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Biochemical and Biological Applications of Isotachophoresis

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A. ADAM, *Centre Hospitalier de Sainte-Ode, Baconfoy, Belgium, and* C. SCHOTS, *LKB Instrument NV SA, Ghent, Belgium (editors).*

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F. M. EVERAERTS, J. L. BECKERS *and* TH. P. E. M. VERHEGGEN, *Department of Instrumental Analysis, Eindhoven University of Technology, The Netherlands.*

Journal of Chromatography, Library 6

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GAS CHROMATOGRAPHIC DETERMINATION OF GLUTAMINE IN THE PRESENCE OF GLUTAMIC ACID IN BIOLOGICAL FLUIDS

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(First received November 19th, 1980; revised manuscript received February 17th, 1981)

SUMMARY

The gas chromatographic determination of glutamine and glutamic acid in biological samples has so far presented considerable difficulties due to rapid conversion of glutamine to glutamic acid during derivatization. Quantitation of glutamine can be based on an intermediate in the above reaction, i.e. pyrrolidone carboxylic acid. However, the percentage formed is strongly dependent on reaction conditions, rendering quantitation unreliable. To overcome this problem D-glutamine, the optical isomer to the natural L-glutamine, is added as internal standard. The enantiomers are chemically identical and form the cyclic derivative to the same extent. The enantiomers of pyrrolidone carboxylic acid ester can easily be separated on a capillary coated with the chiral stationary phase Chirasil-Val. No extra derivatization step is required and quantitation is based merely on the ratio of the peak areas of both enantiomers.

INTRODUCTION

The gas chromatographic (GC) determination of amino acids in biological samples offers a great advantage over the classical Stein and Moore procedure in being faster, more sensitive and less expensive [1]. Until recently, some difficulties remained which hampered the general acceptance of this powerful tech-

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nique. Most of them can be solved by the use of enantiomers as multiple internal standards, a procedure we call enantiomer labelling [2, 3]. However, a problem which still remains is the determination of asparagine and glutamine in the presence of free acids. The amides under the conditions of the common acid-catalyzed esterification are rapidly deamidated and converted to the diesters, the same derivatives as those resulting from the free acids. Consequently, only the sum of both glutamine and glutamic acid or asparagine and aspartic acid could be determined by GC. For an independent quantitation of the amides and free acids the ion-exchange procedure remained indispensible [4, 5].

Especially in clinical chemistry and neurochemistry, accurate separate quantitation of glutamine and glutamic acid is of paramount importance, as their levels in serum and cerebrospinal fluid may be indicative of disorders in the metabolism of amino acids or synaptic transmitters, and may signal neurophysiological consequences of disturbed ammonia detoxication. For a study concerning the role of glutamine and of glutamic acid as a potential neurotransmitter we developed a new, rapid GC procedure. Especially glutamic acid is of increasing interest in neurochemistry [6].

To date only a few alternatives to overcome the difficulty of acid-catalyzed deamidation during derivatization have been proposed. They either depend on mild esterification conditions to keep cleavage of the amide to a minimum [7, 8], or acid treatment is avoided altogether by choosing other derivatives [9]. In the former case esterification time is kept to only a few minutes and the concentration of hydrogen chloride as catalyst is relatively low to ensure maximum formation of the glutamine derivative, pyrrolidone carboxylic acid ester. However, in our hands the reproducibility and accuracy of the procedure were so low that to us it seemed unsuitable as a quantitative method. In addition, we could not follow the argument that on the basis of different kinetics a distinction between glutamine and pyrrolidone carboxylic acid can be made, as the latter is formed from glutamine when heated in acidic medium. In due course [8] this aspect was clarified, but in general the same problem remains, namely the uncertainty in precalculating the amount of glutamine present as pyrrolidone carboxylic acid ester and the amount further converted to the glutamic acid diester. For synthetic amino acid mixtures this procedure might be applicable, but not for biological fluids.

Another approach proposed recently [9] implies the formation of cyclic 1,3-oxazolidinones under weakly basic conditions, leaving the amide group intact and enabling distinction from free acid. However, the method is not compatible with analysis of some other amino acids, especially of arginine and histidine. Another possibility is trimethylsilylation, but again several amino acids cannot be derivatized appropriately or form more than one derivative.

In this paper we describe a method for the accurate determination of glutamine in biological fluids and other samples, without requiring an extra derivatization step. The method relies on the addition of D-glutamine as internal standard for the natural L-glutamine, partial conversion to pyrrolidone carboxylic acid ester, and GC separation of both enantiomers. Consequently, reliable distinction between glutamine and glutamic acid is feasible.

