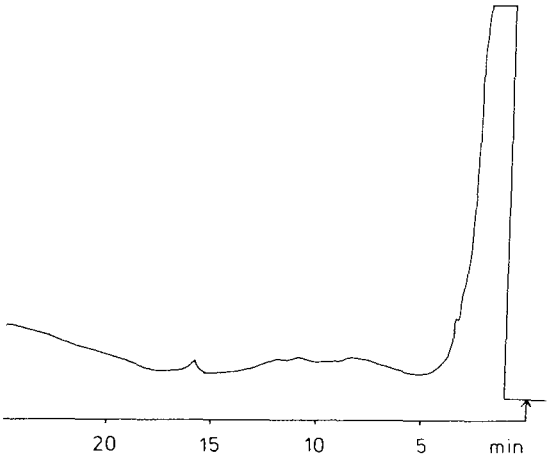

Fig.4. Gas chromatogram of a blank experiment after strict adherence to working instructions. Analytical conditions, see Fig. 2 and text.

especially to extraction or drying. Appropriate cleaning procedures and correctly shaped glassware are important as well.

Low blanks allowed quantitative experiments. In the range 1—10 ppm, recovery rates of barbiturates from 25 µl blood were about 70%. In GC, quantitative experiments are not usually carried out by analysing exactly measured aliquots but by using an internal standard. This method is much more convenient and precise (see ref. 12, for example). 200 ng each of five different barbituric acids were dissolved in 25 µl of blood. Extraction and sample preparation were performed according to the standard procedure. 1 µl of a total of 5 µl was injected into the gas chromatograph. A typical chromatogram is shown in Fig. 5. For quantitative evaluation the peak height ratios

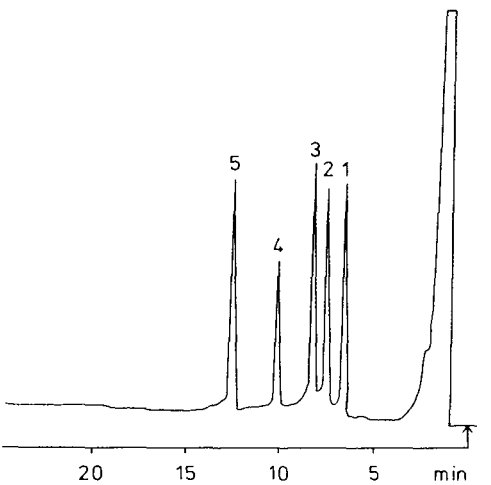

Fig.5. Gas chromatogram of a recovery experiment. 25 µl of blood contained 200 ng each of butalbital (1), amobarbital (2), secobarbital (3), propallylonal (4) and heptabarbital (5). For other conditions, see Fig. 2 and text.

TABLE I

COMPARISON OF GC RESULTS FROM EXTRACTION EXPERIMENTS (A) WITH THOSE FROM CALIBRATION EXPERIMENTS (B)

(A). 200 ng of barbituric acids 1—5 (see Fig. 4) were dissolved in 25 μl blood. Extraction, concentration of extract, methylation in 7-μl end volume, and GC as performed as described in the text. Peak-height ratios of barbituric acids in relation to compound 4 from four experiments are given, together with average values ($\bar{x}$) and standard deviations (S.D.). (B). 200 ng each of barbituric acids 1—5 were dissolved in 7 μl acetone. Methylation and GC were carried out as described.

	Peak-height ratios			
	1:4	2:4	3:4	5:4
Calibration experiments (A)				
1	1.57	1.53	1.60	1.45
2	1.66	1.73	1.83	1.47
3	1.71	1.72	1.94	1.57
4	1.74	1.69	1.84	1.54
$\bar{x}$	1.670	1.665	1.803	1.508
S.D. (absolute)	± 0.074	± 0.091	± 0.144	± 0.058
S.D. (relative)	± 4.4%	± 5.5%	± 8.1%	± 3.9%
Calibration experiments (B)				
1	1.62	1.81	1.87	1.47
2	1.70	1.58	1.70	1.52
3	1.54	1.36	1.61	1.54
4	1.60	1.56	1.70	1.57
$\bar{x}$	1.615	1.578	1.720	1.525
S.D. (absolute)	± 0.066	± 0.184	± 0.109	± 0.042
S.D. (relative)	± 4.1%	± 11.7%	± 6.3%	± 2.8%

of four barbituric acids to the fifth barbituric acid were calculated. Thus, propallylonal (peak 4 in Fig. 5) was used in place of an internal standard. Single values, average values and standard deviations of four series are listed in part A of Table I. They are in good accordance with the values of calibration experiments, which results are shown in part B of Table I. The data of Table I demonstrate the suitability of the method for the determination of barbituric acids in therapeutic concentrations from 25-μl blood samples. Some additional experiments were performed with 25-μl whole-blood samples containing 1-ppm amounts of barbituric acids. Small interfering peaks from impurities were seen in these chromatograms (see Figs. 6 and 7). Recoveries of about 70% and correct peak-area ratios, similar to those in Table I, were obtained.

Analyses with A-FID and column-switching techniques. With conventional GC techniques the detection limits for N,N′-dimethylated barbiturates are about 2 ng per injection (see, for example, Fig. 7). The GC detection limit for nitrogen-containing compounds can be improved with A-FID [8], especially if it is used in conjunction with column-swtiching techniques as described by Deans [9, 10]. Using the heart-cutting mode of operation [12],

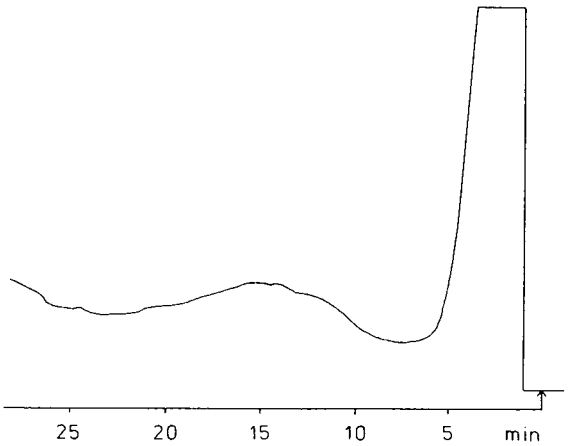

Fig.6. Gas chromatogram of a blank experiment, obtained with maximal GC sensitivity (attenuation 1, range 1) with conventional FID. Injection of 1.5 μl from 6-μl end volume. Other conditions, see text.

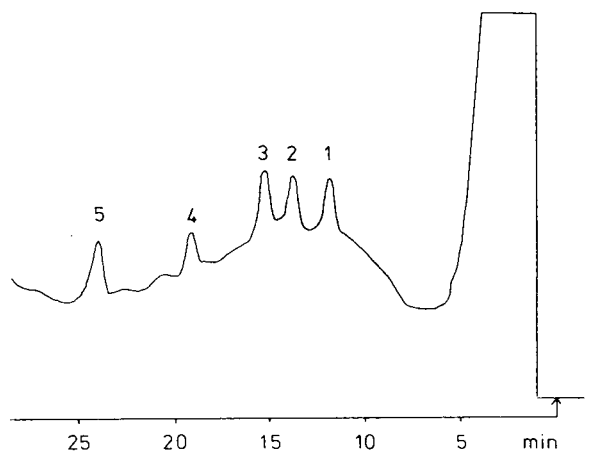

Fig.7. Gas chromatogram of a recovery experiment. Same compounds as in Fig. 4, but 20 ng of each in 25 μl blood. Other conditions, see Fig. 5 and text.

disturbing influences from methyl iodide and acetone on the A-FID are excluded. Thus, up to 10 μl of a reaction mixture can be injected into the gas chromatograph while the A-FID is operated with good sensitivity. With such a special equipment (see Experimental) 16 pg of N,N'-dimethylbarbital could be measured. For the same compound the linearity of detection was found to be between 2×10^{-10} and 1×10^{-4} g.

If 25 μl of human blood are extracted under standard conditions good chromatograms from blanks are obtained (see Fig. 8). Only one disturbing peak was found. It is only present in extracts from human blood and after methylation. It shows the same retention behavior as caffeine.

To 25 μl blood two barbiturates were added (1 ng each). After the application of the described prechromatographic techniques, the total amount of the

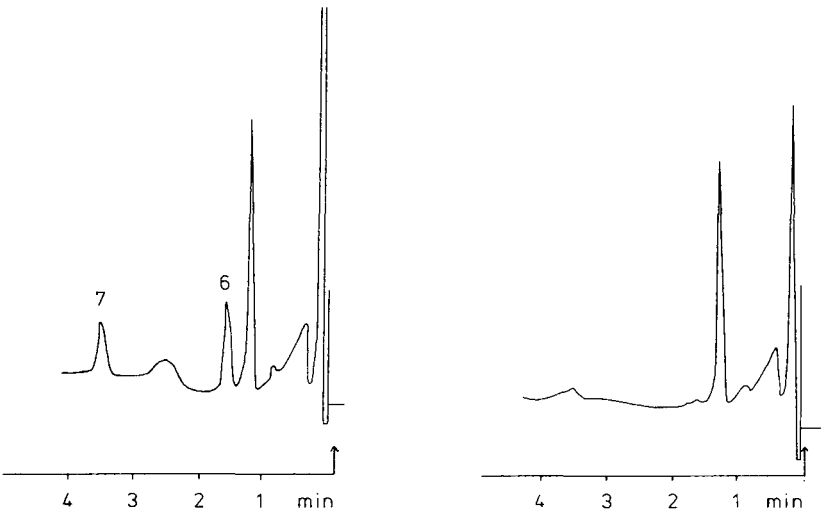

Fig.8. Gas chromatogram of a blank experiment with A-FID and heart-cutting technique. 4.0 μl (i.e. 100%) of the end volume injected. Sensitivity, 8 × 10^{-12} A. Other conditions, see text.

Fig.9. Gas chromatogram of a recovery experiment with A-FID and heart-cutting technique. Dial (7) and cyclobarbital (8) in 1 ng amounts each in 25 μl blood. Same conditions as in Fig. 7 (i.e. total injection of the end volume, here 4.8 μl).

reaction mixtures could be injected into the gas chromatograph (4 to 6 μl). The chromatogram in Fig. 9 shows the possibility of measuring subtherapeutic levels of barbiturates in minute blood samples, as subnanogram amounts can be detected as sharp peaks.

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Biomedical Applications

CHROMBIO. 113

A MICRO LIQUID COLUMN CHROMATOGRAPHY PROCEDURE FOR TWELVE ANTICONVULSANTS AND SOME OF THEIR METABOLITES

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SUMMARY

Solvent extracts of 50 μl volumes of serum were sufficient for the determination of twelve anticonvulsant drugs. The liquid chromatography procedure utilized a C-18 reversed-phase column and isocratic elution with 15% acetonitrile in water. No derivatization was required. Eluted anticonvulsants were detected by UV absorption at 195 nm and quantitated by drug—internal standard peak area ratios. The procedure provided linear working curves over the concentration range from 1 to 100 mg/l of drug in the serum. The procedure for serum provided recoveries of the drugs from 92 to 101%. Within-day precision was about 4% and day-to-day precision was about 6.5%. The procedure has been applied to urine samples to facilitate bioavailability studies. Data are also given for several metabolites. There is a discussion of many practical aspects of the procedure to improve the reliability of the results.

INTRODUCTION

It is becoming well-established that knowledge of the blood levels of anticonvulsant drugs can assist in the clinical management of a patient [1]. The determination of these drugs in clinical samples has utilized many analytical techniques. The two techniques most frequently used, as judged by the number of tests carried out, are microchemistry [2] and gas chromatography [3—5].

Interest is increasing in the use of liquid chromatography for the analysis of anticonvulsant drugs [6—12] because sample manipulation is less demanding than for gas chromatography (GC) and because several of the drugs and some of their metabolites [10] can be determined simultaneously.

Several separation modes are available for the chromatography of anticonvulsant drugs [11] but reversed-phase partitioning offers distinct advantages over most of the others. Since the mobile phase is largely water, it is less expensive and generally more transparent in the far UV than the solvents

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used in normal-phase chromatography.

Utilizing chromatography conditions similar to those we have previously described [6, 9, 10], we have modified the extraction procedure to provide recoveries greater than 90% for all the drugs tested. This and other improvements permits us now to determine the anticonvulsant drugs using only 50 μl of serum.

The procedure is adaptable without modification to the quantitation of the major anticonvulsant drugs and several other anticonvulsant drugs less frequently used. The conditions are set so that the major anticonvulsant drugs (ethosuximide, primidone, phenobarbital, phenytoin, and carbamazepine) may be determined simultaneously. It is not possible with the conditions described to analyze all the other drugs simultaneously. However, clinical samples generally do not contain more than several of the drugs. Data are given so that the analyst will be able to determine what combinations of drugs are feasible to determine.

Because of the significance of metabolites in bioavailability studies, e.g. where a metabolite may possess anticonvulsant activity, several metabolites have been studied and pertinent data are given. This procedure has been used to analyze urine samples for the drug metabolites.

MATERIALS AND METHODS

Apparatus

We used a Perkin-Elmer Model 601 liquid chromatograph, with a Rheodyne 7105 injection valve, a Perkin-Elmer Model LC-55 variable-wavelength UV spectrophotometer detector and a reversed-phase C-18 column (ODS-Sil-X-I, 0.26 × 25 cm; particle size, 13 μm). Special glassware included: 5-ml conical centrifuge tubes, 10 × 75-mm disposable culture tubes, 20-μl and 50-μl disposable pipets and 1-ml disposable pipets.

Reagents and standards

Methanol and chloroform, distilled in glass, were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). The acetonitrile, distilled in glass, UV grade, was also from Burdick & Jackson Labs.

Phenobarbital and phenytoin drug standards were obtained from Applied Science Labs. (State College, Pa., U.S.A.). Phenylethylmalonamide, *p*-hydroxyphenobarbital, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin and carbamazepine 10, 11-epoxide were a gift from Dr. C.E. Pippenger (Columbia-Presbyterian Medical Center, New York, N.Y., U.S.A.). The internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). All other drugs were obtained from the Theta Corp. (Media, Pa., U.S.A.).

Prepared serum standards at six concentrations were obtained from Syva Corp. (Palo Alto, Calif., U.S.A.) and were reconstituted according to the manufacturer's instructions. They include ethosuximide, primidone, phenobarbital, phenytoin and carbamazepine. If drugs not present in the Syva material were to be determined, serum pools were prepared to which concentrations of the drug were added that are appropriate for the expected range. Typically,

three serum levels are chosen, at the lower end, the mid-range and the upper end of the expected range.

Individual standard solutions were prepared from the drugs by dissolving them in methanol to yield 1 mg/ml. A chromatography test mixture was prepared by adding 1 ml each of the standard solutions of ethosuximide, primidone, phenobarbital, phenacetin, phenytoin, carbamazepine and MPPH (internal standard) to a PTFE-lined screw-capped tube and evaporating off the solvent with air. The residue was redissolved in 4 ml of methanol to yield a concentration of 250 mg/l of each drug. All drug standards were stored at 4°.

The mobile phase is prepared by adding 75 ml acetonitrile to 425 ml water and mixing. The chloroform extractant contains 2 mg/l of the internal standard. The phosphate buffer is 0.1 *M*, pH 8.0.

Procedure

Add 50 µl of serum and 50 µl of phosphate buffer to a 5-ml conical centrifuge tube. Mix well for 15 sec and add 0.5 ml of the extractant solution. Mix again for 15 sec and centrifuge for 1 min at 710 *g*. Aspirate off the organic phase using a 1-ml Hamilton syringe (No. 1001) and evaporate at room temperature with a gentle current of air. Redissolve the residue in 20 µl of methanol and inject 10 µl of this into the liquid chromatograph.

Chromatography

The mobile phase consisted of 15% acetonitrile in water, delivered at a flow-rate of 1 ml/min. Column temperature was 65°. The UV detector wavelength was 195 nm.

Calibration

Calibration is accomplished by using the procedure on the six Syva serum standards and the prepared pools in the event that the analysis is required for drugs not in the Syva standards. The peak area is measured for the standards. The area ratio is calculated by dividing the peak area of the drug by the peak area of the MPPH internal standard. A working curve is prepared by plotting the peak area ratio of each drug against concentration. The peak area ratios are calculated for drugs in the patient sera. The concentration of each drug is determined from the working curve.

RESULTS

Quality control

Each day, prior to analyzing samples, we establish that the chromatographic separation is acceptable by injecting the test mixture into the chromatograph. A typical chromatogram of this test mixture is illustrated in Fig. 1A. Both the retention time and the relative retention, r_{is}, for each compound compared to the internal standard are listed in Table I.

We also participated in the very useful quality control program of the Epilepsy Foundation of America (Dr. C.E. Pippenger). This permitted us to compare our results against others, often using different techniques. A chromatogram of a sample from that program is shown in Fig. 1B.

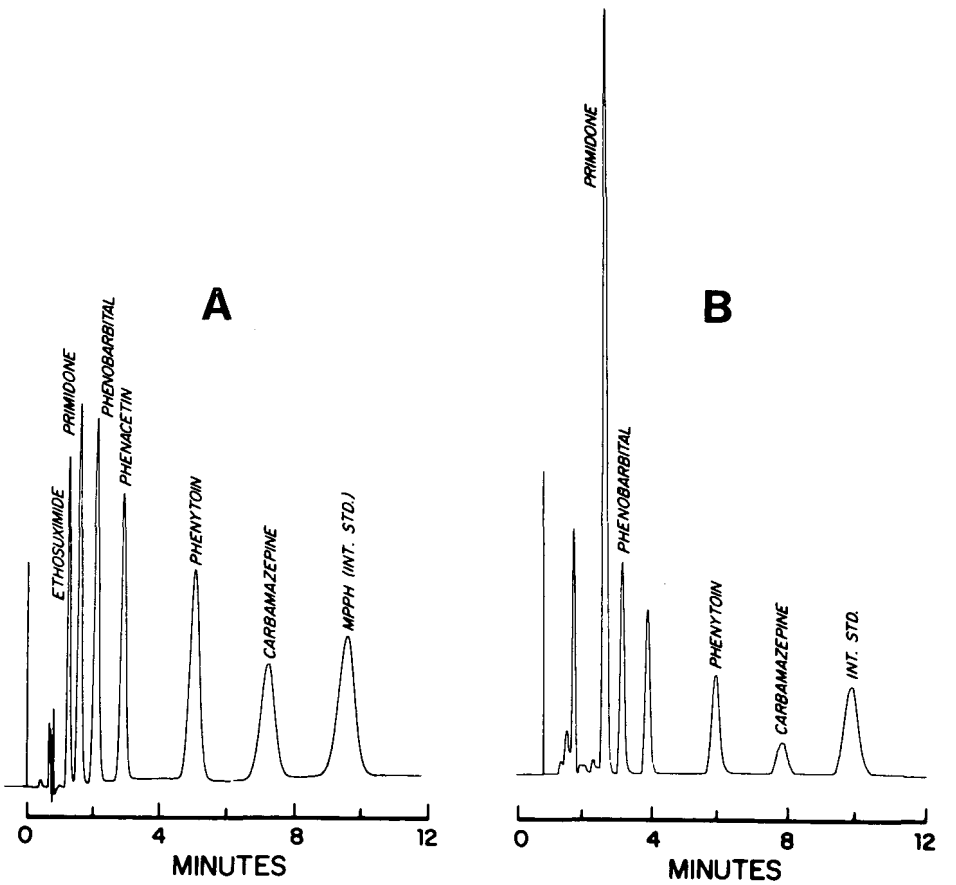

Fig. 1. A, Chromatogram of a mixture of anticonvulsant drug standards, each peak represents 250 ng of each drug. B, Representative chromatogram of a quality control serum; the peak immediately following phenobarbital is phenacetin, which was added to the extractant with the MPPH internal standard.

Recoveries

Recovery studies were made by adding specified quantities of the compounds to pooled sera known to be drug-free. The pool was analyzed in 50-μl aliquots and the peak areas of the extracted drugs were compared with the peak areas obtained by injecting 1 μg of each pure drug standard into the chromatograph. The data are given in Table II for concentrations of 10 and 50 mg/l. The recoveries ranged from 92% to 101%. Table II also includes recovery data for the 10, 11-epoxide metabolite of carbamazepine.

Linearity

We evaluated the linearity of the procedure for each drug by analyzing aliquots of a serum pool to which known quantities of the drugs were added to give a range of concentrations from 1 to 100 mg/l. It was established that for this range a linear relationship existed between the concentration and the

TABLE I

RETENTION TIME AND RELATIVE RETENTION TIME DATA FOR ANTICONVULSANT DRUGS AND METABOLITES

No.	Compound	Retention time (min)	Relative retention*
1	Succinic acid amide	1.86	0.064
2	Acetazolamide	2.07	0.076
3	Ethosuximide	2.66	0.108
4	Trimethadione	2.93	0.123
5	Phenylethylmalonamide	2.94	0.124
6	*p*-Hydroxyphenobarbital	2.96	0.125
7	Primidone	3.28	0.142
8	Paramethadione	3.90	0.177
9	Phenobarbital	4.17	0.192
10	5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	4.48	0.209
11	Ethotoin	4.50	0.210
12	Phensuximide	5.08	0.242
13	Phenacetin	5.70	0.276
14	Mephenytoin	6.72	0.332
15	Mephobarbital	8.18	0.413
16	Metharbital	8.26	0.417
17	Methsuximide	8.28	0.419
18	Carbamazepine 10, 11-epoxide	8.30	0.420
19	Phenytoin	9.80	0.502
20	Carbamazepine	14.41	0.757
21	MPPH	18.81	1.000

*Relative to MPPH, the internal standard, after adjusting the retention time by subtracting 0.70 min mobile phase hold-up time.

peak area ratio of the drug to the internal standard. Of the metabolites, only the 10, 11-epoxide of carbamazepine was studied. A linear relationship was confirmed for this metabolite.

Sensitivity

The quantity of sample that is injected into the chromatograph is equivalent to 25 μl of serum. In this sample volume our procedure readily detected 5 ng (0.2 mg/l) of each of the compounds.

Precision

We studied within-day precision by analyzing 15 aliquots each of two sets of serum pools to which were added 5 and 50 mg/l of each drug, respectively. One serum pool contained ethosuximide, phenylethylmalonamide, primidone, phenobarbital, phensuximide, mephenytoin, methsuximide, phenytoin and carbamazepine. The other serum pool we used contained acetazolamide, trimethadione, paramethadione, ethotoin and metharbital. The other drugs not formulated into one of our serum pools were studied individually. For most compounds, either the 5-mg/l or 50-mg/l concentration is in the therapeutic range. The results are summarized in Table III and indicate that a within-day precision between 3 and 4% is obtainable for concentrations of 5 mg/l and

TABLE II

RECOVERY DATA FOR ANTICONVULSANT DRUGS FROM SERUM

— = Not determined

No.	Compound	Concentration	
		10 mg/l	50 mg/l
1	Succinic acid amide	91	89
2	Acetazolamide	93	96
3	Ethosumixide	96	98
4	Trimethadione	93	93
5	Phenylethylmalonamide	—	—
6	*p*-Hydroxyphenobarbital	—	—
7	Primidone	100	99
8	Paramethadione	96	95
9	Phenobarbital	100	98
10	5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	—	—
11	Ethotoin	94	97
12	Phensuximide	94	96
13	Phenacetin	95	96
14	Mephenytoin	98	101
15	Mephobarbital	96	96
16	Metharbital	93	94
17	Methsuximide	95	95
18	Carbamazepine 10, 11-epoxide	97	98
19	Phenytoin	101	98
20	Carbamazepine	100	98

TABLE III

WITHIN-DAY PRECISION DATA FOR ANTICONVULSANT DRUGS FROM SERUM

$\bar{x}$ = Mean; SD = standard deviation; CV = coefficient of variation.

Compound	Concentration					
	5 mg/l			50 mg/l		
	$\bar{x}$	SD	CV (%)	$\bar{x}$	SD	CV (%)
Ethosuximide	4.6	0.19	4.2	51.3	1.95	3.8
Trimethadione	4.8	0.18	3.8	50.8	1.63	3.2
Primidone	5.1	0.16	3.1	48.9	1.66	3.4
Paramethadione	4.7	0.18	3.9	49.0	1.86	3.8
Phenobarbital	4.7	0.15	3.2	46.8	1.40	3.0
Ethotoin	5.2	0.20	3.8	50.1	1.55	3.1
Phensuximide	4.9	0.19	3.9	48.3	1.64	3.4
Mephenytoin	5.0	0.19	3.8	52.1	1.88	3.6
Mephobarbital	5.0	0.18	3.5	48.5	1.75	3.6
Metharbital	4.7	0.17	3.6	48.9	1.57	3.2
Methsuximide	4.9	0.17	3.4	48.7	1.51	3.1
Carbamazepine 10, 11-epoxide	5.1	0.19	3.7	50.5	1.57	3.1
Phenytoin	5.0	0.20	4.0	48.9	1.66	3.4
Carbamazepine	5.0	0.16	3.1	51.0	1.48	2.9

TABLE IV

DAY-TO-DAY PRECISION DATA FOR ANTICONVULSANT DRUGS FROM SERUM

Compound	Concentration					
	5 mg/l			50 mg/l		
	$\bar{x}$	SD	CV (%)	$\bar{x}$	SD	CV (%)
Ethosuximide	4.8	0.31	6.4	51.2	3.17	6.2
Trimethadione	4.7	0.28	5.9	50.4	2.47	4.9
Primidone	4.9	0.28	5.7	51.0	2.35	4.6
Paramethadione	5.2	0.27	5.2	48.0	2.26	4.7
Phenobarbital	5.1	0.30	5.9	52.2	2.71	5.2
Ethotoin	4.6	0.29	6.2	50.6	2.88	5.7
Phensuximide	4.8	0.29	6.1	50.4	2.42	4.8
Mephenytoin	4.5	0.28	6.3	47.0	2.30	4.9
Mephobarbital	4.9	0.27	5.6	48.9	2.35	4.8
Metharbital	5.2	0.26	4.9	47.3	2.46	5.2
Methsuximide	4.8	0.27	5.6	48.7	2.97	6.1
Carbamazepine 10, 11-epoxide	4.7	0.24	5.1	49.3	3.16	6.4
Phenytoin	5.1	0.30	5.8	48.7	2.78	5.7
Carbamazepine	4.9	0.24	4.8	49.6	2.18	4.4

slightly better precision is found for concentrations of 50 mg/l. Similarly, aliquots of the same pools and individual sera used to determine within-day precisions were analyzed daily for 15 days and the day-to-day precision estimated. Table IV shows that about 5% precision is found at both levels.

Accuracy

Because there are no accepted reference or routine methods for the determination of the less frequently used anticonvulsant drugs, we were unable to compare results obtained by different procedures. In our earlier study [6] comparing a GC procedure and a procedure very similar to the present one for fifteen samples, we found excellent agreement for primidone, phenytoin, phenobarbital and ethosuximide. We were unable to obtain good correlation with a GC procedure for carbamazepine [13]. We believe this was because of the poor precision of the GC procedure, in our hands. We found within-day precision of 21% for the carbamazepine gas chromatography procedure.

Patient sera

Over a period longer than 6 months this procedure has been applied to numerous sera from patients on anticonvulsant therapy. An example is shown in Fig. 2A from a subject on primidone, phenobarbital and phenytoin therapy. The sample was calculated to contain 8, 15 and 6 mg/l each of the compounds, respectively.

Fig. 2B was obtained by the analysis of a clinical serum from a patient on carbamazepine therapy. Carbamazepine was quantitated together with its metabolite, the 10, 11-epoxide of carbamazepine. The quantities calculated

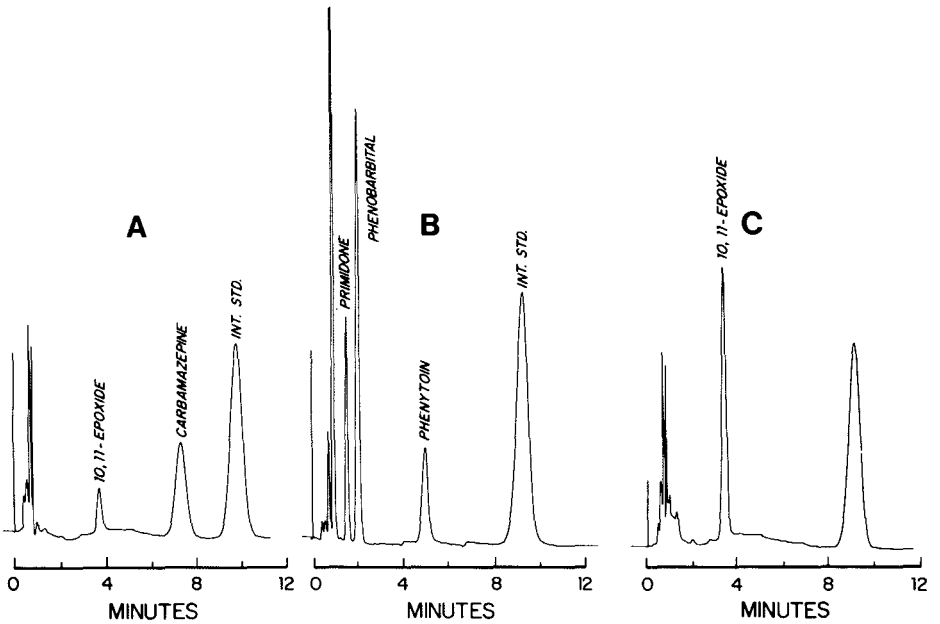

Fig. 2. Several clinical samples. A, Chromatogram of a patient serum containing primidone, phenobarbital and phenytoin at concentrations of 8, 15 and 6 mg/l, respectively. B, Chromatogram of a serum from a patient on carbamazepine; This drug and the carbamazepine 10, 11-epoxide were calculated to be 12 and 2 mg/l, respectively. C, Chromatogram of a urine sample from a patient receiving carbamazepine showing a carbamazepine 10, 11-epoxide concentration of 11 mg/l.

were 12 mg/l and 2 mg/l for the carbamazepine and the 10, 11-epoxide, respectively. The chromatogram in Fig. 2C was obtained from a urine sample from a subject on carbamazepine therapy. A trace of the parent compound was detected, but the 10, 11-epoxide metabolite is strongly evident. The concentration of the 10, 11-epoxide was calculated to be 11 mg/l.

Interferences

The procedure as described is not adequate to separate all the compounds simultaneously. It is adequate to separate all major anticonvulsants and several minor ones simultaneously without interference. Although it is feasible to effect a complete separation of all the drugs simultaneously, this would be at the expense of analysis time. We have chosen to optimize the analysis for the five major anticonvulsants, and the analysis time is about 19 min. Sufficient analysis time is allowed so that metabolites can also be monitored. Significant shortening of the analysis time would most probably reduce resolution to the point where the primidone metabolite, phenylethylmalomide, would overlap the parent compound. It is important to avoid this interference.

To assess the usefulness of the procedure against interfering drug peaks, we injected into the chromatograph the following compounds: amobarbital, pentobarbital, secobarbital, glutethimide, propoxyphene and salicylate. Amobarbital and pentobarbital, if present in the sample, will interfere with the

determination of methsuximide. Propoxyphene, secobarbital and glutethimide have retention times similar to phenytoin and will interfere with the determination of phenytoin, if they are present. No study was undertaken to determine these drugs in physiological samples.

The procedure, equipment and columns that we have used readily permitted identification of compounds that differed in relative retention by more than about 5%. However, if two compounds of similar relative retention were present in the same sample, reliable quantitation required that their relative retention differ by at least 10%.

DISCUSSION

This procedure is a useful approach to the determination of most anticonvulsant compounds, including some metabolites. With the procedure, the analyst will be able to analyze samples containing not only the major anticonvulsant drugs, but also those which are used less frequently. The procedure offers sufficient sensitivity to determine each drug over its entire therapeutic range, all compounds being chromatographed without derivatization.

The growing importance of bioavailability studies, especially where drug metabolites are to be determined, often requires the analysis of urine samples. Urine samples may be analyzed as readily as serum samples.

There are some practical considerations that will improve the reliability of this liquid chromatographic procedure. It is important to monitor on a routine basis the performance of the chromatography system. Periodically injecting a test mixture of drugs into the chromatograph will allow the analyst to confirm the separation capability of the column and to monitor degradation of column efficiency due to usage. A system check of this type should be performed at the beginning of each working day and after every ten analyses.

Clogging of the porous packing retainer located at the top of the column by matrix material extracted from clinical samples will eventually increase the back-pressure of the column to a point where the system becomes unusable. A daily check of system back-pressure will often identify this problem if it arises. Usually, replacement of the packing retainer will reduce the back pressure.

Increasing the acetonitrile percentage in the mobile phase to 100% for 1 h will strip from the column any compounds which have been completely retained on the packing material while operating with the suggested mobile phase for the procedure. This will result in higher column efficiency and reduced back-pressures.

Settling of the column packing will result in loss of column efficiency due to band spreading of the compounds being analyzed. The addition of packing to the top of the column will help correct this.

Carry-over in the injection valve can provide a potential source of error if a previous sample contains large amounts of drug. To a large extent, this problem can be alleviated by routine replacement of the PTFE valve seal every six months. The microliter syringe used for injection should also be inspected routinely for defects. Any analysis immediately following a very high sample should be repeated.

We have listed both the retention time of each anticonvulsant drug and its relative retention as compared to the MPPH internal standard. It should be noted that although the retention time can be used to identify the compound for a particular column under a given set of chromatography conditions, the relative retention is more readily transferred between columns or when chromatographic performance changes.

It is important to evaporate the chloroform extracts at room temperature with an air current not exceeding 50 ml/min. The use of heat during evaporation or continuing to evaporate after the extract is thoroughly dry will result in loss of ethosuximide due to its high volatility.

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Note

The determination of 5-methoxyindole-3-acetic acid in human urine by mass fragmentography

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Appreciable quantities of 5-methoxytryptamine occur in rat hypothalamus [1]. This has led to speculation [2] that 5-methoxytryptamine could be a transmitter within the central nervous system and could possibly be the B-type fluorophore of Bjorklund and coworkers [3]. The major metabolite of exogenous 5-methoxytryptamine in rats and rabbits is 5-methoxyindole-3-acetic acid (5-MIAA) [4]. It is likely that in man too the urinary excretion of 5-MIAA can give an indication of the overall turnover of 5-methoxytryptamine within the body.

With this in mind, we have developed a mass fragmentographic method using an internal isotopic standard to detect and quantitate 5-MIAA in urine and have shown that it is a normal urine constituent. The presence of this acid in humans has not previously been demonstrated. The levels found are probably below the limits of sensitivity of the methods used in earlier searches for this compound, e.g. paper chromatography followed by chemical visualisation [5]. We have measured the urinary output of 5-MIAA in normal subjects and have also confirmed that in man unconjugated 5-MIAA is a major metabolite of exogenous 5-methoxytryptamine.

MATERIALS AND METHODS

Preparation of 5-methoxyindole-3-acetic acid — [methylene-$^2H_2$]

5-Methoxyindole-3-acetic acid (Sigma, St. Louis, Mo., U.S.A.) (20 mg) dis-

solved in 0.75 ml ^2H_2O containing 10% NaO^2H was heated at 125° for 6 h. After acidification with 2 *M* HCl the mixture was extracted with diethyl ether (3 × 10 ml); the extract was dried (Na_2SO_4) and the ether removed by distillation under reduced pressure to leave a yellow oil which crystallised on standing. Thin-layer chromatography on cellulose (*n*-butanol—pyridine—water (4:3:3, v/v); visualisation with dimethylaminocinnamaldehyde [1% in ethanol—conc. hydrochloric acid (1:1)] showed one compound with the same properties as the starting compound. The isotopic composition of the material was calculated from the mass spectrum of the bis(trimethylsilyl) derivative to be 97% $^2H_2$ and 3% $^2H_1$.

Solutions of the compound containing approximately 1 mg per 100 ml water were prepared and standardised by mass fragmentography against solutions of the undeuterated material. These solutions were stable at −14° for several weeks.

Preparation of urine samples. The deuterated standard solution (10 ml) was added to an aliquot (500 ml) of urine which was then acidified to pH 4 with 6 *M* HCl and extracted with ether (500 ml in portions). The extract was dried (Na_2SO_4) and the ether removed by distillation under reduced pressure. The residue in pyridine formate buffer, pH 2.60 (prepared by the addition of constant boiling formic acid to 0.1 *M* aqueous pyridine) (50 ml) was applied to a 15 × 2.5 cm column of Dowex 50W X4 ion-exchange resin (Serva, Heidelberg, G.F.R.) in the pyridinium form, pretreated by washing with two bed volumes (b.v.) of pyridine formate buffer (pH 2.60). The column was washed successively with 2 b.v. of pyridine formate (pH 4.20), 2 b.v. water, and 1 b.v. of 0.5 *M* aqueous pyridine adjusted to pH 11.5 with concentrated ammonia solution. The water wash contains most of the hippuric acid and the aqueous pyridine wash contains indole-3-lactic acid. A further wash with 2 b.v. of the 0.5 *M* aqueous pyridine eluted an obvious dark band which contained indole-3-acetic acid, 5-hydroxyindole-3-acetic acid and 5-MIAA. Evaporation of this eluate under reduced pressure below 40° gave a dark residue containing the acids which were converted to their trimethylsilyl derivatives using pyridine and bis-(trimethylsilyl) trifluoroacetamide with 1% chlorotrimethylsilane. This silylated mixture was generally satisfactory for mass fragmentographic analysis but the 5-MIAA could be further purified by preparative thin-layer chromatography on cellulose Avicel F; (10 × 20 cm; 500 μm; Anachem, Luton, Great Britain) using benzene—propionic acid—water (57:40:3, v/v) of the residue from the column eluate. Indoleacetic acid and 5-MIAA run to the top third of the plate ahead of 5-hydroxyindoleacetic acid and coloured material. This procedure was adopted for some early experiments.

Instrumentation. Gas chromatography—mass spectrometry (GC—MS) was carried out using a Finnigan 3200 GC—MS system (Finnigan, Sunnyvale, Calif., U.S.A.) under the control of a Finnigan 6110 data system. A 5 ft. × 2 mm I.D. glass GC column packed with 3% OV-17 on 100—120 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) and a 4 ft. × 2 mm I.D. column packed with 3% OV-1 on 80—100 mesh Supelcoport were used, programmed from 150° to 280° at 4°/min respectively. The injection temperature was 280°, and the separator oven and transfer line were at 270°. The mass spectrometer was run with 0.30-mA emission at 70-eV ionising energy. Quantitative analysis

was made by selected ion monitoring using the peaks at *m/e* 232 and 234 (M—COOTMS) and the molecular ion peaks at *m/e* 349 and 351 for the 5-MIAA and its deuterated analogue, respectively.

Subjects. Ten normal adult subjects on a free diet collected 24-h urine samples, the urine being placed in a deep freeze immediately after each voiding. An oral load of 5-methoxytryptamine (1 mg) in water (100 ml) was taken in divided dose over 1 h by two normal adults (1 male, 1 female). No mental or systematic effects were noticed. Urine was collected for 24 h from the start of the load.

RESULTS AND DISCUSSION

The mass spectrum of the bis(trimethylsilyl) derivative of 5-MIAA (Fig. 1) is relatively simple with the major peaks being the molecular ion at *m/e* 349, the M—COOTMS ion at *m/e* 232 and low mass silyl fragments. The presence of 5-MIAA in urine without internal standard was shown by the simultaneous monitoring of fragments at *m/e* 202, 232, 306 and 349 in an extract. For quantitation the fragments at *m/e* 232 and 349 were used. The contributions at *m/e* 234 and 351 from the natural compound and the contribution at *m/e* 232 from the deuterated compound (corresponding to the fragment at *m/e* 230 in the spectrum of the unlabelled compound) were corrected for in the calculations.

In the two subjects who took 5-methoxytryptamine orally the urinary unconjugated 5-MIAA excretion over 24 h accounted for 53% and 71% of the administered dose. Thus 5-MIAA is a major metabolite of 5-methoxytryptamine in man and should give some indication of the turnover of this compound.

Although the 5-MIAA was always readily detectable (see Fig. 2), quantitative analysis of those extracts from urines containing the lowest concentrations of the compound (below about 10 ng/ml) presented some difficulties.

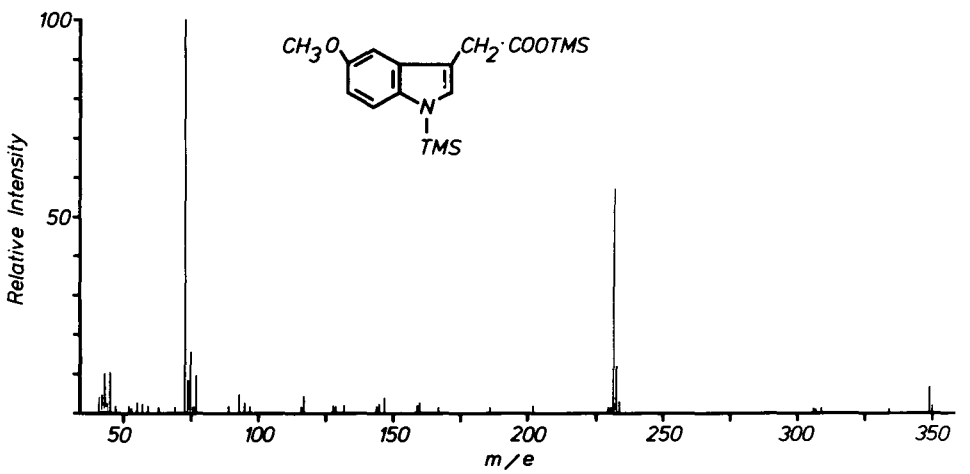

Fig. 1. Mass spectrum of the bis(trimethylsilyl) derivative of 5-methoxyindoleacetic acid.