EXPERIMENTAL

Clean-up and derivatization

Human cerebrospinal fluid (0.5 ml), which was sampled from patients suffering from lumbar disc or multiple sclerosis and stored at −80°C until analyzed, is mixed with a standard solution of D-amino acids in water, containing an amount of glutamine and glutamic acid similar to the amount expected in the sample. Then 2 ml of a solution of 1.2% picric acid are added under vigorous mixing. After standing for 10 min the mixture is centrifuged and the supernatant is transferred to a cation-exchange column (Dowex AG 50W-X8, 100–200 mesh), 2.0 × 0.5 cm. The ion exchanger is washed 4 or 5 times with about 3.0 ml of water each time, waiting about 5 min between each rinsing to allow diffusion of picric acid out of the resin. Finally, the amino acids are eluted from the ion exchanger with 3 ml of 2 *N* ammonia and three times 2 ml of water. The combined eluates are dried in a vacuum centrifuge and esterified with 100 µl of 4 *N* hydrogen chloride in isopropanol at 110°C in an aluminium block. After 5 min an aliquot of 3 µl is withdrawn for GC, and reagents and solvent are evaporated with nitrogen under moderate heating (50°C). The residue is dissolved in 50 µl of methylene chloride.

Gas chromatography

Gas chromatography was performed on a Carlo Erba instrument Model 2900 equipped with split injector, a capillary coated with Chirasil-Val (Applied Science, State College, PA, U.S.A.), 20 m × 0.28 mm, and flame ionization detector. Splitting ratio is 1:30, carrier gas is hydrogen, the linear gas velocity is 35 cm/sec. Injector temperature is 225°C, the column is held isothermally at 160°C and the detector is at 250°C.

Peak areas are integrated electronically with a Spectra Physics integrator, Model 4100.

RESULTS AND DISCUSSION

When glutamine (1) is heated to a temperature of about 100°C in acidic medium, rapid conversion to the glutamic acid diester (3) takes place (Fig. 1). Pyrrolidone carboxylic acid ester (2) has been shown to be a reaction intermediate, which reaches its maximum concentration at between 5 and 10 min at 100°C. A method utilizing the formation of pyrrolidone carboxylic acid has been developed for the quantitative GC determination of glutamine in serum

Fig. 1. Conversion of glutamine (1) to the glutamic diester (3).

and urine. Cyclization and the consecutive ring opening are, however, highly dependent upon time of esterification, temperature, and concentration of acidic catalyst [8]. Consequently, the yield of conversion to pyrrolidone carboxylic acid under the actual conditions of esterification must be known for calculation of the response factor relative to an internal standard; two GC runs are required for the determination of glutamine in one sample. In our hands the procedure yielded irreproducible results for biological samples. This is not surprising if one considers the large changes in yields caused by small changes in the esterification conditions. We see no possibility of explaining the inaccuracy of our determinations other than by fluctuating yields in cyclization and ring opening between sample and pure standards.

We could overcome this specific problem by taking advantage of the separation of optical isomers on a chiral stationary phase, Chirasil-Val [10, 11]. A procedure for the GC determination of amino acids has been proposed recently, the so-called enantiomer labelling [2]. In this approach, for each natural L-amino acid the optical isomer is added as internal standard, which can be separated on Chirasil-Val. Since enantiomers are chemically identical they suffer the same fate during clean-up and derivatization, and thus calculation of recoveries or molar response factors becomes unnecessary. The same, in principle, is applicable to the glutamine problem. When a known amount of D-glutamine is added to the sample prior to clean-up and derivatization, mere calculation of the peak area ratio of both enantiomers allows determination of the amount of L-glutamine present. Both enantiomers undergo cyclization to the same extent even when the amount of D-glutamine added as internal standard does not completely match the amount of L-glutamine in the sample; formation of pyrrolidone carboxylic acid ester is largely independent of concentration [8].

When we injected the N-trifluoroacetyl pyrrolidone carboxylic acid isopropyl ester on to a capillary coated with the chiral stationary phase Chirasil-Val, no separation of the enantiomers could be achieved, nor of the N-pentafluoropropionyl derivative. However, this is to be expected, as the two carbonyl groups adjacent to the ring nitrogen may compete as hydrogen acceptors in forming the association complex through hydrogen bonding; this results in very little or no difference in the association enthalpy of both enantiomers [12].

In contrast, the optical isomers of underivatized pyrrolidone carboxylic acid isopropyl ester itself are well separated, as shown in Fig. 2. Obviously, the hydrogen atom still present at the ring nitrogen now brings about a stereoselective association between selector and selectand with a sufficiently large enthalpy difference. While for the N-perfluoroacyl derivatives of all protein amino acids the D-enantiomers are eluted prior to the L-forms, the sequence for pyrrolidone carboxylic acid is reversed.

As the hydrogen atom of the pyrrolidone nitrogen is slightly acidic, the state of deactivation of the capillaries is important for analysis. Capillaries with residual basicity are not suited for analysis, as they cause strong tailing of the peaks. Capillaries deactivated by the barium carbonate procedure are least suited for this purpose, but acid leaching prior to coating of Chirasil-Val yields capillaries exhibiting only slight tailing [13]. The accuracy of the method is shown with a sample of cerebrospinal fluid spiked with increasing amounts of L-glutamine. The resulting concentration of L-glutamine is determined by

enantiomer labelling and the results are presented in Fig. 2. The slope of the obtained line is 1.0003, indicating a recovery of exactly 100%. The correlation coefficient is 0.9994, and the intercept with the ordinate is at 51.6 μg/ml.

Glutamine values were determined for a group of patients with lumbar disc and multiple sclerosis (Table I). Values reported in the literature for normal subjects are in the range 440—600 μmol/l [14—16] with individual variations between 10 and 20%. Our own results are at the lower end of this range, with a coefficient of variation of 13.6%. Whether this divergence is due to deamidation of glutamine during storage will be investigated. In strong contrast to our findings, for patients suffering from multiple sclerosis a low value of 107 ± 93 μmol/l has been reported previously [17]. We consider this as a result of de-

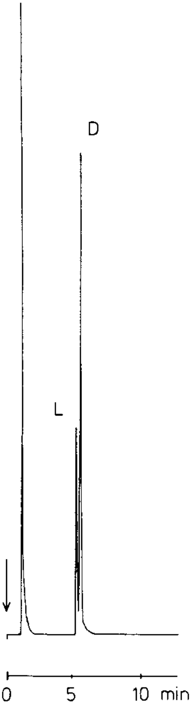

Fig. 2. GC separation of the enantiomers of pyrrolidone carboxylic acid isopropyl ester, formed from L-glutamine in a sample of cerebrospinal fluid, with D-glutamine added as internal standard. Chromatographic conditions are as given in the experimental part.

TABLE I

GLUTAMINE IN CEREBROSPINAL FLUID OF PATIENTS WITH LUMBAR DISC (LD) OR MULTIPLE SCLEROSIS (MS)

	Glutamine found (μmol ± S.D.)	*n*
LD	396 ± 54	5
MS	437 ± 21	4

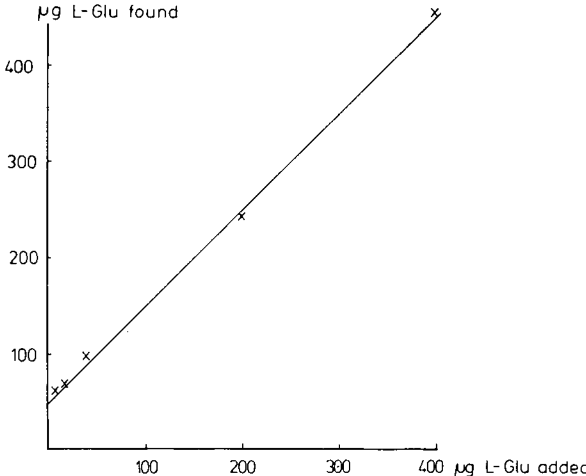

Fig. 3. Calibration line obtained for quantitation of glutamine in a sample of 1 ml of cerebrospinal fluid spiked with increasing amounts of L-glutamine; y (x = 0), 51.6 µg; slope, 1.0003; correlation coefficient, 0.9994.

amidation to glutamic acid during storage, as the latter was found in extraordinarily high concentration. We also determined the glutamine concentration in normal human blood serum. The chromatograms obtained are virtually equal to those of cerebrospinal fluid, i.e. without interference from coeluting compounds.

Obviously, a prerequisite for correct analysis is the absence or at least the exact knowledge of the amount of any endogenous D-glutamine in a sample. In the many samples processed we have never found D-glutamine to be present at a level of more than 1%.