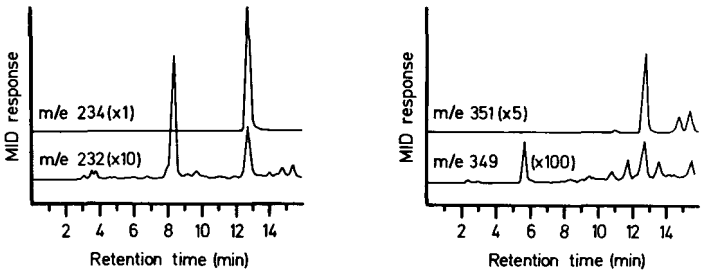

Fig. 2. Mass fragmentogram of a urine extract containing 5-($^2H_2$)-MIAA as an internal standard. The original urine contained the lowest concentration of 5-MIAA found (4.1 ng/ml; 7 μg per 24 h volume of 1700 ml). Amplifications of the MID response relative to *m/e* 234 = 1 are shown in parentheses.

There appeared to be no interference from other components of the extract with the internal standard fragments at *m/e* 351 and 234 and the ratio of these two fragments was fairly constant. However, the single ion profiles of the ions with *m/e* 232 and 349 appeared increasingly complex at the high amplifications necessary to detect the lowest concentrations of 5-MIAA. Interference from other components principally affected the ion at *m/e* 349 rather than the major fragment ion at *m/e* 232 largely because of the difference in their relative intensities. For this reason the concentration of 5-MIAA was generally calculated from the ratio of the ions at *m/e* 232 and 234. The situation was not significantly improved when a 20-m OV-1 glass capillary column was used for the chromatographic separation. The probability of interference also led us to prefer peak heights rather than peak areas for quantitation. Reproducibility of analyses on the GC phase, OV-17, was better than 1% but these results were lower by about 10% from those obtained using an OV-1 column: on this latter phase the peak due to 5-MIAA was often incompletely resolved from other product ions particularly at *m/e* 349. The results in the table were obtained using an OV-17 column.

The very wide range of 5-MIAA excretion in normal individuals suggests that at least in part this compound is of dietary origin. The lower part of this range may represent mainly endogenous production: six of the subjects excreted amounts of 5-MIAA in the range 7—18 μg per 24 h. This quantity of

TABLE I

5-METHOXYINDOLE-3-ACETIC ACID CONTENT OF URINE FROM NORMAL ADULTS (μg per 24 h)

Parameter	Value									
Sex	M	M	M	F	M	F	M	M	F	M
Age (y)	29	29	43	29	24	22	28	35	26	40
5-MIAA (μg)	56	151	9	11	13	11	7	34	18*	65*

*Approximate values calculated from *m/e* 349/351 only.

5-MIAA is about 0.003 that of 5-hydroxyindoleacetic acid excretion in normals, though allowing for some degree of conjugation, by analogy with indoleacetic acid itself [6], the overall turnover of 5-methoxytryptamine could be higher by a factor of up to two. In the rat hypothalamus the 5-methoxytryptamine content is 0.16 that of 5-hydroxytryptamine [1, 2] and a similar concentration would give the human hypothalamus a content of about 0.1 μg of 5-methoxytryptamine. 5-Methoxytryptamine is also present in the rat pineal [7] and by analogy, on a weight for weight basis, the human pineal might contain about 0.5 μg of this amine. Given a turnover time of an hour, this source alone could account for the majority of the urinary output of 5-MIAA in man. 5-MIAA is also a minor metabolite of melatonin [4, 8] and a major metabolite of 5-methoxytryptophol [9] in rats. In this context the demonstration of 5-methoxytryptophol in human cerebrospinal fluid [10] and the very ready methylation of 5-hydroxytryptophol by enzymes in the human pineal [11] are of interest. The lack of precise analytical data for the concentration and distribution of these compounds in human tissue precludes any very definite conclusions being drawn from these figures. However, it would appear that if the analogy with the rat is valid the endogenous production of 5-MIAA in man can be accounted for without involving 5-methoxytryptamine as a neurotransmitter except perhaps in minor and rather specialised roles.

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Note

High-performance liquid chromatographic analysis of biologically important porphyrins

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Determination of biologically important porphyrins and their quantitative analysis in tissues and body fluids plays a significant role in the precise classification of the various types of porphyria in clinical practice.

Until the present time, thin-layer chromatography has been considered the best laboratory separation method for the determination of these compounds in biological materials. It has usually been employed after extraction and methylation, with subsequent elution from the silica gel and spectrophotometric detection [1]. Though this procedure does not require any special instrumental equipment, the performance is usually lengthy and elution of the spots can cause experimental errors as well.

Therefore, high-performance liquid chromatography (HPLC) has recently been utilized for the quantitation of porphyrins, giving increased sensitivity and separation in a considerably shorter time. In all communications published so far, the resolution of porphyrins with 2—8 carboxylic groups has been carried out, after conversion to methyl esters, by adsorption chromatography on silica gel columns using gradient elution [2,3] or programmed flow-rate [4,5] with spectrophotometric detection. In the last paper, a considerable increase in sensitivity was achieved by use of a fluorescence detector [6].

A decrease in the precision of porphyrin analyses can occur, especially in

less concentrated samples, owing to spontaneous formation of copper (II) porphyrin chelates, observed in thin-layer chromatography [7] and HPLC [4] chromatograms and identified by mass spectrometry [4].

In this paper, a chemically bonded stationary phase was used with isocratic elution for the separation of porphyrin methyl esters which were converted to their copper (II) chelates with good chromatographic qualities and increased precision and sensitivity.

EXPERIMENTAL

Apparatus and conditions

A Varian (Palo Alto, Calif., U.S.A.) Model 8500 liquid chromatograph, with a pulseless piston pump, septumless injector and spectrophotometric detector (Variscan) operating at 400 and 402 nm, was used.

A stainless-steel column (25 cm × 0.2 cm I.D.) was packed with MicroPak CN, 10 μm (Varian), and the mobile phase, consisting of ethyl acetate — *n*-heptane — isopropanol (40:60:0.5), was pumped through at a flow-rate 50 or 80 ml/hour. Pressure, 70 kg/cm^2 ; chart speed 0.5 cm/min.

Chemicals

All solvents were of reagent-grade quality or for use in spectrophotometry. Standards of protoporphyrin-IX dimethyl ester, coproporphyrin-I tetramethyl ester and uroporphyrin-I octamethyl ester were obtained from Sigma (St. Louis, Mo., U.S.A.).

Sample preparation from urine

The method of Doss [8] was employed, with adsorption on talc, esterification with 5% sulphuric acid in methanol, and extraction with chloroform.

Preparation of cupric porphyrin chelates

The procedure of Doss [8] was modified as follows. A tenfold molar excess of the chelating agent (0.1% solution of copper (II) acetate in chloroform — methanol (19:1)) was added to porphyrins which had been isolated and esterified as described above. The sample was kept for 60 min at laboratory temperature and than evaporated to dryness. The residue was dissolved in a known volume of chloroform and injected into the column.

RESULTS AND DISCUSSION

Under the chromatographic conditions described abcve the mixture of porphyrin methyl esters with 2—8 carboxylic groups was successfully separated on a new column with polar chemically bonded stationary phase. This column did not require lengthy washing with the mobile phase in order to obtain a correct initial equilibrium, as is the case with silica gel columns. The period necessary for the resolution of porphyrins with 2—8 carboxylic groups using isocratic elution did not exceed 16 min (flow-rate 50 ml/h). The separation of porphyrins with 4—8 carboxylic groups required only 10 min (flow-rate 80 ml/h).

Fig. 1 shows the separation of five porphyrins occurring in a urine extract, the retention times of which increase with increasing number of carboxylic groups in the porphyrin molecule. Besides the five main elution peaks of the corresponding porphyrins, the chromatogram shows additional interfering peaks, with shorter elution times, which in some cases significantly reduce the precision of the quantitative evaluation. It is known from literature data [7] , and mass spectrometry has proved [4], that these small peaks belong to copper (II) complexes which result from the reaction of porphyrins with copper (II) ions contained in the chemicals used. Chloroform [4] and distilled water [7] used in the preparation of samples of clinical materials were stated as possible sources of these ions.

The interference of copper (II) complexes was low and virtually negligible in clinical samples that had been analysed immediately after extraction and methylation. In the case of a longer time interval (several days), or in samples with a low porphyrin content, the formation of complexes was manifested in a significant way. This was the reason for the additional treatment of the samples before the actual chromatographic analysis. Considering the fact that complexed methyl esters of porphyrins have similar chromatographic properties to free compounds, the methyl esters of porphyrins were purposely converted to their complexed analogues which were then chromatographed. Under the same chromatographic conditions, the mixture of porphyrin chelates with 4—8 carboxylic groups was entirely separated within a time interval that was even shorter than that of non-complexed compounds. Intentional conversion of free methyl esters of porphyrins to copper (II) chelates resulted at the same time in an increase in sensitivity of the analysis, since the millimolar absorption

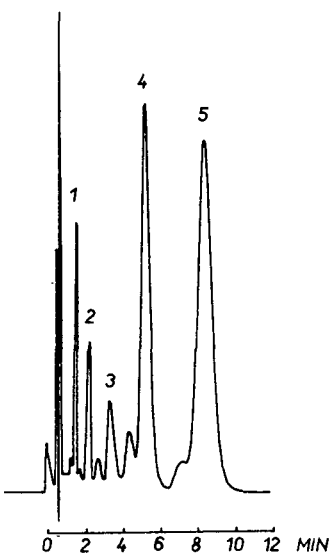

Fig. 1. Recording of a separation of porphyrin methyl esters from a urine sample of a porphyric patient. 1, coproporphyrin methyl ester; 2, pentacarboxylicporphyrin methyl ester; 3, hexacarboxylicporhyrin methyl ester; 4, heptacarboxylicporphyrin methyl ester; 5, uroporphyrin methyl ester. Detection at 402 nm.

coefficients of chelated porphyrins are on average more that 50% higher than non-complexed porphyrins [8].

Fig. 2 shows two chromatograms of a week-old extract of porphyric urine before (A) and after (B) conversion to copper (II) complexes. It is evident that chelation of the sample has yielded a better chromatogram with increased sensitivity of the analysis.

The above findings have been utilized in the detection and determination of porphyrins in clinical samples of skin of porphyric patients in which the porphyrin levels lie in the nanogram region [9].

Possible removal of contaminating trace amounts of metal ions and utilization of complexed porphyrins in the HPLC analysis clinical materials are the subject of our next studies.

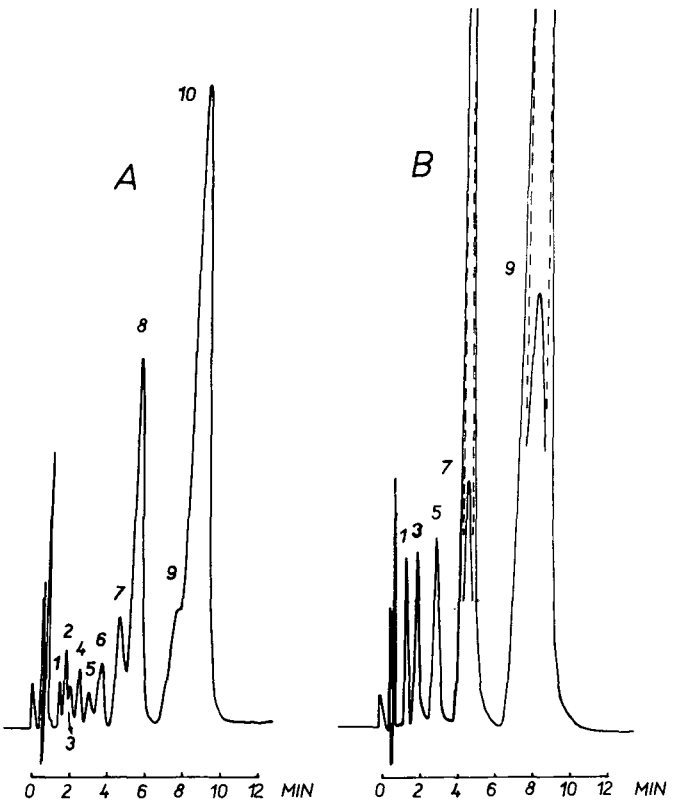

Fig. 2. Chromatogram of porphyrin methyl esters from a week-old sample of porphyric urine before (A) and after (B) conversion to cupric complexes. 1, cupric chelate of coproporphyrin methyl ester; 2, coproporphyrin methyl ester; 3, cupric chelate of pentacarboxylicporphyrin methyl ester; 4, pentacarboxylicporphyrin methyl ester; 5, cupric chelate of hexacarboxylicporphyrin methyl ester; 6, hexacarboxylicporphyrin methyl ester; 7, cupric chelate of heptacarboxylicporphyrin methyl ester; 8, heptacarboxylicporphyrin; 9, cupric chelate of uroporphyrin methyl ester; 10, uroporphyrin methyl ester. Detection at 400 nm.

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Biomedical Applications

CHROMBIO.124

Note

Application of paired-ion high-pressure liquid column chromatography to the analysis of L-3,4-dihydroxyphenylalanine metabolites

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Since their discovery in mammals, catecholamines and their metabolites have posed a considerable challenge to the analytical chemist with respect to both their chromatography and quantitation. Of the various techniques developed to measure catechols in biological fluids and tissue [1, 2], most single analytical methods permit quantitative analysis of only a few compounds. Yet the diversity of catechol metabolism dictates development of analytical methods with greater scope, and only gas—liquid chromatography (GLC) with detection by flame ionization [3, 4], electron capture [4—7] or mass spectrometry [6—8] has allowed simultaneous assay of a broad range of metabolites. Volatilization for GLC requires derivatization, a process which for this group of compounds often demands careful, time-consuming preliminary purification, and is prone to error due to the lability of the products obtained [3—8]. With the advent of high-pressure liquid chromatography (HPLC), it was apparent that the method would lend itself to the resolution of a range of underivatized catechols and their metabolites with a speed and efficiency comparable to that of GLC. This has been borne out in a number of studies. Liquid chromatography of catechols, as mixtures of standards or in biological fluids and tissues, has been accomplished using ion exchange [9, 10], reversed-phase columns [11], as well as by ion-pair liquid—liquid partitioning [12]. We wish to report a variation of the HPLC technique which affords the separation of a range of catechols and their methyl ethers on octadecylsilica reversed-phase columns by paired-ion chromatogrpahy [13]. Pretreatment on alumina, which permits fractionation of catechols from non-catechols [14], was not necessary for standard mixtures but facilitated urinary analyses by this method. Using UV spectrophotometric detection the technique proved to be a simple, rapid means of quantitative analysis of L-3,4-dihydroxyphenylalanine (L-DOPA) and its major metabolites in glusulase-hydrolyzed urine of L-DOPA-treated parkinsonian patients.

MATERIALS AND METHODS

All catechols and their methyl ethers were purchased from Sigma (St. Louis, Mo., U.S.A.) with the exception of 3,4-dihydroxyphenylpyruvate, which was synthesized from L-DOPA [15]. 1-Heptanesulfonic acid (HSA) was obtained from Fischer Scientific (St. Louis, Mo., U.S.A.) or Waters Assoc. (Milford, Mass., U.S.A.) whereas alumina (neutral, EM Reagents) was purchased from Brinkmann Instruments (Des Plaines, Ill., U.S.A.). Glusulase is a product of Calbiochem (San Diego, Calif., U.S.A.). All other chemicals and solvents were reagent grade quality. All solvents were redistilled and, if used in the HPLC system, were passed through a 0.2-μm filter.

High-pressure liquid chromatography

The Waters Assoc. HPLC system used included a Model 6000A solvent delivery system, a Model U6K loop injector and a Model 440 absorbance detector which was operated at 280 nm. The column, also from Waters Assoc., was a 30 cm × 3.9 mm μBondapak C_{18} column (octadecylsilane chemically bonded to a silica solid support, 10-μm particle size range). All chromatograms were obtained at ambient temperatures.

Urinary analysis

Urine specimens (24 h) were collected from six male parkinsonian patients on Sinemet (0.3—0.8 g of L-DOPA and 30—80 mg of carbidopa, a peripheral decarboxylase inhibitor, per day), two female and one male parkinsonian patients on L-DOPA alone (1.5—4.0 g of L-DOPA per day) and two male parkinsonian patients who were neither receiving L-DOPA nor Sinemet. Control urine samples were obtained from five male laboratory workers. Urine was collected in specimen bottles which contained either 5 ml of 6 *M* hydrochloric acid or 10 g of sodium metabisulfite and were immediately stored at −20°.

To 1—3-ml aliquots of urine were added 0.1 volumes of 0.1 *M* sodium acetate buffer containing EDTA and ascorbate both at a final concentration of 3 m*M*. This solution, which was adjusted to a pH of 5.2, was incubated with 0.2 ml of glusulase (1.34 I.U. of glucuronidase and 2.07 I.U. of sulfatase) for 20 h at 37°. The sample was then made 0.4 *M* in perchloric acid and sodium metabisulfite was added to a final concentration of 50 m*M*. After shaking for 1 min, the mixture was centrifuged at 13,000 rpm for 15 min in a Sorvall SM-24 rotor at 4°. Upon cooling the supernatant to 0° in an ice bucket, 3 *M* potassium carbonate was added to bring its pH to 5. It was then centrifuged at 5000 rpm for 5 min at 4°. To the supernatant were added 100—300 mg of alumina (depending upon the initial volume of urine), 50 mg of sodium metabisulfite, and 1 ml of 0.15 *M* EDTA. After adjusting the pH of the slurry to 8.6 with 1 *M* sodium hydroxide, it was shaken for 3 min. The slurry was then placed onto a column (disposable Pasteur pipet) of 100—300 mg of alumina which had been pre-equilibrated with 0.2 *M* sodium acetate buffer, pH 8.6, containing 3 m*M* EDTA. After washing the column with 4 ml of the same buffer to remove non-catechols, the catechols were eluted with a 1 *M* hydrochloric acid solution containing 0.05 *M* sodium metabisulfite. The non-catechol and catechol fractions were dried and taken up in 1% aqueous acetic acid and passed through a 0.2-μm filter for HPLC analysis.

Data treatment

Metabolite levels were determined by peak height analysis using authentic compounds for comparison. Recovery of [^{3}H] dopamine (^{3}H-DA) from the alumina—HPLC method was 59.6 ± 1.3% in seven determinations. Using non-radioactive authentic standards the following recovery values were estimated: DA, 58%; DOPA, 55%; 3,4-dihydroxyphenylacetic acid (DOPAC), 76%; homovanillic acid (HVA), 83%; vanillyllactic acid (VLA), 62% and 3-methoxy-4-hydroxyphenylalanine (MDOPA), 41%.