In conclusion, we wish to point out that determination of glutamine by this method does not require any extra treatment of the sample, such as for alternative derivatization. Only during esterification of the amino acids is an aliquot withdrawn after 5 min heating and, after evaporation of reagents and solvent and dilution with an appropriate volume of methylene chloride, directly injected for GC in the isothermal mode. The rest of the sample can be carried through the whole derivatization sequence as required for the other amino acids. Advantageously, no other peaks appear in the gas chromatogram, as the non-acylated amino acid esters are not sufficiently volatile. Thus free glutamine is selectively determined in biological samples without interference from other compounds.

The method is not applicable for the determination of asparagine, which does not cyclize to the corresponding 4-membered lactam. The derivative partially formed after a few minutes' treatment with hydrogen chloride—isopropanol is presumably the asparagine isopropyl ester, which is not sufficiently volatile and stable for GC. N-Acylation is required to arrive at a suitable derivative. The possibility of differentiating between glutamine and glutamic acid also enables us to determine free glutamic acid in the samples by GC. Previously, usually only the sum of the amide and acid were given.

We consider the proposed method to be a further improvement of amino acid analysis by GC, rendering the powerful technique more generally applicable. Gas chromatography is thus a truly alternative if not superior method to ion-exchange chromatography [4].

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CHROMBIO. 859

ISOLATION AND IDENTIFICATION OF SOME GUANIDINO COMPOUNDS IN THE URINE OF PATIENTS WITH HYPERARGININAEMIA BY LIQUID CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Liquid column chromatographic studies of monosubstituted guanidino compounds, which are excreted in the urine of patients with hyperargininaemia are reported. The guanidino-positive peaks, with the highest excretion values, were isolated from urine and the isolated compounds were identified by thin-layer chromatography and gas chromatography—mass spectrometry. Guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid, arginine and α-keto-δ-guanidinovaleric acid were found to be excreted at high levels in the urine of patients with hyperargininaemia compared with controls.

INTRODUCTION

Patients with hyperargininaemia have an arginase deficiency, which leads to blockage of the urea cycle in the last step with symptoms of coma, epilepsy, spasticity and vomiting.

Owing to the arginase deficiency, patients with hyperargininaemia accumu-

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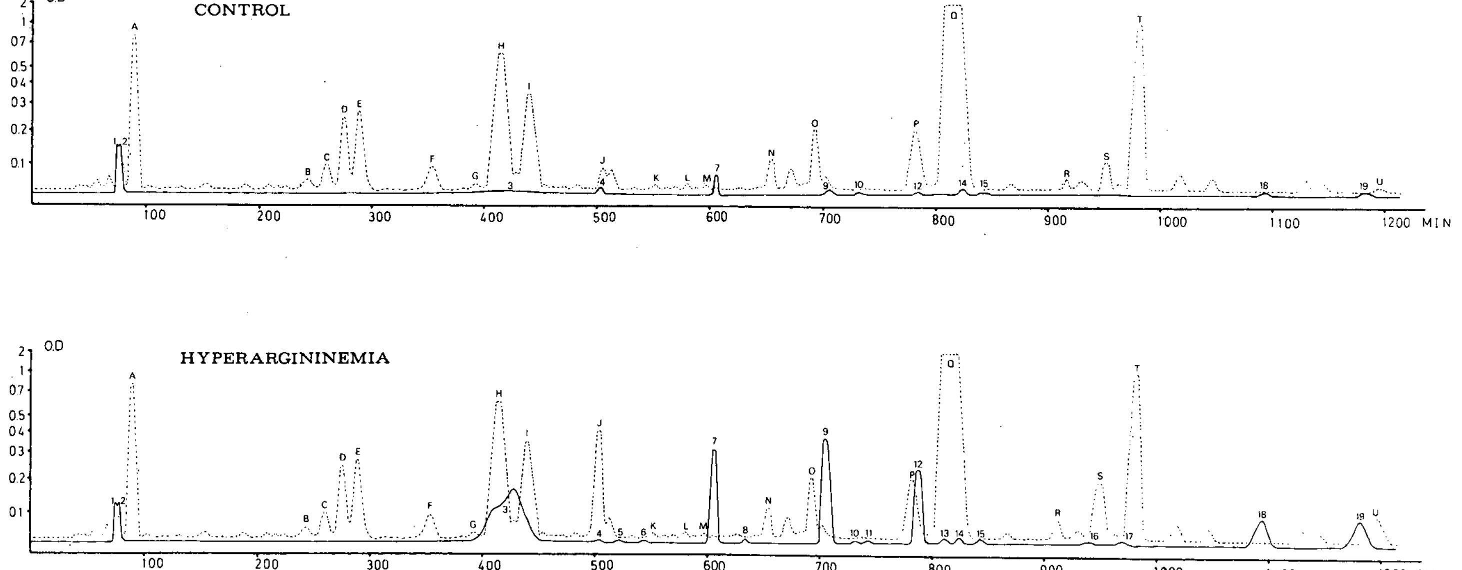