RESULTS AND DISCUSSION

Elution profiles of a mixture of L-DOPA metabolites in the absence and presence of HSA are illustrated in Fig. 1. The change in the elution pattern reflects the interaction of the counter-ion with amino groups of the catechol derivatives to form hydrophobic ion pairs. Both the loss of charge and the introduction of the heptane group of HSA enhance the retention of the ion pair on the non-polar stationary phase (C_{18} hydrocarbon) of the μBondapak column, thereby increasing retention times of compounds with amino groups. For the group of catecholamines, amino acids and methyl ethers examined the retardation effect increased with retention time. As seen in Fig. 1, metab-

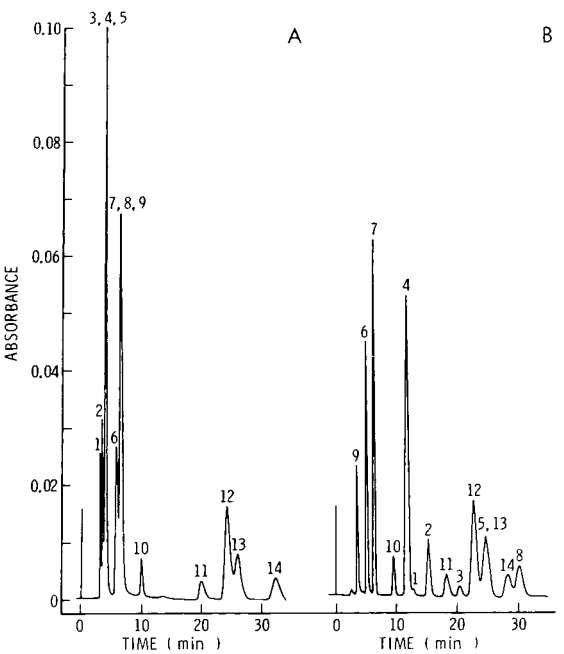

Fig. 1. Chromatogram of a standard mixture of L-DOPA metabolites in the absence (A) and presence (B) of 5 m*M* HSA. Column, μBondapak; mobile phase, water—methanol—acetic acid (95:5:0.95); flow-rate, 1.0 ml/min; inlet pressure, ca. 1000 p.s.i. Peaks: 1, norepinephrine; 2, epinephrine; 3, normetanephrine; 4, DOPA; 5, DA; 6, vanillylmandelic acid; 7, 3-methoxy-4-hydroxyphenylglycol; 8, MDOPA; 9, DHPP; 10, DOPAC; 11, VLA; 12, HVA; 13, vanillic acid (VA); 14, isovanillic acid (3-hydroxy-4-methoxy-benzoic acid). PHP is not shown; if present it would have had retention times of ca. 7 and 4 min in A and B, respectively.

TABLE I

URINARY LEVELS (MEAN ± S.E.) OF L-DOPA METABOLITES IN CONTROLS AND PARKINSONIAN PATIENTS

Urine samples were collected from controls and parkinsonian patients on drug regimens described in *Urinary analysis.* ND=not detectable.

Drug regimen	Number of subjects	Excretion (mg/24 h)						
		DOPA	DA	VLA	MDOPA	DOPAC	HVA	VA
Controls	5	1.51±0.61	0.98± 0.2	ND	ND	3.2 ±0.11	9.36± 1.0	3.3±1.06**
Sinemet	6	25.7 ±2.2	15.2 ± 3.3	23 ±6.2*	35.8±6.1	18.7 ±1.7	54.9 ± 8.6	10 ±3.5
L-DOPA	3	11 ±2.3	204 ±66	6.25±2.4*	129.2±7.8	623 ±30	774 ±91	4.6±1.8
None	2	0.39±0.11	0.66± 0.18	ND	ND	0.98± 0.02	8.1 ± 3.9	3.9±0.3

*$P < 0.1$.

**VA levels were measured on only three of the controls.

olites which do not contain amino groups were only slightly affected by addition of HSA. Two exceptions were *p*-hydroxyphenylpyruvate and 3,4-dihydroxyphenylpyruvate (DHPP) (Fig. 1), both of which exhibited two major peaks in all chromatograms. In both cases only the peak which had the shorter retention time exhibited a decrease in retention time when HSA was included in the mobile phase ($\triangle t$=2.8 and 2.6 min, respectively, in the system described in Fig. 1). The height of this same peak increased in the presence of HSA at the expense of the second peak (at 13 min in Fig. 1A). Since these phenomena were only observed with the pyruvates, it appeared that they might be attributable to keto—enol tautomerism. If so, the peak with the short retention time, which was more polar and predominated in the presence of 5 m*M* sodium borate, should be the enolate form. By measuring the change in absorbance of 330 nm it was ascertained that borate but not HSA shifts the equilibrium toward the enolate form [16]. Also HSA did not affect the pH of the mobile phase. Hence, a reason for the appreciable change in retention time of the pyruvates attributable to HSA is not apparent.

Despite the resolution obtained with standards, it was clear that given their complexity even hydrolyzed urine samples would require a prefractionation of free catechols from non-catechols by alumina chromatography [14]. As seen in Fig. 2, the combination of alumina and paired-ion reversed-phase HPLC allowed good resolution of the detectable metabolites present in a glusulase-hydrolyzed urine specimen from a Sinemet-treated patient. Using this technique urinary analyses were conducted for parkinsonian patients on L-DOPA

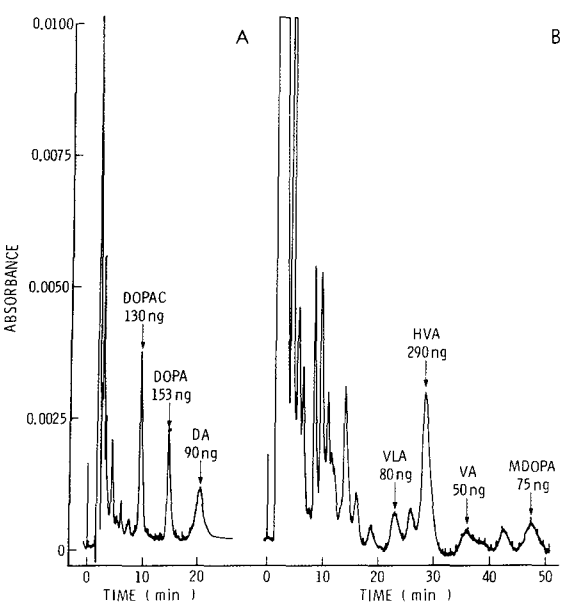

Fig. 2. Chromatogram of catechol (A) and non-catechol (B) fractions from a urine extract. Column, μBondapak; mobile phase, water—acetic acid (99:1) with 5 m*M* HSA; flow-rate, 1.5 ml/min; inlet pressure, ca. 1000 p.s.i. Results from a 3-ml urine sample from a parkinsonian patient on 0.44 g Sinemet per day.

or Sinemet as well as controls. Peak height measurements afforded values (Table I) which were comparable to amounts reported previously for urinary metabolites of parkinsonian patients on L-DOPA with or without a peripheral decarboxylase inhibitor taking into consideration variations in dosage [3, 17—19]. The changes in dopamine and vanillyllactic acid levels produced by the introduction of carbidopa confirmed the report of Sandler et al. [19] that this peripheral decarboxylase inhibitor shunts L-DOPA catabolism from decarboxylation to transamination.

The high molar absorptivity of catechol derivatives affords a sensitivity in the 10-ng range with the UV detector monitoring at 280 nm. Thus concentrations of most catechol metabolites in controls and non-treated parkinsonian patients were near the minimal level of detection (0.1 μg/ml of urine). The fact that neither DHPP nor 3-methoxy-4-hydroxyphenylpyruvate were detected in patients or controls under conditions in which added standards were recovered in 60% yields establishes that these compounds are not major urinary metabolites even when large doses of L-DOPA are administered.

The method described is a rapid, simple technique for analysis of the major metabolites of L-DOPA in urine of parkinsonian patients on L-DOPA chemotherapy. It would also be useful in other neurologic disorders in which L-DOPA metabolism is excessive [9]. Its adaptation to routine screening of normal urine, serum, and tissue samples awaits development of a detector with a sensitivity comparable to that of electron capture or mass fragmentography. Kissinger and co-workers [20—22] have demonstrated the excellent response of an electrochemical detector which is compatible with solvents used on reversed-phase columns [21]. It has proven useful for the measurement of hydrolyzed catechol levels (10—700 μg per 24 h) in normal unloaded urines [10] as well as in serum and brain [22, 23]. Although the electrochemical detector is more sensitive than UV spectrophotometry, it requires special sample pretreatment to eliminate electroactive contaminants and variable detector potential settings depending upon the types of catechol derivatives present [20—23]. Fluorimetric detectors have been used with HPLC instruments although in this case one must resort to derivatization with a fluorescent reagent. Fluorescamine has been reacted with the amino group of catecholamines, norepinephrine and dopamine, to yield a fluorescent derivative detectable at a lower limit of about 10 ng using HPLC with a fluorimetric detector [24, 25]. Optimal conditions for dansylation of the same catecholamines and separation of their dansylated derivatives by HPLC have also been reported [26].

A major advantage of HPLC over GLC is the ability to collect the eluate and analyze it further by more sensitive methods such as mass spectrometry. It has also been possible to study hepatic metabolism of DL-[7-^{3}H] norepinephrine by adding carrier metabolites to tissue homogenates, separating them by the HPLC technique described above, collecting the fractions and subjecting them to radioassay [27].

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Biomedical Applications

CHROMBIO. 101

Note

A study of serum folate by high-performance ion-exchange and ion-pair partition chromatography

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The term "folic acid" designates a family of related compounds that vary by functional group, degree of reduction, and number of glutamic acids. Identification of the various forms of folate in biological materials has been a problem due to inadequate separation techniques. Microbiological assays, although commonly used, are difficult to perform, are time-consuming, and show wide variation.

Standard N^5- and N^{10}-substituted pteroylglutamic and pteroylpolyglutamic acids have been separated by ion exchange with high-pressure liquid chromatography (HPLC) [1, 2], and a recent preliminary study described the use of this method to characterize polyglutamate folates from mammalian cells in culture [3]. To our knowledge however, HPLC techniques have not been used to study serum folate.

Pteroylglutamic acid is the compound used clinically to alleviate folic acid deficiency, but there is confusion concerning the identity of the folate present in the serum after the oral administration of this compound. The present work describes a preliminary study utilizing high-performance ion-exchange and ion-pair partition chromatography to characterize the predominant serum folate in subjects pretreated with pteroylglutamic acid.

MATERIALS AND METHODS

Crystalline pteroylglutamic acid, N^5-methyltetrahydrofolic acid, and dihydrofolic acid were purchased from Sigma, N^5-formyltetrahydrofolic acid was a product of ICN—K & K Labs., and [^{14}C]-N^5-methyltetrahydrofolic acid-barium salt was a product of Amersham-Searle. Standards were made in 0.5% sodium ascorbate.

Ion-exchange separations were performed with a potassium perchlorate gradient on a column (1 m × 2.1 mm I.D.) prepacked with Permaphase AAX (DuPont), Ion-pair partition chromatography was performed with a 0.05 *M* Pic A (Waters Assoc.) methanol—water (30:70) mobile phase on a column (30 cm × 4 mm I.D.) prepacked with μBondapak C_{18} (Waters Assoc.). The columns were attached to a Model 4100 liquid chromatograph (Varian) equipped with a gradient elution device and a 280-nm detector.

Serum samples were taken from volunteers who had received 0.169 mg/kg Folvite (Lederle) 4 h prior to the experiment. For ion-exchange separations, the serum was mixed with sodium ascorbate to a final concentration of 35.4 m*M*, autoclaved for 3 min, and centrifuged at 1000 × *g* for 5 min. Fifty microliters of the serum supernatant fraction were chromatographed in the presence and in the absence of 100 ng of [^{14}C]-N^5-methyltetrahydrofolic acid (0.0125 μCi). For ion-pair chromatography, the serum samples were mixed with 5 mg/ml ascorbic acid and filtered through a 0.45-μm Millipore filter (HAWPO 1300). Recovery experiments with standard folate did not show significant adsorption on Millipore filters of this pore dimension.

The eluted samples were collected in sodium ascorbate and assayed with *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043), and *Pediococcus cerevisiae* (ATCC 8081). The microbiological assays were performed to the methods of Herbert et al. [14] and Scott et al. [5]. Pteroylglutamic acid was used in standards curves for the *L. casei* and the *S. faecalis* assays; N^5-formyltetrahydrofolic acid was used in standard curves for the *P. cerevisiae* assay. Ascorbate assays were performed by the method of Bessey et al. [6].

In isotope experiments, the eluted samples were counted in a Beckman LS-100 scintillation counter in 15 ml of a scintillation cocktail which contained 4.5 g of 2,5-diphenyloxazole, 1.5 g of 1,4-bis-2-(5-phenyl-oxazolyl) benzene, 1.5 l of toluene, and 0.5 l of Triton X-100.

RESULTS AND DISCUSSION

Fig. 1A shows the elution pattern for the reference folate compounds separated by ion exchange. The large peak at the solvent front can be identified as the ascorbate which was added to the standard solutions to prevent oxidation. After the ascorbate was eluted, the $KClO_4$ gradient was started and all three reference compounds were separated. The chromatographic identities of N^5-formyltetrahydrofolic acid (peak I), N^5-methyltetrahydrofolic acid (peak II), and pteroylglutamic acid (peak III) were confirmed by microbiological assay of the eluted peak fractions.

Two major peaks were evident in the gradient-elution pattern of serum samples taken from subjects pretreated with folate (Fig. 1B). The first peak had a retention time similar to that of N^5-formyltetrahydrofolic acid, but the results of microbiological assays were not confirmatory. Only a small and variable growth response was observed with the three organisms. It has been demonstrated that autoclaving in a neutral environment leads to the chemical conversion of $N^{5\text{-}10}$-methenyltetrahydrofolate and N^{10}-formyltetrahydrofolate to N^5-formyltetrahydrofolate [7, 8]. If the serum contained significant quantities

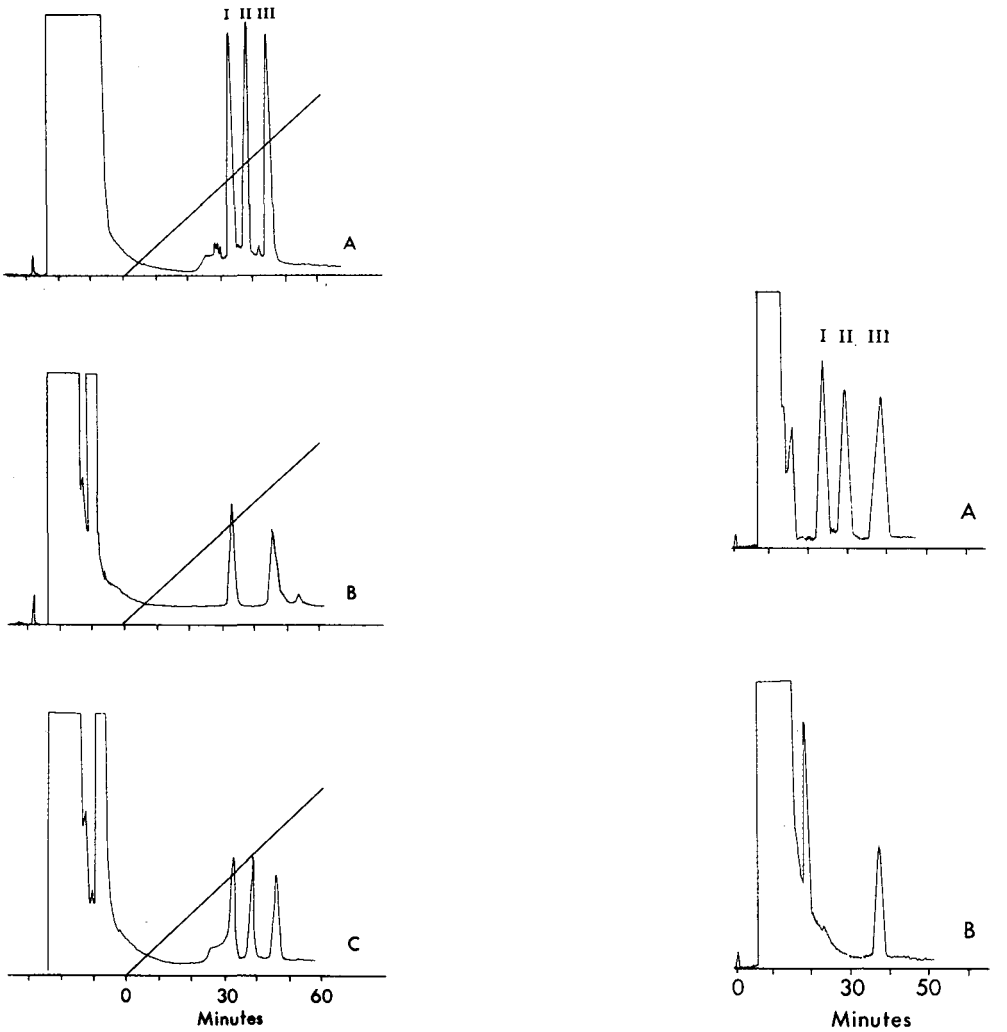

Fig. 1. Ion-exchange separations of folic acid compounds. Standards and serum samples were chromatographed on a Permaphase AAX column as described in Materials and methods. Conditions: mobile phase, potassium perchlorate gradient (0—0.006 *M* at 1%/min); flow-rate, 30 ml/h; column temperature, ambient; detector, UV photometer (280 nm). (A) Chromatography of reference compounds; I = N^5-formyltetrahydrofolic acid (100 ng); II = N^5-methyltetrahydrofolic acid (100 ng); III = pteroylglutamic acid (100 ng). (B) Chromatography of serum samples from subjects pretreated with folate. (C) Chromatography of serum samples to which [^{14}C]-N^5-methyltetrahydrofolic acid has been added.

Fig. 2. Ion-pair partition chromatography of folic acid compounds. Compounds were chromatographed on a μ Bondapak C_{18} column as described in Materials and methods. Conditions: mobile phase, 0.005 *M* Pic A reagent (pH 7.5) in meth[illegible]-water (30:70); flow-rate, 30 ml/h; column temperature, ambient; detector, UV photometer (280 nm). (A) Chromatography of reference compounds; I = dihydrofolic acid (100 ng); II = N^5-formyltetrahydrofolic acid (100 ng); III = pteroylglutamic acid or N^5-methyltetrahydrofolic acid (100 ng). (B) Chromatography of serum samples from subjects pretreated with folate.

of these convertible forms, the resultant N^5-formyltetrahydrofolate peak would have produced a greater growth response than we observed. The identity of this peak is still in question.

The second peak had a retention time similar to that of our pteroylglutamic acid standard and its identity as pteroylglutamic acid was confirmed by microbiological assay. The eluted material supported the growth of *L. casei* and of *S. faecalis*, but not of *P. cerevisiae*. To confirm that our material was pteroylglutamic acid and not N^5-methyltetrahydrofolic acid, a sample of [^{14}C]-N^5-methyltetrahydrofolic acid was added to serum samples prior to our chromatographic separation. The counts were recovered in the elution fraction representing a peak (Fig. 1C) which did not exist with serum alone.

In the course of our ion-exchange separations, we discovered that dihydrofolic acid eluted in a position identical to that of pteroylglutamic acid. Both folate forms support the growth of *L. casei* and *S. faecalis*. To confirm the identification of the serum peak as pteroylglutamic acid and not dihydrofolic acid, we chromatographed our materials on an ion-pair partition system.

Fig. 2A shows the elution pattern for dihydrofolic acid (peak I), N^5-formyltetrahydrofolic acid (peak II) and pteroylglutamic acid or N^5-methyltetrahydrofolic acid (peak III) in the partition system. N^5-Methyltetrahydrofolic acid and pteroylglutamic acid cannot be separated by this technique, but they can be differentiated by microbiological assays.

When the sera from the subjects pretreated with pteroylglutamic acid were chromatographed in this system, the results in Fig. 2B were obtained. The peak eluting after ascorbic acid has a retention time comparable to that of either N^5-methyltetrahydrofolic acid or pteroylglutamic acid, but not to that of dihydrofolic acid. Results from microbiological assays suggest that the material is pteroylglutamic acid and not N^5-methyltetrahydrofolic acid; the peak fraction supported the growth of *L. casei* and *S. faecalis* but not *P. cerevisiae*.

In summary, our experiments were designed to determine the feasibility of using HPLC techniques to study serum folate and to resolve the controversy concerning the identity of the folate appearing in the serum after the oral administration of pteroylglutamic acid. Some reports have suggested that pteroylglutamic acid is absorbed unchanged [9, 10, 11]; some suggest that it appears as N^5-methyltetrahydrofolic acid [12]; some have measured a combination of both pteroylglutamic acid and N^5-methyltetrahydrofolic acid [13, 14]; and some have suggested that there is a gradual conversion to N^5-methyltetrahydrofolic acid 3 h after the administration of folate [15, 16].

Our preliminary investigations show that liquid chromatographic techniques can be used to study serum folates after the oral administration of folic acid; our results with both ion-exchange and ion-pair partition chromatography suggest that most of the administered folate appears unchanged in the serum 4 h later. The presence of smaller amounts of other folate forms is not excluded since the lower limit of detection in our system is 3 ng per 50 μl.

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO. 105

Note

The diminution of the myelin ethanolamine plasmalogen in brain of the *Jimpy* mouse and brain and spinal cord of the *Quaking* mouse as visualized by thin-layer chromatography

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Within the nervous system of vertebrates it has been long established that the glycolipids are a characterizing component of myelin and are quantitatively related to the degree of myelination as demonstrated by morphological criteria. On the other hand the ethanolamine plasmalogens are both more ubiquitous in nature and uniquely abundant in the myelin sheath; they are histochemically demonstrated by the Feulgen plasmalogen reaction which is also used as the thin-layer chromatographic (TLC) spot test. We have recently shown [1] that the ethanolamine plasmalogens of brain, spinal cord and optic and sciatic nerves can be resolved by TLC into two components, only one of which is peculiar to myelin.