Fig. 1. Elution pattern of urinary monosubstituted guanidino compounds, in parallel with amino acids, of patients with hyperargininaemia and controls. The peak numbers correspond to those in Table I. A, Urea; B, aspartic acid; C, threonine; D, serine; E, glutamine; F, glutamic acid; G, citrulline; H, glycine; I, alanine; J, cystine; K, methionine; L, isoleucine; M, leucine; N, tyrosine; O, β-aminoisobutyric acid; P, ethanolamine; Q, ammonia; R, ornithine; S, lysine; T, histidine; U, arginine.

TABLE I

ABSOLUTE RETENTION TIMES OF MONOSUBSTITUTED GUANIDINO COMPOUNDS ON THE AAI AMINO ACID ANALYSER

Standard*	Hyperargininaemic urine peak number	Retention time (min)	Concentration in urine** (μmole/g creatinine)			
			Patient A.W.***	Patient M.W.***	Patient I.W.***	Controls §
Opheline	—	68	—	—	—	—
§§	1	76	138	N.D.	59	106—180
Urea	2	78	229	N.D.	98	269—469
Taurocyamine	—	86	—	—	—	—
α-Keto-δ-GVA	3	422	5650	6410	904	Trace
Guanidinosuccinic acid	4	505	Trace	—	—	14—97
§§	5	522	Trace	13	—	—
Octopine	—	538	—	—	—	—
§§	6	545	Trace	32	—	—
α-Guanidinoglutaric acid	—	577	—	—	—	—
Guanidinoacetic acid	7	605	1764	2764	1182	182—1213
α-Guanidinopropionic acid	8	634	—	19	—	—
α-Guanidinobutyric acid	—	677	—	—	—	—
N-α-Acetylarginine, α-G-β(CH_3)-BA	9	706	1037	4271	649	19—50
§§	10	730	—	6	10	Trace—19
§§	11	745	21	16	—	—
Argininic acid, α-G-β(CH_3)-VA	12	789	909	1069	61	3—36
§§	13	810	Trace	Trace	Trace	—
§§	14	823	50	280	Trace	28—78
γ-Guanidino-β-OH-butyric acid	—	827	—	—	—	—
§§	15	843	26	75	Trace	Trace—9
β-Guanidinopropionic acid	—	882	—	—	—	—
β-Guanidinoisobutyric acid	—	920	—	—	—	—
L-Arginylaspartic acid	16	940	Trace	Trace	Trace	—
α-Guanidino-β-phenylpropionic acid	—	950	—	—	—	—
§§	17	968	13	52	Trace	—
α-NH_2-β-guanidinopropionic acid	—	1057	—	—	—	—
γ-GB.$CONH_2$, γ-OH-Arg, γ-GBA	18	1093	167	233	19	Trace—31
α-NH_2-γ-guanidinobutyric acid	—	1118	—	—	—	—
Arginine	19	1181	86	127	497	10—73

*α-Keto-δ-GVA = α-keto-δ-guanidinovaleric acid; α-G-β(CH_3)-BA = α-guanidino-β-methylbutyric acid; α-G-β(CH_3)-VA = α-guanidino-β-methylvaleric acid; γ-GB.$CONH_2$ = γ-guanidinobutyramide; γ-OH-Arg = γ-hydroxyarginine; γ-GBA = γ-guanidinobutyric acid.

**N.D. = not determined.

***Excretion values on low-protein diet.

§ Range for eight control children.

§§ Unknown monosubstituted guanidino compound.

late arginine [1,2], which leads to excretion of high levels of guanidino compounds, catabolites of arginine, in the urine [3,4]. Fig. 1 shows the elution patterns of the different monosubstituted guanidino compounds in parallel with the amino acids in the urine of patients and controls. As can be seen in Table I, some guanidino compounds are excreted in 10—100-fold greater amounts than in controls. In addition, some monosubstituted guanidino compounds are excreted at much lower levels but always in higher concentration than in controls. The guanidino compound that is present in the greatest amount has been identified as α-keto-δ-guanidinovaleric acid [5] by liquid, thin-layer (TLC) and gas chromatography—mass spectrometry (GC—MS).