The present report describes our observations on brain and spinal cord of the myelin-deficient mouse mutants *Jimpy* and *Quaking* and may serve to explain the observations of others with regard to the diminution of plasmalogens in the brains of these mutants [2, 3, 4]. Similar plasmalogen diminution has been reported for the demyelination of multiple sclerosis [5] and in Wallerian degeneration of rabbit sciatic nerve [6].

MATERIAL AND METHODS

Normal Swiss mice were used to serve as a base-line for interpreting the observations on the *Jimpy* and *Quaking* mutants. This normal series consisted

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of adult, new-born, 15-, 20-, 25- and 32-day post-natal specimens. The myelin-deficient mouse mutants, all males, consisted of 6 each 17-day *Jimpy* and litter-mate controls and 2 each 40- and 50-day, respectively, *Quaking* and litter-mate controls. The *Jimpy* and *Quaking* series were obtained directly from the Jackson Laboratory (Bar Harbor, Maine, U.S.A.). The mice were killed by exsanguination, the spinal cord cut at the level of the foramen magnum and the brains removed, frozen with dry ice and lyophilized; the spinal cords of the *Quaking* series were also removed, frozen and lyophilized. Chloroform—methanol (2:1, v/v) extracts were made of each sample with a ratio of 5 ml solvent per 100 mg dry weight of tissue. The chromatographic analysis of the phosphatides and glycolipids was done as explained elsewhere [1], mainly using Schleicher & Schuell F-1500 silica gel thin-layer plastic sheets and the solvent system CEW (chloroform—ethanol—water, 65:25:3).

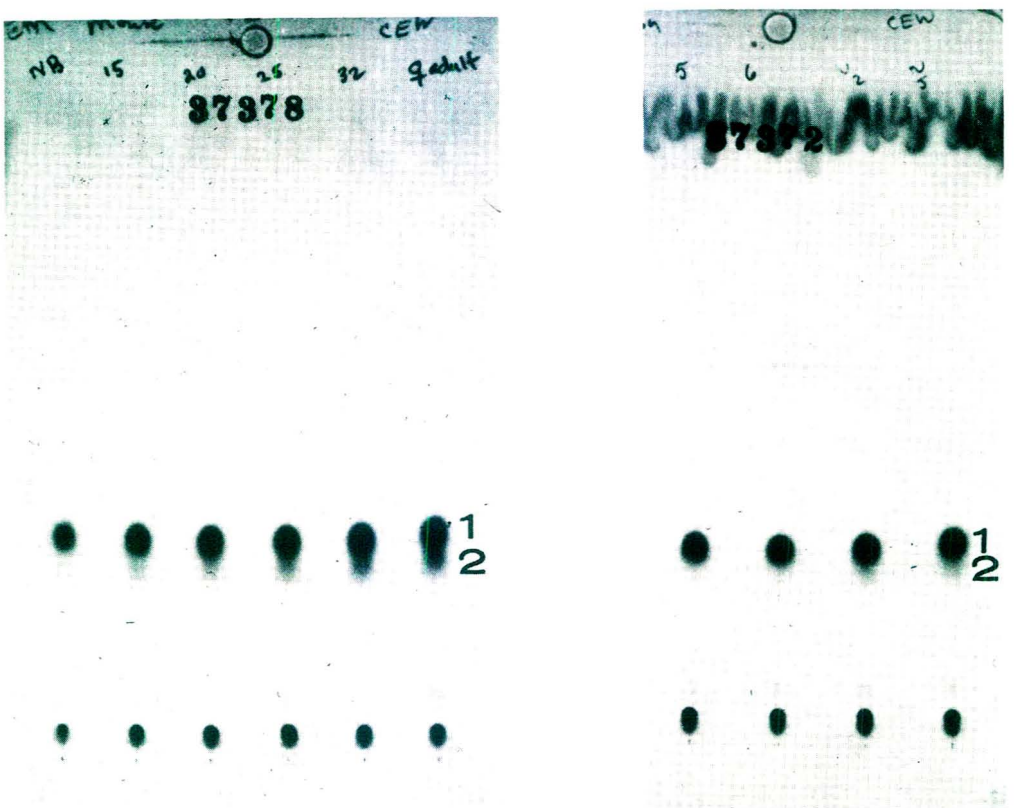

Fig. 1. Brain of normal mice; from left to right: new-born, 15-, 20-, 25- and 32-day post-natal, adult. E. Merck silica gel plastic back sheet with chromatographic development in CEW (see text) and stained by immersion in the Feulgen plasmalogen reagents. The major phosphatide in all specimens is PE-1 (1), mainly as plasmalogen, with PE-2 (2) clearly increasing with age.

Fig. 2. Same chromatographic and staining conditions as Fig. 1; brain of 17-day *Jimpy* (left pair) and litter-mate controls (right pair) showing the increased PE-2 (2) in the controls. The massive solvent front is largely derived from material within the silica gel plate itself.

Validation of the myelin phosphatide was accomplished by co-chromatography with both commercial PE* samples of known purity and the silicic acid column PE-1, PE-2 isolates described earlier [1]. Recognition of the amine component was by use of the fluorescent amine reagent fluorescamine (Roche, Nutley, N.J., U.S.A.) and of the alk-1-enyl (plasmalogen) component by the Schiff leuco-fuchsin reagent. Recognition of the accompanying lipids was readily accomplished by their Rhodamine 6G stainability.

RESULTS

Fig. 1 shows the progressive appearance, in mouse brain, of the characteristic myelin ethanolamine plasmalogen species, PE-2, with increasing post-natal age. Fig. 2 compares two brain specimens each of *Jimpy* and their litter-mate controls revealing the near absence of PE-2 in the myelin-deficient animals and diminished PE-1. Figs. 3a and 3b show the more marked PE-2 differences in the *Quaking* mouse brain, 40 and 50 days post-natal, as compared to 17 days

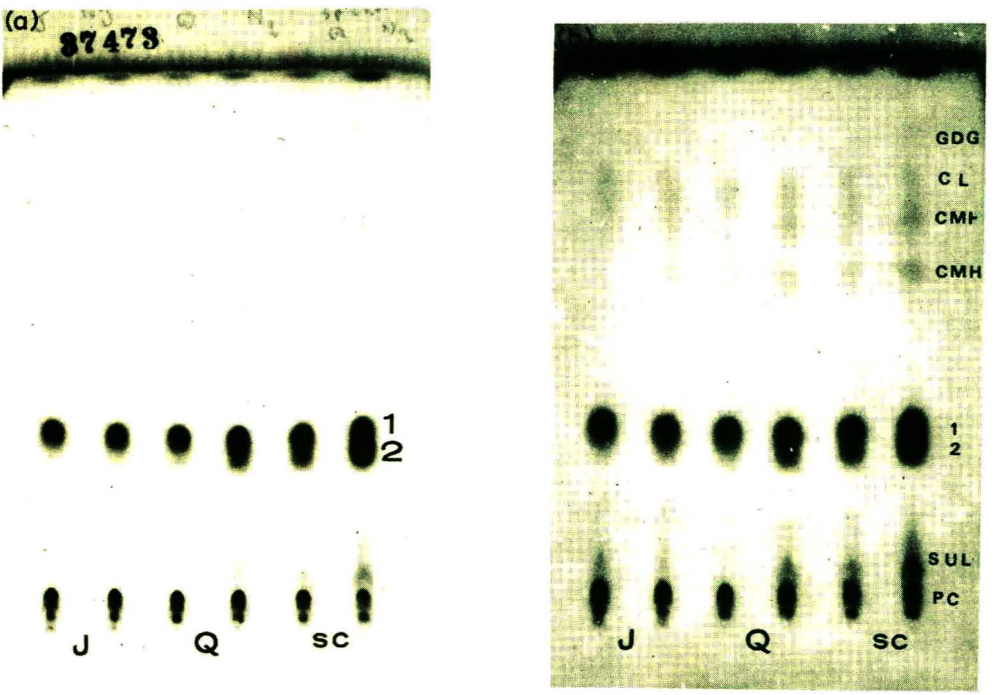

Fig. 3. (a) Schleicher & Schuell F-1500 chromatogram with CEW development of (left to right) *Jimpy* brain and control (J), *Quaking* 40-day brain and control (Q), and *Quaking* 40-day spinal cord and control (sc). The plamalogen reaction stains the PE-1 (1) and PE-2 (2) and the Rhodamine 6G counterstaining reveals the glycolipids and various other phosphatides. (b) The Rhodamine spots are much more visible while the chromatogram is still wet after staining.

*Abbrevations: PE-1 and PE-2 refer to the two phosphatidyl ethanolamine plasmalogens chromatographically resolved from lipids of myelinated nerve tissue. *n*-CMH and *h*-CMH are the major nerve tissue ceramide monohexosides. GDG is galactose diglyceride and CL is the mitochondrial phosphatide cardiolipin.

for *Jimpy*, and in the spinal cord samples of the *Quaking* series. The 50-day *Quaking* is approximately at the myelination level of the 17-day *Jimpy* littermate control. It is clear from fig. 3 that spinal cord samples are to be preferred for this demonstration because of their greater proportion of myelin as compared to the whole brain. Fig. 3b (wet chromatogram) shows more clearly the diminished sulfatide, *n*-CMH, *h*-CMH and GDG of the myelin-deficient specimens as well as what may be ester-CMH (cf. ref. 7). In the CEW chromatographic system CL and *n*-CMH coincide thereby distorting the interpretation of the relative amounts of *n*- and *h*-CMH. Replacement of some of the water with NH_4OH corrects this overlapping by selectively retarding the migration of CL allowing the two major CMH species to be more clearly expressed. The clear identity of the ester-CMH needs to be yet established so that a correct interpretation can be made as to its relationship with the myelination process; similarly the place GDG has in this over-all scheme needs to be more clearly determined.

As described earlier [1], and confirmed here, PE-1 more readily reduced OsO_4 than did PE-2 and therefore represented the more unsaturated molecular species. The specific cleavage by $HgCl_2$ is characteristic of the alk-l-enyl linkage and forms the basis for the plasmalogen reaction with the Schiff leuco-fuchsin reagent.

DISCUSSION

We have interpreted our earlier studies [1] to indicate that the major difference between PE-1 and PE-2 plasmalogen is that the PE-2 seems to be largely 18:1 with respect to both the 1-alk-1′-enyl and 2-acyl groups. Its diminution in the myelin-deficient brain and spinal cord, then, would be generally consistent with current notions [8, 9, 10] that the metabolic defect of *Jimpy* and *Quaking* mice affects one or more enzymes of fatty acid synthetic pathways within the oligodendroglia. The demyelination of multiple sclerosis and Wallerian degeneration and associated loss of plasmalogen (presumably mainly PE-2) would of course have a different explanation relating rather to the damage of the oligodendrocyte itself (or only of its plasma membrane) with subsequent lytic activity occurring. The products of this lysis could show the sequence of the plasmalogen degradation and whether or not the response is different for PE-1 than for PE-2.

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Biomedical Applications

CHROMBIO.119

Note

An improved gas—liquid chromatographic assay for 5-fluorouracil in plasma

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5-Fluorouracil plays an important role in the chemotherapeutic management of certain forms of cancer [1]. Detailed pharmacokinetic studies could provide the clinician with information necessary to devise optimal drug schedules, combining maximum therapeutic response with minimum toxicity. Such studies require a rapid, specific and sensitive method of drug assay. The spectrophotometric assay for 5-fluorouracil lacks specificity and sensitivity [2], whilst the microbiological method lacks accuracy and is unsuitable for large numbers of samples [2].

In recent years three gas—liquid chromatographic (GLC) assay methods for 5-fluorouracil have been described. Two of these techniques [3, 4] were based on detection of a silyl derivative of the drug using a flame ionisation detector. A third method by Rao et al. [5] entailed flash-methylation of 5-fluorouracil followed by GLC separation and flame ionisation detection.

The sensitivity of all these methods was of the order of 1 μg 5-fluorouracil per ml of plasma. Recent pharmacokinetic studies in our own laboratory have shown that a more sensitive assay was required. We therefore investigated the possibility of preparing a derivative of 5-fluorouracil which would be sensitive to electron-capture detection. In this paper we wish to report a GLC assay method which is capable of detecting 10 pg 5-fluorouracil as a chloromethyldimethylsilyl derivative. This technique results in a lower limit of detection of 5-fluorouracil in plasma of 20 ng/ml.

MATERIALS AND METHODS

Gas—liquid chromatography

GLC was carried out using a Pye Unicam GCV chromatograph equipped with a ^{63}Ni electron-capture detector (Pye-Unicam, Cambridge, Great Britain). The column was a glass tube, 213 cm × 4 mm I.D., packed with 80—100 mesh Chromosorb W HP coated with 3% OV-1, (Pierce, Rockford, Ill., U.S.A.). Operating conditions were: column oven temperature, 230°; injection port temperature, 230°; electron-capture detector temperature 280°. Nitrogen was

used as the carrier gas at a flow-rate of 40 ml/min. The column was conditioned for 16 h before use at the operating temperature.

Extraction of 5-fluorouracil from plasma

5-fluorouracil (Sigma, St. Louis, Mo., U.S.A.) was extracted from 1-ml plasma samples with 15 ml of a solution of 16% *n*-propanol in ether according to the method of Cohen and Brennan [4]. The extract (0.5 to 7.5 ml depending on the concentration of 5-fluorouracil in the plasma) was evaporated to dryness in sample tubes under a gentle stream of nitrogen. Standards were prepared by evaporating aqueous solutions (50 μl) of the drug. Thymine (Calbiochem, San Diego, Calif., U.S.A.) was used as an international standard. Depending upon the anticipated concentration of 5-fluorouracil, 50 or 625 ng thymine in aqueous solution (50 μl) was added to each sample tube. The water was removed by evaporation prior to derivatization of 5-fluorouracil and internal standard.

Derivatization

1,3-Bis (chloromethyltetramethyldisilazane) (100μl) and chloromethyldimethylchlorosilane (50μl) (Pierce and Warriner, Chester, Great Britain), was added to the evaporated sample. The reaction mixture was heated at 75° for 10 min in a water bath. The tubes were cooled to room temperature and 2 ml redistilled spectroscopic-grade ethyl acetate (Koch-Light, Colnbrook, Great Britain), was then added. Addition of this solvent was necessary in order to prevent saturation of the detector by chlorinated reaction by-products. The resultant precipitate of ammonium chloride was removed by centrifugation and 1 μl of the supernatant was injected into the chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of derivatized extracts of plasma and of plasma-containing 1.25-μg/ml 5-fluorouracil. In agreement with Cohen and Brennan [4] we have found that 80—85% of 5-fluorouracil added to plasma is recovered in a single extraction with 16% *n*-propanol—ether. The peak corresponding to the derivative of 5-fluorouracil has a retention time of 9.4 min and the internal standard, thymine, emerges 10.0 min after injection. Although thymine is not well separated from 5-fluorouracil under the conditions used, reproducible calibration curves based on peak height ratios are obtained. No interfering peaks arising from endogenous plasma components or reaction by products were observed. A linear relationship between 5-fluorouracil concentration and peak height ratio was obtained provided the amount of 5-fluorouracil injected into the chromatograph fell within the range of 10—1000 pg. The standard deviation of the mean peak height ratio for a given sample was less than 3% of the mean within a run and less than 7% of the mean between runs.

We have been using this technique to monitor plasma levels of 5-fluorouracil in patients undergoing chemotherapy for breast cancer and the results of these studies will be reported separately. Although we have found thymine to be an acceptable internal standard it would clearly be inappropriate in studies directed towards quantitative analysis of 5-fluorouracil in biological specimens containing the naturally occuring pyrimidine.

The method described in this paper allows quantitative analysis of a wide range of drug concentrations in plasma, with a lower limit of detection of

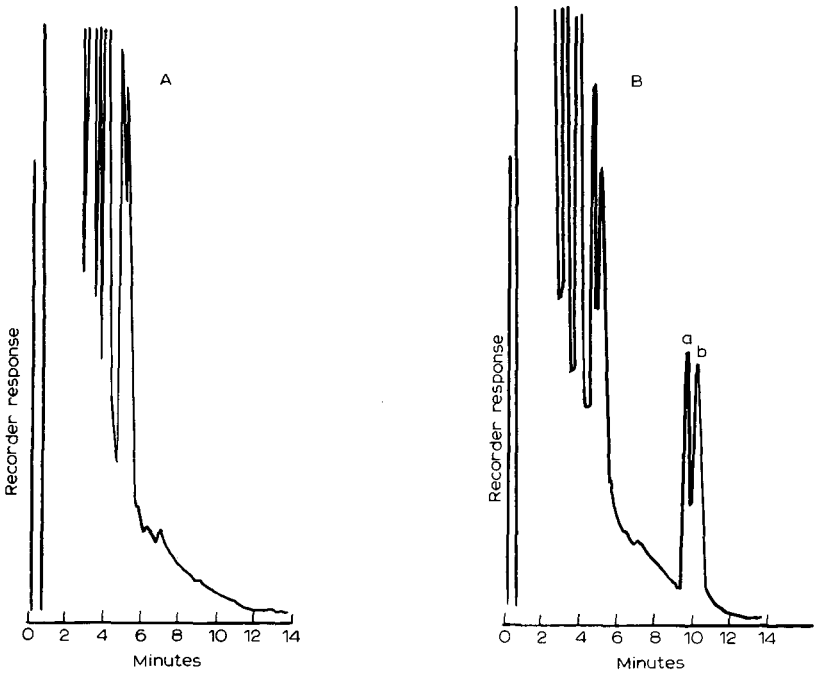

Fig. 1. Gas chromatograms of plasma extracts. A, Plasma blank; B, plasma containing 1.25 μg/ml 5-fluorouracil (peak a), with 625 ng thymine as internal standard (peak b). 7.5 ml of the *n*-propanol—ether extract was evaporated prior to derivatization.

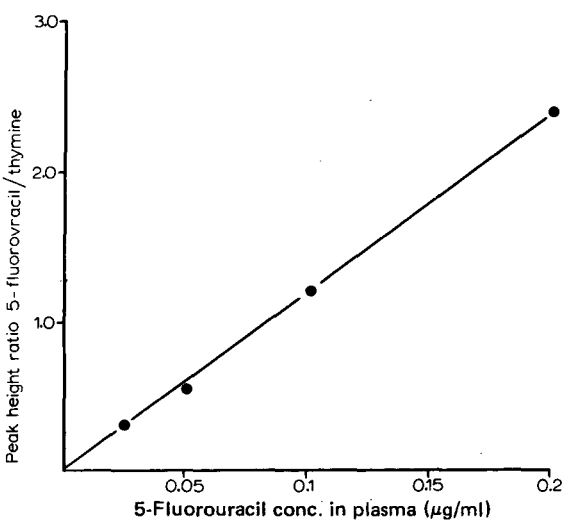

Fig. 2. Calibration curve for 5-fluorouracil extracted from serum at low 5-fluorouracil concentrations. 7.5 ml of the *n*-propanol—ether extract was evaporated prior to derivatization and the final reaction mixture contained 50 ng thymine as the internal standard.

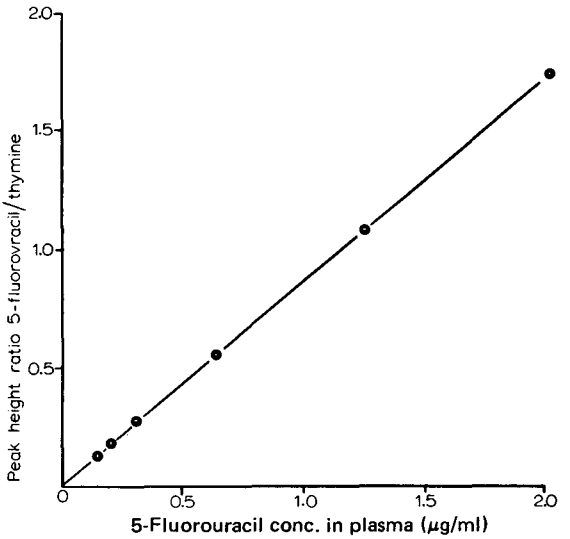

Fig. 3. Calibration curve for 5-fluorouracil extracted from serum over a wide concentration range. 7.5 ml of the *n*-propanol—ether extract was evaporated prior to derivatization and the final reaction mixture contained 625 ng thymine as the internal standard.