In this work the structures of other guanidino-positive urine peaks were elucidated by using the above-mentioned techniques. These identification techniques were necessary as different guanidino-positive compounds can have identical retention times in liquid column chromatography (Table I). The isolated compounds were identified by TLC and GC—MS.

EXPERIMENTAL

Apparatus

A Technicon AAI amino acid analyser (Technicon Instruments, Tarrytown, NY, U.S.A.) was used. The liquid chromatograph was equipped with a column (140 cm × 6.2 mm I.D.) packed with Dowex 50-X8 resin (Technicon Chromobeads, Type A, particle size 21 μm). The flow-rate was 0.5 ml/min.

GC—MS analyses were carried out on a Nermag R 10-10 quadrupole mass spectrometer. The mass spectrometer was connected with a Girdel gas chromatograph, and a PDP/8a computer system (Nermag, Rueil-Malmaison, France).

TLC was carried out on glass plates coated with cellulose, layer thickness 0.1 mm (E. Merck, Darmstadt, G.F.R.).

Reagents

For liquid and thin-layer chromatography, all reagents were of analytical-reagent grade. The silylating agents hexamethyldisilazane and trimethylchlorosilane were obtained from Pierce (Rockford, IL, U.S.) and the acetylating agent trifluoroacetic anhydride from Aldrich (Milwaukee, WI, U.S.A.). The dimethylpyrimidyl-forming agent was acetylacetone (Merck).

Specimens

The patients were hyperargininaemia were three sisters.

Monosubstituted guanidino standard compounds

Guanidinosuccinic acid, urea, octopine, guanidinoacetic acid, N-α-acetylarginine, argininic acid, β-guanidinopropionic acid, L-arginylaspartic acid, γ-guanidinobutyric acid and arginine were purchased from Sigma (St. Louis, MO, U.S.A.) and α-amino-β-guanidinopropionic acid and α-amino-γ-guanidinobutyric acid from Calbiochem (Lucerne, Switzerland).

Taurocyamine, α-guanidinopropionic acid, α-guanidinobutyric acid, α-guanidino-β-methylbutyric acid, α-guanidino-β-methylvaleric acid and α-guanidino-β-phenylpropionic acid were prepared starting from the corresponding

amino derivative and S-methylisothiourea, according to the method described by Schütte [6]. α-Keto-δ-guanidinovaleric acid was prepared enzymatically as described by Cooper and Meister [7].

γ-Guanidino-β-hydroxybutyric acid, γ-guanidinobutyramide and γ-hydroxyarginine were gifts from Prof. D.J. Durzan (Dept. of Fisheries and Forestry, Petawawa Forest Experiment Station, Chalk River, Ontario, Canada). Opheline and β-guanidinoisobutyric acid were kindly supplied by Prof. Y. Robin (Biochimie Marine, Collège de France, Paris, France). α-Guanidinoglutaric acid was a gift from Prof. A. Mori (Institute for Neurobiology, Okayama, Japan).

Liquid column ion-exchange chromatography

Urine of patients with hyperargininaemia and the standard products were applied either separately or together on the amino acid analyser. The free monosubstituted guanidino compounds were analysed in parallel with amino acids according to the method of Durzan [8]. Liquid ion-exchange chromatography was performed according to the procedure described by Efron [9].

The monosubstituted guanidino compounds were detected with Sakaguchi reagent, prepared as described by Durzan [8]. This reagent is specific for these compounds.

Desalting of the fractions corresponding to the guanidino-positive compounds

The eluent from the amino acid analyser, containing the guanidino-positive peaks 3, 7, 9, 12, 18 and 19 (Table I), was collected and desalted on Dowex 50W-X8 (H^+) ion-exchange resin (50—100 mesh). The guanidino compounds were eluted with 0.5 mol/l ammonia solution, except for arginine, for which 0.75 mol/l ammonia solution was used. Ammonia was removed immediately from the eluent by means of a rotary evaporator. The eluent was then lyophilized and aliquots were used for TLC and GC—MS studies.

Thin-layer chromatography

One-dimensional TLC was performed in a saturated chamber. In order to obtain identical saturation conditions, a device in the lid was made for holding the loaded plates before lowering them in the solvent.