20 ng 5-fluorouracil per ml of plasma. The volumes of *n*-propanol—ether extract evaporated and ethyl acetate added prior to sample injection can be adjusted so that the amount of the 5-fluorouracil derivative injected into the chromatograph falls within the range of 10—1000 pg, for which linear detection response is obtained. The sensitivity afforded by the technique described has a number of important practical advantages over pre-existing GLC methods. For most analyses the amount of endogenous plasma material or reaction by products injected into the chromatograph can be kept to a minimum by the appropriate dilutions described. This greatly facilitates quantitative assay and reduces the incidence of detector contamination, a severe problem with earlier methods based on detection of the silyl derivative of 5-fluorouracil [3, 4]. In addition the method could be adapted to allow 5-fluorouracil measurement in plasma volumes of less than 1 ml, an important consideration in many clinical situations.

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Biomedical Applications

CHROMBIO. 102

Note

Simple and sensitive method for the determination of cyclophosphamide by means of a nitrogen—phosphorus-selective detector

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(First received March 29th, 1977; revised manuscript received July 4th, 1977)

Cyclophosphamide (Cy) (Fig. 1) is widely used as an anti-tumoral agent in various clinical protocols [1]. The need for information on the bioavailability of this compound makes it important to have at our disposal sensitive analytical methods for measuring Cy plasma levels. Pantarotto et al. [2] have published a Gas Chromatographic (GC) method based on derivatization of the drug with trifluoroactive anhydride. Although this method satisfies the need for high sensitivity, apart from derivatization problems, it also calls for an expensive electron capture detector.

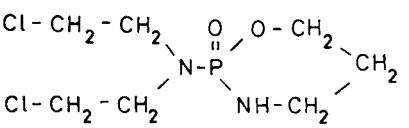

Fig. 1. Structure of cyclophosphamide.

In this paper we present a GC method using a nitrogen—phosphorus-selective detector (NPSD) which can detect as little as 10 ng/ml of the compound without any derivatization. It is possible to measure plasma levels of patients treated with a single intravenous dose of 100 mg of Cy.

MATERIALS AND METHODS

Chemicals

Cyclophosphamide, as the hydrate salt, was kindly donated by the Chester Beatty Institute (London, Great Britain). Imipramine, used as internal stan-

dard, was obtained from Ciba-Geigy (Milan, Italy). All reagents were of the highest purity grade.

GC conditions

GC analysis was carried out with a Fractovap 2300 apparatus (Carlo Erba) equipped with an NPSD (Carlo Erba). A glass column, 2 m × 4 mm I.D. packed with 100—120 mesh Gas-Chrom Q coated with 3% OV-17, was used. The standard operation conditions were: column oven temperature 250°, injector temperature 300°, detector temperature 300°. The gas flow-rates were: nitrogen 40 ml/min; hydrogen, 35 ml/min; air, 220 ml/min.

Extraction procedure from plasma and urine

Plasma and urine were extracted twice with ethyl acetate in the ratio 1:5 (v/v). The solvent phases were collected and evaporated to dryness in a water-bath at 40° under a gentle stream of nitrogen. The samples were submitted to GC analysis adding the necessary volume of ethanol containing 20 μg/ml of imipramine, as internal standard. After the samples were taken up, 1 μl of this solution was injected into the gas chromatograph.

RESULTS

Fig. 2 shows the chromatogram of a plasma blank and of a sample containing

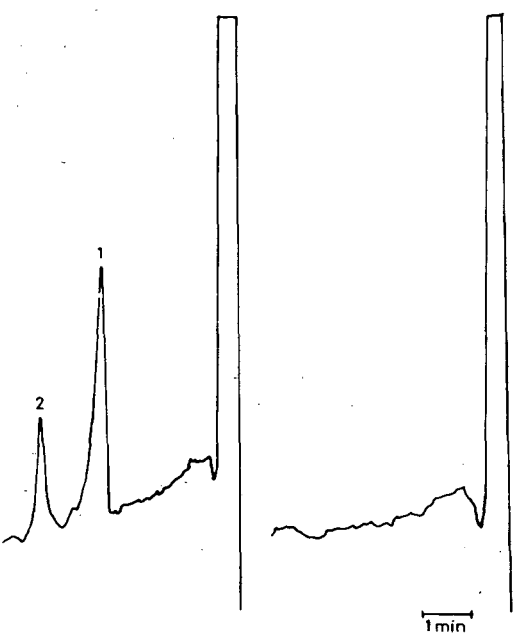

Fig. 2. Gas chromatogram of a blank from plasma (right) and of a biological sample (left) containing 3 μg/ml cyclophosphamide together with 20 μg/ml of the internal standard, imipramine. 1, Cyclophosphamide; 2, imipramine.

about 3 μg/ml of Cy. It is noteworthy that urine extracted with ethyl acetate caused no important peak disturbance in the analysis.

Calibration curves showed a linear response from 0.05 to 0.50 μg/ml. At a concentration of 0.1—10 μg/ml the recovery of Cy from plasma and urine was about 88%. The extractions were carried out on 1 ml of plasma, and on 10 ml of urine.

Fig. 3 shows the disappearance curves of cyclophosphamide from the plasma of three patients after intravenous injection of 100 mg of the drug. Concentration vs. time fit well in a bioexponential function. Analysis of regression shows a high significance ($P < 0.01$).

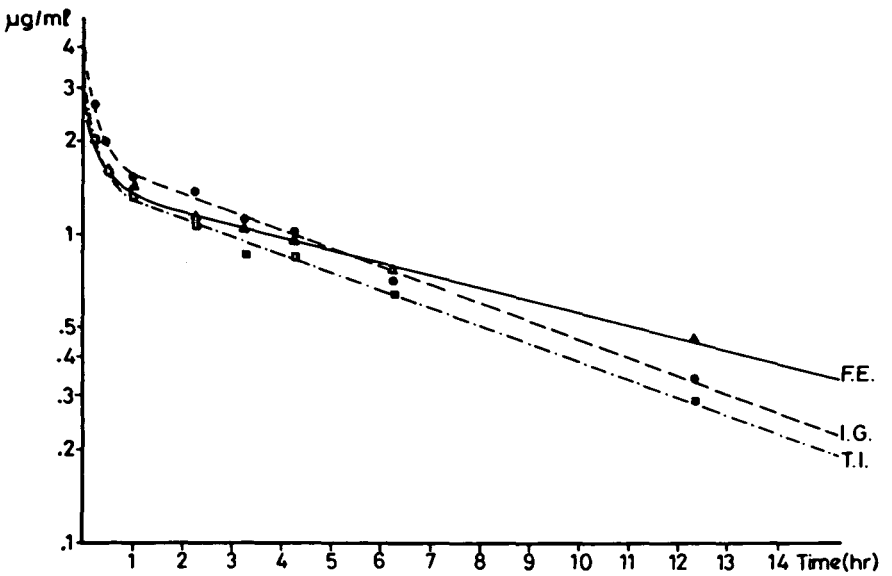

Fig. 3. Plasma levels of cyclophosphamide (μg/ml) in three patients after a single intravenous injection of 100 mg of the drug.

DISCUSSION

To date, the NPSD has mainly been employed to determine organophosphorus pesticides, although it has also been widely used for measuring the levels of some anti-epileptic drugs [3—5]. Its major feature, selectivity for nitrogen and phosphorus, helps overcome one of the biggest problems of quantitative assay: sample cleaness. From a general point of view, extraction with a polar solvent, such as ethyl acetate, from plasma and especially from urine may produce interfering peaks during the GC analysis. With our experimental conditions, however, we were able simultaneously to achieve high recovery of the drug and clean biological samples. Furthermore, the simplicity of the extraction and analytical methods makes it possible to perform about 30—40 determinations per day.

Table I shows the pharmacokinetic parameters in the three patients studied. The mean half-life is 5.87 ± 0.74 h; the volume of distribution (V_d) is 62.2 ± 4.3 l. Total body clearance (ClB = $V_d \times \beta$)* is 124.5 ± 11.1 ml/min. These values are in good agreement with those described by other investigators using ^{14}C-labelled Cy [6,7].

Study of the mechanism of action of Cy depends on our knowing the levels of some of its metabolites, which are the true active compounds [8—10]. However, these must be derivatized in advance to permit their detection. It is also important to note here that most reagents available for derivatization contain one or more nitrogen atoms which saturate the detector since they are generally added in large excess. Studies are in progress in our laboratory in order to stabilize some of the Cy metabolites and evaluate then quantitatively by means of the NPSD, since we believe that this detector may be a useful tool.

TABLE I

PHARMACOKINETIC PARAMETERS OF CYCLOPHOSPHAMIDE IN THREE PATIENTS

Each patient was treated once (intravenously) with 100 mg cyclophosphamide.

Patient	Half-life	V_d (l)	ClB (ml/min)
F.E.	7.36	68	106.5
I.G.	5.08	53.7	122.1
T.I.	5.18	65.1	145.0
Mean ± S.E.	5.87 ± 0.74	62.2 ± 4.3	124.5 ± 11.1

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*β= Final slope of the biphasic plot based on the equation for a two-compartment model.

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Note

Quantitative assay of rifampicin and three of its metabolites in human plasma, urine and saliva by high-performance liquid chromatography

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Rifampicin (R) is partly metabolised in man, its principal metabolite being 25-desacetylrifampicin (DR) [1]. It was recently reported [2] that rifampicin could also give sizable amounts of 3-formylrifamycin SV (FR), while 3-formyl-25-desacetylrifamycin SV (FDR) and N-desmethylrifampicin could be expected in low concentrations. A quinone metabolite has also been suspected, but not detected because it would have been reduced in the presence of the ascorbic acid required to protect rifampicin from air oxidation.

The microbiological assay of rifampicin and most of the physicochemical procedures described [1, 3 5] do not permit the separate determination of R and its metabolites. A high-performance liquid chromatography (HPLC) technique for the quantitative analysis of R and DR down to 0.1 μg/ml of plasma has been reported [6], but its specificity as regards FR and FDR has not been demonstrated.

This paper describes a simple and sensitive HPLC assay procedure for the simultaneous and specific determination of R, FR, DR and FDR in biological fluids.

EXPERIMENTAL

Materials

R, FR, DR and FDR were supplied by Ciba-Geigy, (Basle, Switzerland). The solvents and reagents used were all of analytical grade: dichloromethane

*To whom correspondence should be addressed.

(E. Merck, Darmstadt, G.F.R., Cat. No. 6050), isooctane (Merck, Cat. No. 4727), ethanol (Prolabo, Paris, France, Cat. No. 20821).

The buffer solution used consisted of 2 g ascorbic acid (Merck, Cat. No. 127) and 10 g anhydrous sodium sulphate (Merck, Cat. No. 6649) made up to 50 ml with concentrated buffer solution pH 6 (Merck, Cat. No. 9886) and continuously mixed in a 50-ml volumetric flask. Fresh batches were prepared each day.

Instruments

Chromatography was performed on a Model 1010 high-performance liquid chromatograph (Hewlett-Packard), equipped with a U6K valve injector (Waters Assoc.) and a Model 1036 A fixed-wavelength (254 nm) UV absorbance detector (Hewlett-Packard). The detector was connected to a CRS 204 electronic integrator (Infotronics) and to a 3012 potentiometric recorder (W+W, Kontron).

Column

The chromatographic column was a stainless-steel tube (10 cm × 7.5 mm I.D.) filled with LiChrosorb Si 60 (5 μm particles) (Merck) by the slurry technique [7, 8]: 3 g of LiChrosorb were suspended in 15 ml of the mobile phase and the slurry was forced into the column under pressure. The flow-rate must be high enough to give a pressure around 150 bars at the end of filling. The column is ready for use as soon as filled. Its efficiency is 2000—3000 theoretical plates for the DR peak.

Assay procedure

To 1 ml of plasma, urine or saliva in a 5-ml glass centrifuge tube are added 50 μl of methanol, 1 ml of buffer solution and 1 ml of isooctane—dichloromethane (3:2, v/v). The tube is stoppered and mechanically shaken for 10 min at 350 rpm with an Infors shaker, then centrifuged for 5 min at 2000 *g*. A 100-μl portion (5 μl when the concentration of R is higher than 20 μg/ml and 200 μl when it is lower than 0.1 μg/ml) of the supernatant organic phase is injected into the chromatographic column.

The mobile phase, dichloromethane—isooctane—ethanol—water—acetic acid (36.6:45:16.8:1.65:0.002), is pumped at a constant flow-rate of about 3 ml/min under a pressure of about 40 bars at room temperature.

Every sample is analysed in duplicate, and one aliquot of each assay is chromatographed. If the peak areas of the compounds are not within ± 5%, a third analysis is performed.

Calibration

Calibration samples are prepared by measuring 100 μl of convenient R, FR, DR and FDR dichloromethane solutions into 10-ml tubes. Dichloromethane is evaporated under nitrogen, the compounds are redissolved in 50 μl of methanol and 1 ml of plasma, urine or saliva is added. Three to four samples containing 0.2—20 μg of the compounds are prepared. The calibration graphs (peak area against concentration on a log—log graph) are straight lines. The complete calibration is repeated every day.

RESULTS AND DISCUSSION

Specificity

Fig. 1A shows that R is clearly separated from its three metabolites. Under the prescribed conditions, some batches of LiChrosorb may result in a poor separation of either R and FR or FR and DR. In such cases, a good separation is achieved by increasing or decreasing the dichloromethane content of the mobile phase by 1—2%: increasing the dichloromethane content lenthens the retention times of FR and FDR. Plasma components do not interfere (Fig. 1B and C). The same is true of urine and saliva.

Reproducibility and accuracy

Various spiked human plasma, urine and saliva solutions were analysed repeatedly. Table I shows that the proposed procedure permits the quantitative assay of R, FR, DR and FDR down to 0.1 μg/ml; if a higher sensitivity is needed, it is possible to inject a larger volume of extract (200—300 μl). The absolute detection limit is 1 ng R per injection when the peak height is about five times the background noise.

Table II demonstrates the good reproducibility of the chromatographic step. It is to be noted that the coefficient of variation calculated from the ratio (peak area of R/peak area of DR) is not better than when it is obtained from either the R or DR peak area. This shows that an internal standard would not improve accuracy. The yields (± S.D.) of extraction from plasma or urine are 96 ± 5% for R, 86 ± 6% for FR, 72 ± 6% for DR and 75 ± 6% for FDR.

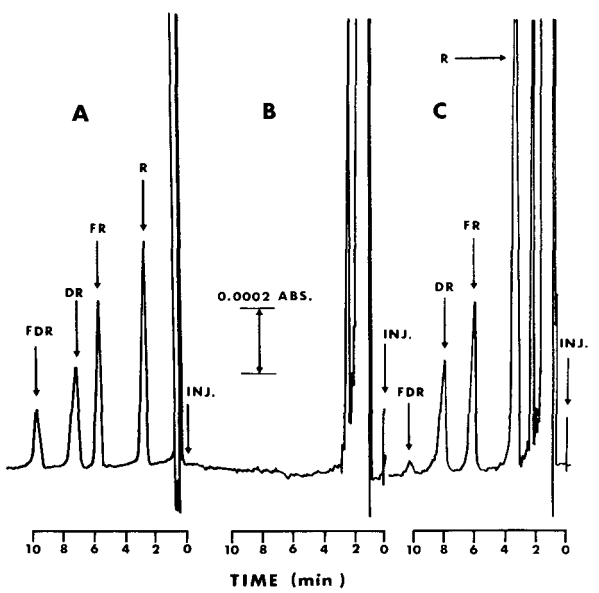

Fig. 1. (A). Chromatography of a synthetic mixture of rifampicin and three of its metabolites. R, Rifampicin; FR, 3-formylrifamycin SV; DR, 25-desacetylrifampicin; FDR, 3-formyl-25-desacetylrifamycin SV. (B). Chromatography of a blank plasma extract (C). Chromatography of a plasma extract of a subject given an oral dose of rifampicin. R, found: 1.45 μg/ml; FR, found 0.41 μg/ml; DR, found: 0.45 μg/ml; FDR, found: 0.05 μg/ml.

Stability of R, FR, DR and FDR solutions

The presence of ascorbic acid in the organic solution is necessary to prevent oxidation of R and its metabolites. Dichloromethane calibration solutions can be stored for one month at +5°. Aqueous solutions were found not to be stable enough to be used for calibration, even with ascorbic acid. No decrease in the R content was observed in plasma and urine samples when stored frozen for three months at −20°.

Speed of analysis

The analytical technique is fast: one single extraction is needed before chromatography, which takes about 10 min.

Application

The described procedure was used in the quantitative assay of R, FR, DR

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ASSAY IN HUMAN PLASMA, URINE AND SALIVA

100 μl of extract injected.

Compound	Sample (1 ml)	Amount of compound added (μg)	Average of 6 assays (μg)	Coefficient of variation (%)
Rifampicin	Plasma	0.05	0.05	23.0
		0.10	0.10	7.6
		0.50	0.50	4.7
		1.00	0.96	4.9
		5.00	4.90	4.9
		20.00	19.90	5.9
	Urine	9.00	9.10	1.2
		18.00	18.50	2.3
	Saliva	2.00	2.00	8.6
25-Desacetylrifampicin	Plasma	0.10	0.11	9.2
		0.20	0.20	7.0
		0.50	0.50	6.5
		2.50	2.49	4.0
		5.00	4.84	4.0
		10.00	10.40	4.5
	Urine	4.50	4.60	1.8
		9.00	9.20	1.8
	Saliva	1.00	1.01	4.7
3-Formylrifamycin SV	Plasma	0.05	0.05	12.0
		0.10	0.10	5.4
		2.50	2.45	3.8
3-Formyl-25-desacetyl-rifamycin SV	Plasma	0.10	0.11	10.6
		0.20	0.21	7.2
		0.50	0.52	4.2

TABLE II

INJECTION OF TEN SUCCESSIVE SAMPLES OF THE SAME R AND DR DICHLOROMETHANE SOLUTION

100 μl solution injected.

Injection No.	Rifampicin peak area (arbitrary units)	25-Desacetylrifampicin peak area (arbitrary units)	Rifampicin peak area/ 25-Desacetylrifampicin peak area
1	184208	92369	1.99426
2	183845	91599	2.00706
3	185639	92321	2.01079
4	185746	91451	2.03109
5	184873	92167	2.00584
6	183810	92240	1.98876
7	184299	91891	1.99803
8	185390	94983	1.95182
9	184765	91161	1.98635
10	181082	92424	2.01069
Average	184366	92261	1.99847
Coefficient of variation	0.7	1.1	1.0

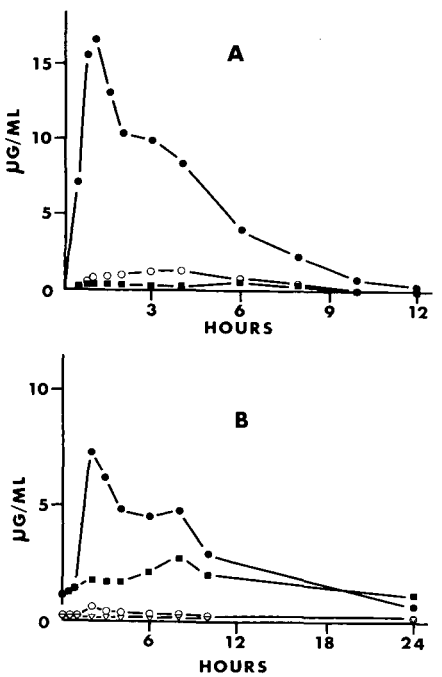

Fig. 2. Serum concentrations obtained: (A) in one healthy subject on the 1st day of treatment; (B) in one cirrhotic patient on the 7th day of treatment after daily administration of 600 mg of rifampicin. •, Rifampicin; ▪, 3-formylrifamycin SV; ○, 25-desacetylrifampicin; △, 3-formyl-25-desacetylrifamycin SV.

and FDR in the serum of normal subjects and cirrhotic patients given 600 mg of rifampicin daily for seven days. Fig. 2 shows the serum concentrations obtained in one healthy subject on the first day and in one of the patients on the seventh day of treatment.

CONCLUSION

The proposed technique permits the separate determination of rifampicin and three of its metabolites in human plasma, urine and saliva. It is fast and more specific than the existing assay procedures. It can be used to monitor rifampicin concentrations in patients, and to ascertain whether any accumulation of metabolites takes place.

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Note

Rapid assay of tinidazole in plasma by high-performance liquid chromatography

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Tinidazole (Fasigyn®, Pfizer), ethyl 2-(2-methyl-5-nitro-1-imidazolyl) ethyl sulphone, is used in the treatment of trichomoniasis in man. For the measurement of nitroimidazole-related compounds in human plasma, polarographic methods based on that described by Kane [1] have been used [2]. De Silva et al. [3] described methods for the determination of N-substituted nitroimidazole in blood and urine which involved a preliminary separation of unchanged drug by thin-layer chromatography (TLC) before quantitation by an absorptiometric, polarographic or gas chromatographic procedure. This TLC procedure was improved by Welling and Monro [4]: tinidazole was quantitated at the TLC stage by measuring the quenching of plate fluorescence. This method has been used for pharmacokinetic studies both in laboratory animals [5] and in man [4, 6]. However, the TLC procedure remains lengthy and is difficult to use for serial analyses of large number of samples.

In this paper a new high-performance liquid chromatographic (HPLC) assay is described which is quicker than the TLC method and no less specific.

EXPERIMENTAL

Instrumentation

Chromatography was performed on a Model 841 high-performance liquid chromatograph (DuPont) equipped with the Model 837 variable wavelength spectrophotometric detector set at 315 nm. Samples were injected through a Valco sample valve set for 50 μl volumes. The detector was connected to a Model 3380A recorder—integrator (Hewlett-Packard).

The chromatographic column was a stainless steel tube (1 m × 2.2 mm I.D.) filled with ETH Permaphase (25—37 μm; DuPont, Wilmington, Del., U.S.A.).

Reagents

All reagents were of analytical grade and used without further purification: chloroform RP (Prolabo, Paris, France) containing 0.5% ethanol as stabilizer, hexane (Merck, Darmstadt, G.F.R.), ethanol RP (Prolabo).

Assay procedure

0.3 ml of plasma in a 12-ml centrifuge tube is diluted to 1 ml with water. After addition of 3 ml chloroform the tube is shaken for 20 sec on a Vortex mixer. The tube is then centrifuged for 5 min at 5000 *g* and the lower layer is transferred to a second 12-ml tube. The extraction is repeated once. The combined organic extracts are evaporated to dryness under a stream of nitrogen at 40°. The dry residue is dissolved in 0.75 ml of a hexane—chloroform—ethanol (90:15:0.5) mixture and 50 μl are injected into the chromatographic column.

The above mixture is used as mobile phase and pumped at a constant flow-rate of about 1 ml/min under a pressure of about 450 p.s.i. at room temperature. The retention time of tinidazole is about 3 min.

Two injections of each extract are performed.

Calibration

Each day, calibration samples are prepared by adding up to 700 μl of a suitable aqueous solution of tinidazole to 0.3 ml plasma. Three or four calibration samples containing 2—10 μg of tinidazole are prepared.

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 1. The first peak corresponds to normal plasma constituents and is relatively constant irrespective of the time elapsed between tinidazole administration and blood sampling. No other peak was observed after that of tinidazole.

Comparing tinidazole peak areas from organic solutions to those from extracts of spiked plasma, the recovery of the extraction process was close to 99% whatever the concentration used. The coefficient of variation determined

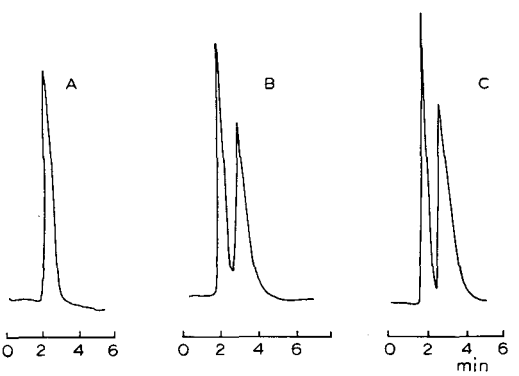

Fig. 1. A, Chromatogram of a blank plasma extract. B, Chromatogram of an extract of plasma spiked with tinidazole (5 μg/ml). C, Chromatogram of an extract of plasma from a volunteer after tinidazole administration. For HPLC conditions, see text.

from peak areas of four different assays on the same samples was about 4%. The minimum detectable concentration using 0.3-ml plasma aliquots was about 0.2 μg/ml, whereas the minimum measurable concentration was about 1 μg/ml. This sensitivity can be improved using higher plasma volumes up to 1 ml without peak overlap. The present conditions are satisfactory, however, since the range of tinidazole plasma levels encountered in clinical use is far above 1 μg/ml. Both recovery of the extraction and linearity of reference curves are satisfactory up to concentrations as high as 100 μg/ml.

As shown in Fig. 2, a good correlation between the present HPLC assay and the TLC procedure of Welling and Monro [4] was obtained. This suggests that the two techniques exhibit similar specificity, the HPLC method, however, being faster and less tedious: about 30 samples/day can be processed by one technician.

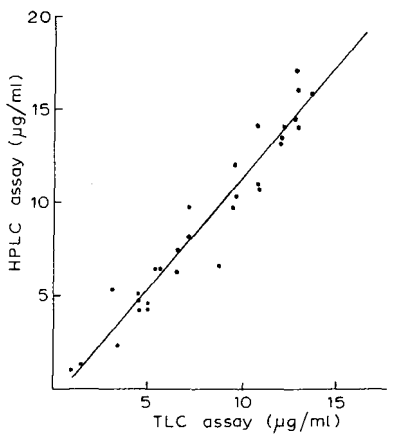

Fig. 2. Correlations between HPLC and TLC tinidazole assays in human plasma obtained at various time intervals after a single pessary (100 mg) administration.

This method has also been used successfully for assay of tinidazole in dog plasma; in this case, the first peak is higher than in human plasma but does not overlap with the tinidazole peak.

A similar chromatographic pattern was observed with metronidazole, suggesting that the present procedure could also be used for assay of metronidazole in plasma.

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Note

Determination of acetaminophen in plasma by high-performance liquid chromatography with electrochemical detection

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The lack of extreme sensitivity in detectors for high-performance liquid chromatography (HPLC) often limits its application in the determination of drugs in blood and plasma. The development of an electrochemical detector by Kissinger et al. [1] provides for very sensitive detection of compounds that can be oxidized at the carbon-paste electrode. By using this electrochemical detector, these authors were able to detect picogram quantities of norepinephrine and dopamine. Subsequently this detector has been used in the analysis of epinephrine [2], isoproterenal [2], L-dopa [2], α-methyldopa [2], phenylephrine [2], norepinephrine [2—4], dopamine [3, 4], ascorbic acid [5] and homogentisic acid [6]. Recently, this detector was used in the analysis of acetaminophen (N-acetyl-*p*-aminophenol) in commercial dosage forms, urine and plasma [7]. While these authors reported the detection of acetaminophen in plasma they did not demonstrate the application of this method in clinical studies.

This paper reports the application of HPLC with electrochemical detection to a study on acetaminophen bioavailability which required an analytical method capable of quantitating acetaminophen in plasma at a concentration of 0.2 μg/ml.

MATERIALS AND METHODS

Materials

Reagent grade potassium dihydrogen phosphate, disodium hydrogen phosphate, ethyl acetate and methanol were used directly. Acetaminophen (Chem. Service Co., Westchester, Pa., U.S.A.) and *p*-butoxyphenol (McNeil Labs.,

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Ft. Washington, Pa., U.S.A.) were also used directly. All water was double distilled from glass.

Extraction procedure

3 ml of pH 7.4 phosphate buffer, prepared by mixing 19.6 ml of solution of KH_2PO_4 (9.08 g/l) and 80.4 ml of a solution of $Na_2HPO_4 \cdot 2H_2O$ (11.8 g/l), were added to 1 ml of plasma in a round-bottom centrifuge tube. 1 ml of internal standard solution (*p-n*-butoxyphenol, 5.0 μg/ml) and 8.0 ml of ethyl acetate were added to the tube. The tube was rocked for 10 min followed by centrifuging for 5 min at 300 *g*. The ethyl acetate layer was transferred to a conical centrifuge tube and evaporated to dryness under reduced pressure at 40°. The residue was redissolved in 70 μl of methanol with 2—5 μl of the resulting solution being used for HPLC.

Instrumentation

All samples were analyzed by HPLC with electrochemical detection as previously described by Kissinger et al. [1]. Stainless-steel columns (0.5 m × 2.6 mm I.D.) packed with Pellidon (Pellicular polyamide, 37 μm; Reeve Angel, Clifton, N.J., U.S.A.) were used with an aqueous mobile phase consisting of 0.04 *M* NaH_2PO_4 adjusted to pH 7.4 and 5% methanol (v/v). The flow-rate was 60 ml/h. Solvent pumping was accomplished with either a Varian Model 4100 syringe pump or a Milton Roy reciprocating piston Minipump®.

A three-electrode system consisting of a carbon-paste electrode (working electrode), platinum electrode (auxiliary electrode), and saturated calomel electrode (reference electrode) was utilized to measure the current arising from the oxidation of the phenol group at a potential of +0.7 V (vs. SCE).

Analysis of acetaminophen

Acetaminophen concentrations in plasma were determined from a standard curve obtained by plotting drug/internal standard peak height ratio versus acetaminophen concentration. The standard curve was obtained by carrying through the assay procedures, samples prepared by adding known amounts of acetaminophen to pooled human plasma.

Clinical studies

Nine normal, healthy male subjects ranging in age of 22—26 years and weight of 68—92 kg were administered three dosage forms of acetaminophen in a three-way crossover study. The dosage forms used in this study were an experimental suppository dosage form consisting of a gelatin encapsulated water-soluble solution of acetaminophen, a glycol base suppository and an oral elixir. A 300-mg dose of acetaminophen was administered in each case. Following dosing, venous blood samples (15 ml) were withdrawn from the right or left antecubital fossa into heparinized vacutainers. The plasma was harvested immediately and frozen until analysis.

RESULTS AND DISCUSSION

Acetaminophen and the internal standard appeared as well-resolved symmetrical peaks under these conditions with retention times of 2 and 4.25 min,

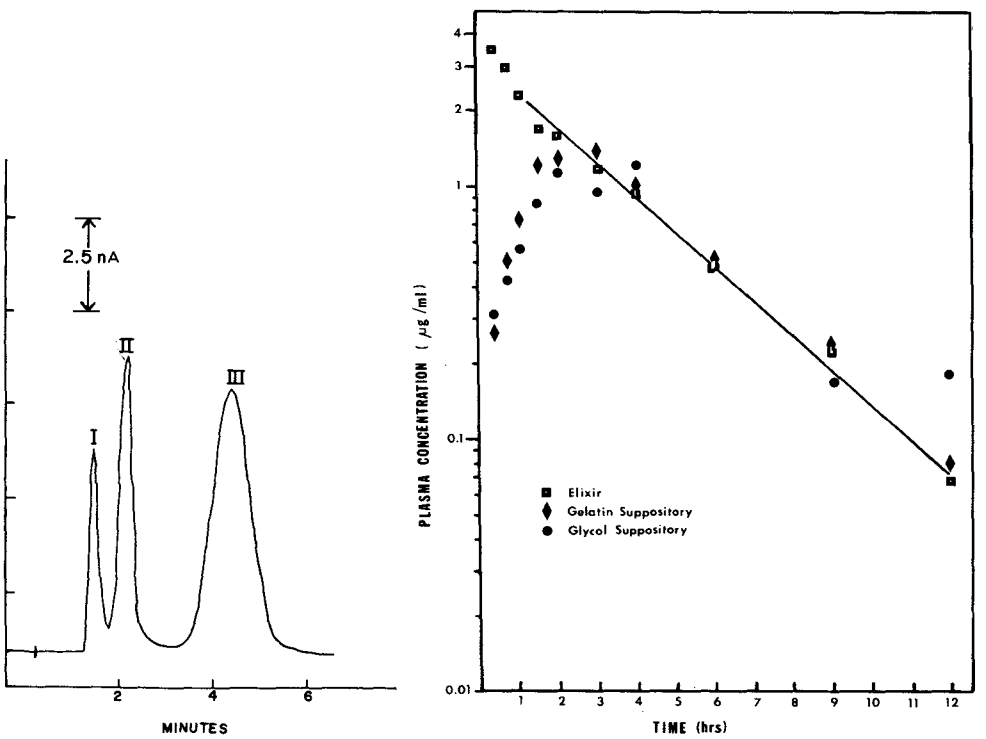

Fig. 1. Typical chromatogram for acetaminophen (II) and internal standard (III). Peak I represents the solvent front.

Fig. 2. Averaged plasma acetaminophen concentrations in nine human subjects following administration of 300 mg of acetaminophen as an oral elixir, glycol base suppository or gelatin encapsulated suppository. Straight line corresponds to an elimination half-life of 2.2 h.

respectively. A typical chromatogram is shown in Fig. 1. The standard curve is described by the following regression equation: peak height ratio = 0.180 X concentration (μg/ml) + 0.005. The correlation coefficient for this line is 0.997 with a standard error of the estimate ($S_{x.y}$) of 0.0099. Analysis of multiple standards gave coefficients of variation (n = 3) of 4.8, 9.6, 4.2 and 4.1% at concentrations of 0.2, 1.0, 2.0, and 4.0 μg/ml, respectively. During the course of the study standard curves were determined on a daily basis. This was necessary since the carbon-paste in the detector lost sensitivity with use and was changed periodically. Fig. 2 shows the average data for all subjects for all three dosage forms. The straight line represents an elimination half-life of 2.2 h for acetaminophen.

HPLC with electrochemical detector has been shown to be useful for measuring plasma concentrations of acetaminophen at low levels. However, it should be noted that the electrochemical detector is quite sensitive to solvent flow variation. Because of this, extensive pulse dampening is necessary when the reciprocating piston type pump is used. Also, the electrochemical detector

is quite sensitive to fluctuations in the line voltage. Consequently, best performance of the detector is achieved when no other electrical devices are used on the same circuit.

Preliminary results obtained in this laboratory also indicate that acetaminophen levels can be assayed in very small volumes of plasma using electrochemical detection. Plasma samples (50 μl) with concentrations ranging from 0.5 to 8 μg/ml have been measured in our laboratory using a variation of this method. Since this type of method would be quite suitable for measurement for acetaminophen in neonates and children, where only small plasma samples are available, further investigations along these lines are in order.

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Note

Quantitative and qualitative analysis of the anticoagulant acenocoumarol in human plasma

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Recently we described a method for the determination of the coumarin anticoagulant phenprocoumon (Liquamar, Marcoumar). The reasons presented by us for that investigation are also valid for another coumarin anticoagulant, acenocoumarol, namely to obtain information on the presence of intoxication, malabsorption, non-compliance or hereditary resistance, and on the pharmacokinetics of the drug.

As, in contrast to phenprocoumon, acenocoumarol does not display natural fluorescence because of its nitro group, a reduction step and a labelling step were introduced which resulted in the formation of a fluorophore. This enables us to use the plasma as its own blank by omission of the reduction step. Only other compounds containing nitro groups might interfere with the assay.

MATERIALS AND METHODS

Acenocoumarol (Sintrom) and its reduced derivative "aminosintrom" [(CGP 8 435, 3-(α-acetonyl-4-aminobenzyl)-4-hydroxycoumarin)] were gifts from Ciba-Geigy (Arnhem, The Netherlands). Fluorescamine (Fluram) was purchased from Hoffmann-La Roche (Mijdrecht, The Netherlands). All other chemicals were of analytical grade. Thin-layer chromatography, fluorescence densitometry, preparation of blood plasma, and thrombotest determinations were performed as described previously [1], with the exception of the development of the thin-layer plates which will be described in the final procedure.

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RESULTS

Extraction

Of a number of organic solvents examined, the best results were obtained with a mixture of light petroleum (b.p. 40—60%) and dichloromethane. Recovery for the extraction of 50 ng/ml acenocoumarol, added to blank plasma, was 68 ± 1.2% (mean ± S.E.M., n=7).

Reduction

After extraction and evaporation the nitro group was reduced to an amino group by tin (II) chloride. The efficiency of the reduction was 77 ± 1% (mean ± S.E.M., n=7), as compared to known amounts of aminosintrom. No destruction of aminosintrom was observed under the experimental conditions.

Formation of the fluorophore and thin-layer chromatography

After evaporation of the reaction mixture the residue was dissolved in toluene which is used instead of the commonly employed ethanol since tin(II) chloride, which interferes with the chromatography, is insoluble in toluene. Formation of the fluorophore with fluorescamine was performed by derivatization at the origin of the thin-layer plate by developing with a fluorescamine solution [2].

Procedure

To 1 ml of plasma in a Sovirel culture tube (110 × 16 mm) with a PTFE-lined screw cap, add 1 ml of 0.1 mole/l sodium acetate buffer (pH 4.0) and 1 g NaCl. After mixing, add 7 ml of light petroleum (b.p. 40—60°)—dichloromethane (1:1, v/v). Treat in the same manner a calibration series containing 0, 0.1, 0.5 and 0.9 mg acenocoumarol per litre added to blank plasma. Shake the tubes mechanically for 15 min and centrifuge at maximum speed in a clinical centrifuge for 10 min. Filter the upper layer in conical tubes through Whatman No. 1 PS phase-separating filter paper that has been washed thoroughly with chloroform to prevent the impurities present in the paper [3] interfering with the subsequent chromatographic procedure. Wash the filters with 1—2 ml of the solvent mixture. Evaporate the filtrate in a hot water bath under a stream of nitrogen. Dissolve the residue in 70 µl of ethanol and add 20 µl of 30 m-moles/l $SnCl_2$ in 0.4 mole/l acetate buffer (pH 4.0). Mix thoroughly and incubate for 30 min at 60°. Evaporate again under nitrogen and add 90 µl of toluene. Centrifuge and spot 30 µl of the supernatant solution on a thin-layer plate (maximally 13 spots per 20 cm × 20 cm plate). Place the plate in a tank containing a 1-cm depth of derivatizing reagent, prepared by dissolving 10 mg of fluorescamine in 20 ml acetone and then adding hexane to a final volume of 100 ml. After the solvent front has moved at least 10 cm, remove the plate and evaporate the solvent. Then develop the plate in ethyl acetate—methanol—triethanolamine (70:30:3, v/v/v), until the solvent has ascended 10 cm from the starting point. After drying at room temperature, inspect the plate under ultraviolet light at 366 nm. Mark the position and diameter of the spots with a pencil at both vertical rims of the plate (R_f 0.35). Scan the plate with a Vitatron TLD 100 Flying Spot densitometer. The instrument settings are: mode,

lin II+; level, e 7; zero, C 7; damping, 2; span, 7—10; lamp, Hg; diaphragm, 0.50 mm; primary filter, 366 nm; secondary filter, U12, cut off below 540 nm; scanning speed, 1 cm/min; integrator sensivity, position 7.

Draw a calibration curve by plotting the number of integrator units vs. concentration; calculate from this straight line unknown concentrations by interpolation. Use as a blank the unknown serum to be investigated submitted to the same procedure but without addition of the reducing reagent.

The lower limit of detection under the conditions presented above is about 0.010 mg/l. The determination is reproducible to about 3%. Nine samples can be analyzed within 5 h.

When acenocoumarol was given to patients in the evening and the blood was drawn the following morning for a thrombotest and acenocoumarol assay, plasma concentrations between 0.015 and 0.080 mg/l were observed.

Qualitative demonstration of acenocoumarol in plasma

The formation of a fluorescent product from acenocoumarol can be used in the screening of a suspected case of intoxication with anticoagulant coumarin derivatives. Plasma extracts can be chromatographed on thin-layer plates with chloroform—methanol (97:3, v/v) as described by Daenens and Van Boven [4]. Dicoumarol (R_f 0.29), warfarin (R_f 0.45) and phenprocoumon (R_f 0.55) are visible under ultraviolet light at 254 nm. It is possible to visualize also acenocoumarol (R_f 0.40) on this plate by spraying with the buffered tin(II) chloride solution, followed, after incubation of the plate for 30 min at 60° in a closed tank saturated with water vapour, by spraying with a fluorescamine solution in acetone. The fluorescent product is visible under light of 366 nm.

Interference by other compounds

For those compounds that contain no nitro group, a correction is made by using as the blank a non-reduced extract from the same plasma. Only the few nitro compounds that are in use as therapeutic agents may cause serious interference. We submitted all the drugs containing nitro groups known to us to the same procedure as acenocoumarol and observed the following R_f values: acenocoumarol, 0.35; chloramphenicol, 0.34; nitrazepam, 0.28, hydroxymethylnitrofurantoin, 0.28; clonazepam, 0.23; nitrofurantoin, invisible.

Hydroxymethylnitrofurantoin is not extracted from plasma under the circumstances used here. The only serious interference may be caused by chloramphenicol, the presence of which is known in most cases.

Stability of acenocoumarol in serum

When a serum spiked with 100 ng/ml acenocoumarol was kept at -20° or +4° for one week, no decrease in concentration was observed. Kept on the laboratory bench for one week the concentration of acenocoumarol was diminished by about 30%.