The solvents used were *n*-butanol—glacial acetic acid—water (BuA) (120:30:50) and *n*-butanol—pyridine—water (BuP) (65:65:65). After chromatography the plates were dried at room temperature and the guanidino compounds were located spraying the plates with Sakaguchi reagent prepared according to Robin [10].

Derivatization and GC—MS conditions

As compounds containing a guanidino function are not suitable for GC—MS analysis, the isolated urine fractions were converted into dimethylpyrimidyl derivatives using the method described by Mori et al. [17]. The dimethylpyrimidyl derivatives were then silylated or acylated.

The silylation procedure applied to the dimethylpyrimidyl derivatives of urine peaks (9 and 19) was not satisfactory. Therefore, these dimethylpyrimidyl derivatives were acylated: the dimethylpyrimidyl derivatives were dissolved in 10 ml of *n*-butanol, saturated with dry hydrogen chloride gas and refluxed for 3 h in a water-bath.

The syrup-like substance obtained after drying in vacuo was trifluoroacetylated with 10% trifluoroacetic anhydride in ethyl acetate and applied to the gas chromatograph.

The dimethylpyrimidyl derivatives of peaks 3, 7, 9 and 12 (Table I) were silylated: the dimethylpyrimidyl derivatives were treated with a mixture of 0.5 ml of pyridine, 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane. The silylation occurred at room temperature.

The gas chromatograph was fitted a 2.5-m column of 3% SE-30 on Chromosorb W and a helium flow-rate of 20 ml/min was used. After injection of an aliquot of 1 μl, the oven was programmed from 80 to 220°C at 8°C/min. The mass spectral conditions were as follows: source temperature, 150°C; ionization voltage, 70 eV; emission current, 200 μA; and integration time, 6 msec/peak.

RESULTS

The absolute retention times for monosubstituted guanidino compounds, standards and urine compounds on the amino acid analyser are given in Table I.

Comparison of the retention times of a set of guanidino standards with those of the products eluted from the urine sample showed that peaks 3, 7, 9, 12, 18 and 19 could be attributed to α-keto-δ-guanidinovaleric acid, guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid and arginine, respectively. These designations were confirmed by spiking the urine sample with the standard guanidino compounds, identical peaks being observed.

The identities of peaks 3, 7, 9, 12, 18 and 19 were first investigated by TLC. The hR_F values of the standard products are the same as those of the corresponding isolated urine fraction. Using BuA as solvent, the hR_F values for α-keto-δ-guanidinovaleric acid, guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid and arginine were 45, 39, 51, 46, 58 and 18, respectively. Using BuP as solvent, the hR_F values were 43, 32, 41, 38, 41 and 5, respectively.

Fig. 2 shows the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 3. The molecular ion $M^{+\cdot}$ at m/z = 291 was due to the dehydrated cyclic silylated dimethylpyrimidyl derivative of α-keto-δ-guanidinovaleric acid. Loss of water occurs during the derivatization procedure. Typical fragment ions are at m/z = 276, 202, 174, 107 and 73, the origin of which is shown in Fig. 2. The ion at m/z = 200 (base peak) can be explained by the elimination of $(CH_3)_3SiOH$ from the $(M - H)^+$ fragment ion (m/z = 290). Loss of a $(CH_3)_3$-SiOCO radical from the $(M - H)^+$ ion leads to m/z = 173.

Fig. 3 shows the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 7. The molecular ion $M^{+\cdot}$ at m/z = 253 corresponds to the silylated dimethylpyrimidyl derivative of guanidinoacetic acid. The typical fragment ions are at m/z = 238, 180, 163, 136, 107–108 and 73, the origin of which is shown in Fig. 3.

Fig. 4 shows the mass spectrum of the trifluoroacetylated dimethylpyrimidyl derivative of peak 9. The molecular ion $M^{+\cdot}$ at m/z = 336 corresponds to the dimethylpyrimidyl derivative of N-α-acetylarginine. The typical fragment ions are at m/z = 263, 164, 150, 136, 123 and 107–108, the origin of which is shown in Fig. 4.