DISCUSSION

We have developed a highly specific and sensitive method for the determination of acenocoumarol in human plasma. The specificity is a result of the use

of a reduction step, necessary for the development of a fluorophore, which eliminates interference from all compounds not containing nitro groups, if the proper blank is used. The fluorogenic labelling enhances the sensitivity of the method, which is necessary in view of the very low blood levels of acenocoumarol under therapeutic circumstances. A major advantage is the use of derivatization at the origin of the thin-layer plates, as derivatizing by spraying lowers both sensitivity and reproducibility. However, the procedure of reduction on the plate, followed by spraying with fluorescamine can be useful to visualize acenocoumarol next to other coumarin derivatives which display natural fluorescence. So far, no correlation can be demonstrated between thrombotest values and acenocoumarol concentration, unlike our recent findings with phenprocoumon [5].

In summary, this method is useful for the simultaneous qualitative and quantitative analysis of acenocoumarol in human plasma in the therapeutic as well as in the toxic range.

ACKNOWLEDGEMENTS

This study was initiated by Professor Dr. E.A. Loeliger. The investigations were financially supported by Ciba-Geigy B.V., Arnhem, The Netherlands.

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Book Review

Quantitative mass spectrometry in life sciences (Proc. 1st Int. Symp., Ghent, June 16—18, 1976), edited by A.P. De Leenheer and R.R. Roncucci, Elsevier, Amsterdam, Oxford, New York, 1977, VIII + 253 pp., price Dfl. 74.00, ISBN 0—444—41557—2.

This book, the proceedings of an international symposium held at the State University of Ghent in June, 1976, is a successful blend of four long and sixteen short chapters (based on the plenary lectures and short communications, respectively). The former are state-of-the-art reviews of work from four laboratories acknowledged to be among the leaders in the field of analytical mass spectrometry (MS), and are highly complementary. Each offers readers the opportunity to "visit" the authors' laboratories and to catch the flavor of quality research. E.C. Horning, J.-P. Thenot and M.G. Horning (Baylor College of Medicine), drawing upon their wealth of expertise, succinctly discuss in a highly readable fashion many aspects of the quantification of drugs by MS. H. Adlercreutz (University of Helsinki), in a chapter sure to be of interest to workers in the steroid field, makes refreshingly candid comments about several mass spectrometers, and lists sound reasons for and against using mass fragmentography instead of other techniques (e.g., radioimmunoassay). C.C. Sweeley, J.F. Holland and co-workers (Michigan State University) authoritatively discuss the use of computer techniques in gas—liquid chromatography—MS studies, with special emphasis on comparison of cyclic scanning versus selective ion monitoring. The use of stable isotopes as carriers/internal standards is a recurring theme in the first three chapters. This is also true in the informative and wide ranging chapter by P. Padieu and B.F. Maume (University of Dijon), but these authors also introduce the use of ^{14}C-labeled compounds of high specific activity (i.e., approximately 50 mCi/mmole) as internal standards in metabolic studies using cell cultures.

The remaining sixteen short chapters, many also written by well-known and experienced workers, cover a wide variety of compound types and MS-based analytical methods. Sullivan and McMahon present an illuminating report on the use of deuterated drugs in a pharmacokinetic study of propoxyphene; R.W. Kelly describes the use of *tert*-butyldimethylsilyl ether derivatives in the measurement of prostaglandins in tissues and semen; the University of Bonn group discusses quantitation of dopamine and cyclophosphamide by field desorption MS; and Cattabeni and co-workers discuss using deuterated sub-

strates to measure enzyme activities in the presence of large excess of the endogenous unlabeled species. The latter article reports focusing on specific ions, e.g. 174.304, with a commercially available quadrupole instrument, and such third decimal place accuracy is fanciful. Although a few of the short papers do little more than report on methods potentially applicable to certain problems, together they offer a highly useful compendium of MS-based methods of quantitative analysis.

This volume can be heartily recommended to analytical and bio-analytical chemists, biologists, clinical pharmacologists and others who need sensitive, selective methods for the analysis of compounds which can undergo gas—liquid chromatography. University teachers would find it highly useful in preparing lectures on modern methods of analysis. It is a valuable volume for anyone concerned with the analytical use of stable isotopes. This reviewer has only one general criticism of the book (even the cost is not unreasonable): the contrast between the light type of the text and the page background leaves something to be desired in most of the chapters, rendering some of the pages somewhat difficult to read except in strong light. It is hoped that this relatively minor defect can be corrected in any future books on the lively topic of quantitative MS in biological research. Perhaps by then the workers in the field will have settled on a single term which encompasses "mass fragmentography," "selective ion monitoring," etc.

Rahway, N.J. (U.S.A.) W.J.A. VANDENHEUVEL

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Book Review

Chromatographic and electrophoretic techniques, Vol. I, Paper and thin-layer chromatography, edited by I. Smith and J.W.T. Seakins, William Heinemann Medical Books, London, 4th ed., 1976, IX + 465 pp., price £ 9.00, ISBN 0-433-30504-5.

This is a book that needs no recommendation. The popularity of its preceding editions, especially among clinical biochemists, leaves no doubt about its practical value, and it is one of the most often quoted sources in chromatographic papers. This review will therefore concentrate on the main differences between the third and fourth editions.

Whereas in the preceding edition the first volume was designated "Chromatography", although it included mainly paper (PC) and thin-layer chromatography (TLC), the title of the present volume no longer has this inaccuracy. The emphasis has shifted from PC to TLC. Even more so than in the previous editions, stress has been laid on clinical biochemistry and toxicology; thus the book has become more uniform and balanced in scope. The number of pages has decreased from 1080 to 465, as the newly included material occupies less space than that left out. Part of the content that is not relevant to chromatography, *i.e.*, the chapter on the Biobit models for macromolecules (45 pp.), has been shifted to Volume II, which deals with electrophoresis.

The senior editor, now a Reader in Biochemical Education at the Cour-Tauld Institute, Middlesex Hospital, University of London, has shared the editorship of the 4th edition with J.W.T. Seakins, working in the Department of Chemical Pathology of the Institute of Child Health. Of the 38 authors of the preceding edition, 6 have remained (R.W.H. Edwards, J.V. Jackson, I.S. Menzies and Margaret J. Smith, in addition to the Editors) and 10 have been added to the list. This already shows the extent of the changes. Interestingly, unless Nieuwe Gracht is a town, the city has been omitted from the address of P.K. de Bree and S.K. Wadman. The other authors are from Great Britain.

A brief authoritative historical introduction by an expert, T.I. Williams, has been added. The part on general techniques now includes a section on screening for inborn errors (J.W.T. Seakins and R.S. Ersser) and a chapter on sources of error in PC and TLC (R.S. Ersser and I.S. Menzies). Radioactive techniques (except for the spark chamber, now by P.D. Mitchell and I. Smith), ion-exchange celluloses, Sephadex in thin layers, preparative layer chromato-

graphy, automated quantitative PC, the brief special section on non-adhering thin layers and the hypertrophic section on pre-coated layers have been omitted. A certain amount of advertizing for the Unikit tools and apparatus has remained.

The sequence analysis of peptides and nucleotides and the chromatography of their amino acid derivatives have been excluded. Fluorescamine is among the new reagents in the amino acid chapter. "Indoles and related Ehrlich reactors" and "Kynurenine pathway metabolites of tryptophan" have been replaced by E.W.A. Oliver's chapter on tryptophan metabolites. Iodoamino acids, guanidines, plant phenols, catecholamines, inorganic ions and dyes have been omitted and there are now no illustrations in colour.

Toxicological applications (J.V. Jackson and A.J. Clatworthy) have been extended to 75 pages, treated in a more systematic and more practical manner and subdivided into (1) initial screening and tentative identification, (2) confirmation of acidic and neutral drugs (including salicylates, barbiturates, benzodiazepines etc.) and (3) confirmation of alkaloids and basic drugs. Alkaloids have been given less prominence. Both chapters lack references.

Among the speculative, but highly stimulating, sections, that on the identification of new compounds has disappeared, while that on the use of R_M and ΔR_M values has been brought up-to-date and included in the excellent "Steroids" chapter (W.H. Edwards). The clinical implications of steroid chromatography have been extended.

In conclusion, this is an entirely new book, which does not replace the preceding edition, but supplements it. Most users would do well to have both available.

Hradec Králové (Czechoslovakia) I.M. HAIS

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Book Review

Hormones in human blood: detection and assay, edited by H.N. Antoniades, Harvard University Press, London, Cambridge, Mass., 1976, XVII + 810 pp., price £37.15, ISBN 0-674-40635-4

The book "Hormones in human blood: detection and assay" edited by H.N. Antoniades describes the state of the art of hormone assays in human blood. It consists of 46 separate chapters written by different authors, which are experts in their field. The first part deals with protein separation techniques, with special chapters on the powerful techniques of isoelectric focusing and affinity chromatography. Thereafter radio-immunoassay, which has become the standard method of hormone determination is treated in a general way. This section describes practical aspects, such as radio-iodination, production of antisera, separation of bound and free ligand, and includes also a theoretical chapter on the kinetics of radio-ligand assays.

A third section describes the assays used for various polypeptide hormones. In this part the choice of techniques has not been limited to radio-immunoassays, but great attention is given to radio-receptor assays and bio-assays. Indeed, the current awareness that radio-immunoassays have not always the ultimate specificity in terms of active hormone, has led to a new interest in these methods.

The final part is devoted to the assay of non-polypeptide hormones (thyroid hormones, catecholamines and steroid hormones). The approach of each author to his chapter is not always the same, but the general emphasis on practical aspects and on critical evaluation of various alternative methods makes this book a very useful and informative guide in a rapidly evolving field.

Leuven (Belgium) W. HEYNS

Vol. 145, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

APPARATUS

N-1071

PROTEIN CONCENTRATION APPARATUS

Available from Schleicher & Schuell is an instructional bulletin describing the company's dialysis bags and matching glass apparatus for the concentration or desalting or protein solutions. The collodion bags are available in two grades: one with an average retention of about 25,000 mol. wt. and above, the other with an average retention of about 75,000 mol. wt. and above. The bulletin gives full details of the apparatus and operating instructions.

N-1082

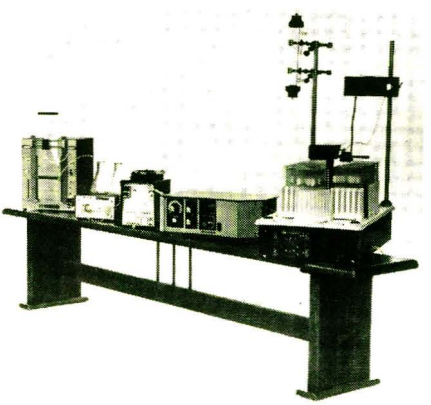
ELECTROPHORESIS POWER SUPPLY

Now available from Bio-Rad Laboratories is a new Model 500 power supply suitable for both tube and gel electrophoresis. Voltage output is 500 V d.c. and current output 200 mA, in many cases suitable for powering two cells at one time. Four operation modes can be used: constant voltage, constant current, current limited constant voltage, and voltage limited constant current.

N-1077

LIQUID CHROMATOGRAPHY SYSTEM

Gilson Medical Electronics announces a bench-top liquid chromatography system with components that can be substituted or eliminated to meet different applications. The system for low-pressure applications contains a Mixograd gradient former, Minipuls peristaltic pump, flow-through spectrophotometer, column, valve, fittings, shut-off valve, and Race Track fractionator.



N-1083

AUTOMATIC GAS CHROMATOGRAPH–MASS SPECTROMETER SYSTEM

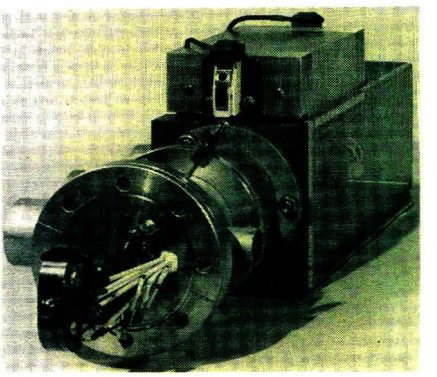
Universal Monitor announce a fully automatic gas chromatograph–mass spectrometer system (OLFAX IIA) incorporating a microprocessor. All operating parameters for an assay are incorporated in a panel on a magnetic tape cassette. Included are names of compounds to be searched for, their reference selected-ion mass spectra, calibration data, chromatographic retention times, concentration scale factors, designated internal standards, temperatures and procedures for sample preparation. Up to three concentration scale factors which compensate for extraction efficiency, column adsorption, etc. can be stored and averaged, and several internal standards can be designated. The information is readily transmitted to the microcomputer to prepare the system automatically for an assay. The user can, however, modify operating parameters, add or delete compounds, or create his own automatic identification panels. In the automatic mixture analysis mode of operation, the microcomputer completely controls operating parameters including system temperatures and GC temperature programming. It also determines data to be acquired for each compound and controls data acquisition, analysis and print-out of results. OLFAX IIA employs a special form of reverse-search selected-ion monitoring (probability based matching) combined with GC retention time screening for identification and quantitation.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1076

LIQUID CHROMATOGRAPH–MASS SPECTROMETER INTERFACE

Finnigan Instruments announces the development of an interface to combine LC and MS systems. The interface allows choice of electron impact or chemical ionization and does not interfere with GC–MS or solid probe operation. The interface can be added to any Finnigan mass spectrometer with a differential pumping system. It can be installed on a new mass spectrometer or retrofitted to an existing system. The interface should find wide application in pharmaceutical and biomedical research, and in petroleum, energy, pollution and pesticide studies.



N-1115

AMINO ACID ANALYSER

New from Biotronik is the amino acid analyser LC 2000, which was designed to handle protein hydrolysates as well as physiological fluids. Single-column run for protein hydrolysates can be achieved in 90 min, and in 4 h for physiological fluids. The high-pressure glass column (0.6 x 35 cm) is filled with cation-exchange resin Durrum DC-6A. The two-channel photometer has one lamp and one cuvette and is designed for simultaneous measurement at 570 and 440 nm (16 mm light path). The instrument can be operated up to 75 bar and includes a two-channel line recorder, programmer with punch card system. It is adaptable to a computing

CHEMICALS

N-1091

LDH ISOENZYME MARKER

Recently introduced by the Sigma Chemical Company is a new electrophoretic marker for lactate dehydrogenase isoenzymes. LDH ISOTROL is derived from human tissue containing LDH-1, -2, -3, -4 and -5, freeze-dried in a human serum matrix. It is suitable for electrophoretic media and buffer systems and is designed for interpreting fluorometric or colorimetric patterns.

N-1101

"POCKET MASS SPEC KIT"

PCR Research Chemicals have introduced a pocket-sized kit containing the four most frequently used markers in mass spectrometry. There are 1 g each of perfluorokerosene-H-MASS SPEC, perfluorotributylamine MASS SPEC, tris-(perfluoroheptyl)-*s*-triazine MASS SPEC, and 500 mg of Ultramark 443 MASS SPEC.

N-1098

NATURAL PHOSPHOLIPIDS WITH PROFILE ANALYSIS

Analabs are now supplying phosphatidyl choline (egg) and ethanolamine (egg) with a profile analysis. Every batch of each compound is accompanied with a specification sheet showing a qualitative analysis of the fatty acid composition

N-1103

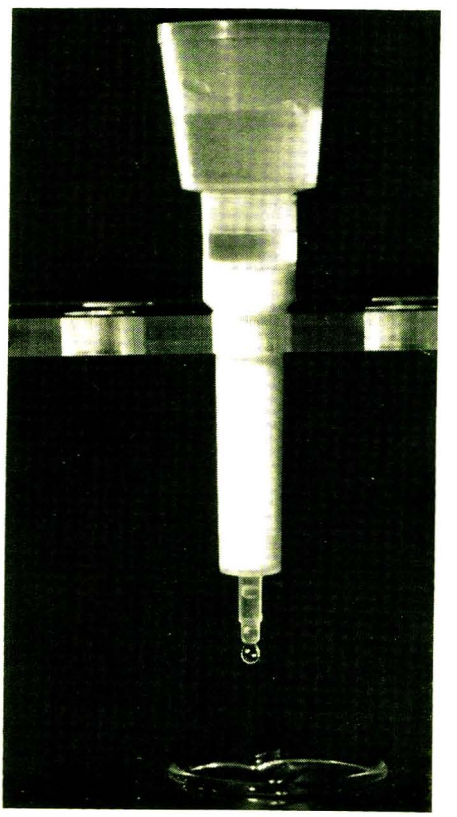
SCINTILLATION COCKTAILS

Now available from Packard-Becker are two brochures describing their new liquid scintillation cocktails Pico-Fluor 15 and 30. Pico-Fluor 15 is compatible with aqueous buffer solutions such as radioimmunoassay supernatants and accepts aqueous solutions containing acids, bases, salts or biological material. A continuous single phase is retained from zero to maximum acceptable sample load. It shows high quench resistance and its low diffusion rate into polyethylene gives greater counting stability when plastic scintillation vials are used. Pico-Fluor 30 has the additional properties of low photo- and chemiluminescence, and low viscosity for faster mixing.

N-1088

DRUG EXTRACTION COLUMNS

Disposable drug extraction columns plus procedures for their use are now available from Isolab. The new Quik-Sep® XAD-2 resin columns concentrate and extract drugs from urine by gravity flow. The resin is specially pre-conditioned Amberlite XAD-2, a non-ionic material which absorbs drugs of all classes. Elution is with methanol and overall recovery is 63–78% of the drug originally present in the urine. Pre-packaged kits for analysing the isolated drugs are also available.



MEETING

CHROMATOGRAPHY '78

INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The 13th International Symposium on Advances in Chromatography will be held October 16–19, 1978 at the new Sheraton-St.Louis Hotel, St. Louis, Mo., U.S.A. The scope of the meeting will cover papers and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. In particular, new developments in gas, liquid and high-performance thin-layer chromatography will be included. There will also be an exhibition of the latest instrumentation and books. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers must submit 500-word abstracts by March 3, 1978. The deadline for receipt of manuscripts of accepted papers is April 3, 1978. Program and registration forms will be available in June. Special, separate intensive 2-day short courses in (a) high-resolution capillary columns, (b) liquid chromatography and (c) high-performance thin-layer chromatography will be available on October 14–15 just prior to the meeting at the same hotel. The staff for these courses will include Dr. R.E. Kaiser, Institute for Chromatography, Bad Dürkheim, Germany, Dr. Cs. Horvath, Yale University, New Haven, Conn., U.S.A. and Dr. U. Hezel, Carl Zeiss, Oberkochen, G.F.R. All correspondence concerning the symposium and short courses should be directed to Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A.

NEW BOOKS

Formulation and preparation of dosage forms (Proc. 37th Int. Congr. Pharmaceutical Sciences of F.I.P., The Hague, Sept. 5–9, 1977), edited by J. Polderman, Elsevier/North-Holland, Amsterdam, New York, 1977, VIII + 308 pp., price Dfl. 87.00, US $ 35.50, ISBN 0-444-80033-6.

Biological activity and chemical structure (Proc. IUPAC-IUPHAR Symp., Noordwijkerhout, August 30–September 2, 1977; Pharmacochemistry Library, Vol. 2), edited by J.A. Keverling Buisman, Elsevier, Amsterdam, Oxford, New York, 1977, X + 314 pp., price Dfl. 107.00, US $ 44.60, ISBN 0-444-41659-5.

Analysis of drugs and metabolites by gas chromatography–mass spectrometry, Vol. 2, Hypnotics, anticonvulsants, and sedatives, by B.J. Gudzinowicz and M.J. Gudzinowicz, with the assistance of H.F. Martin and J.L. Driscoll, Marcel Dekker, New York, Basel, 1977, XI + 493 pp., price SFr. 144.00, ISBN 0-8247-6585-0.

Analysis of drugs and metabolites gas chromatography–mass spectrometry, Vol. 3, Antipsychotic, antiemetic, and antidepressant drugs, by B.J. Gudzinowicz and M.J. Gudzinowicz, with the assistance of H.F. Martin and J.L. Driscoll, Marcel Dekker, New York, Basel, 1977, X + 268 pp., price SFr. 92.00, ISBN 0-8247-6586-9.

Side effects of drugs annual 2 – 1978, edited by M.N.G. Dukes, Excerpta Medica, Amsterdam, New York, 1978, XVIII + 428 pp., price Dfl. 110.00, US $ 44.95, ISBN 0-444-90023-3.

Progress in medicinal chemistry 14, edited by G.P. Ellis and G.B. West, North-Holland, Amsterdam, New York, 1977, XX + 308 pp., price Dfl. 110.00, US $ 44.95, ISBN 0-7204-0645-5.

Research on steroids, Vol. VII (Trans. 7th Meet. Int. Study Group for Steroid Hormones), edited by A. Vermeulen, A. Klopper, F. Sciarra, P. Jungblut and L. Lerner, North-Holland, Amsterdam, New York, 1977, XVI + 618 pp., price Dfl. 220.00, US $ 89.95, ISBN 0-7204-0639-0.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (full-length papers), short communications and notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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