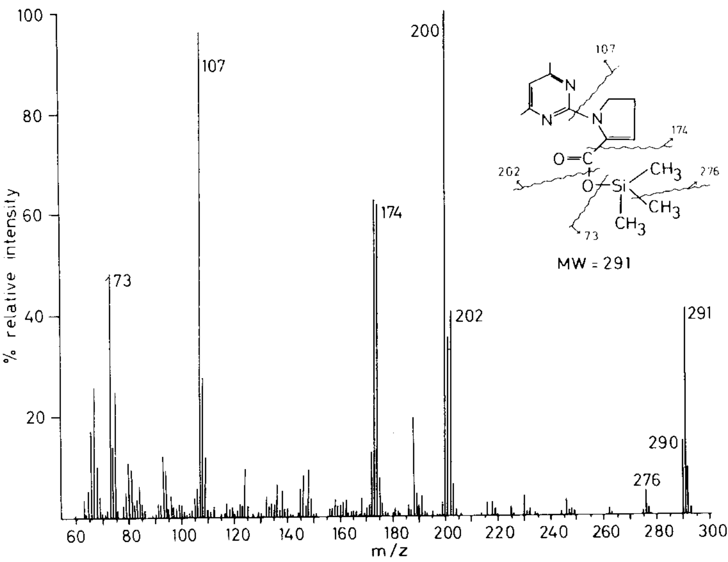

Fig. 2. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 3 (dimethylpyrimidyl derivative of α-keto-δ-guanidinovaleric acid).

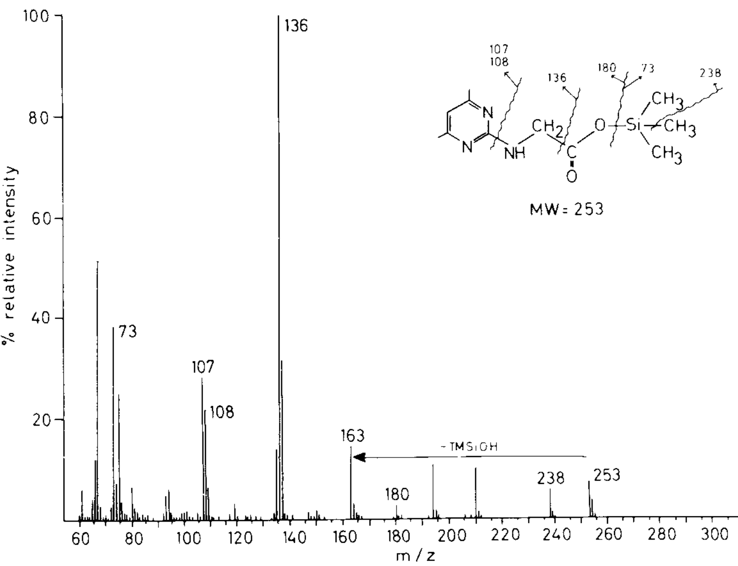

Fig. 3. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 7 (silylated dimethylpyrimidyl derivative of guanidinoacetic acid).

Fig. 5 illustrates the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 12. The molecular ion $M^{+\cdot}$ at m/z = 383 corresponds to the silylated dimethylpyrimidyl derivative of argininic acid. The typical fragment ions are at m/z = 368, 278, 266, 176, 164, 150, 136, 123 and 107—108, the origin of which is shown in Fig. 5.

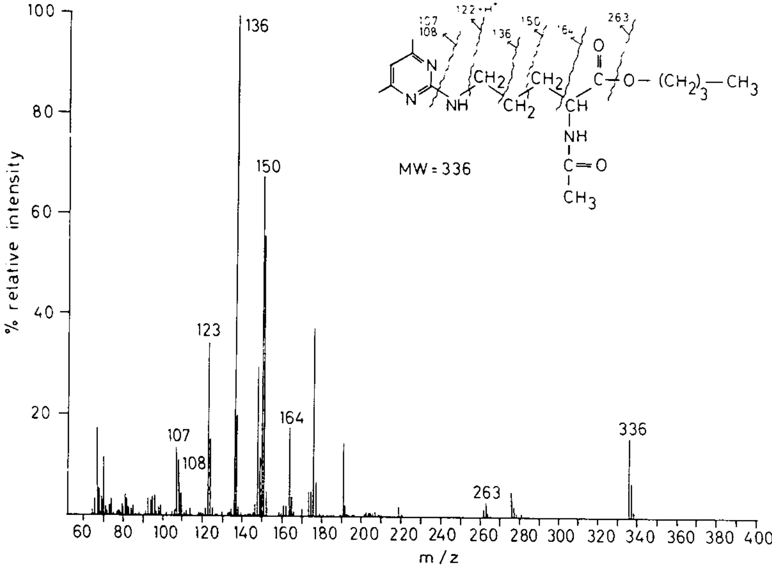

Fig. 4. Mass spectrum of the dimethylpyrimidyl derivative of peak 9 (dimethylpyrimidyl derivative of N-α-acetylarginine).

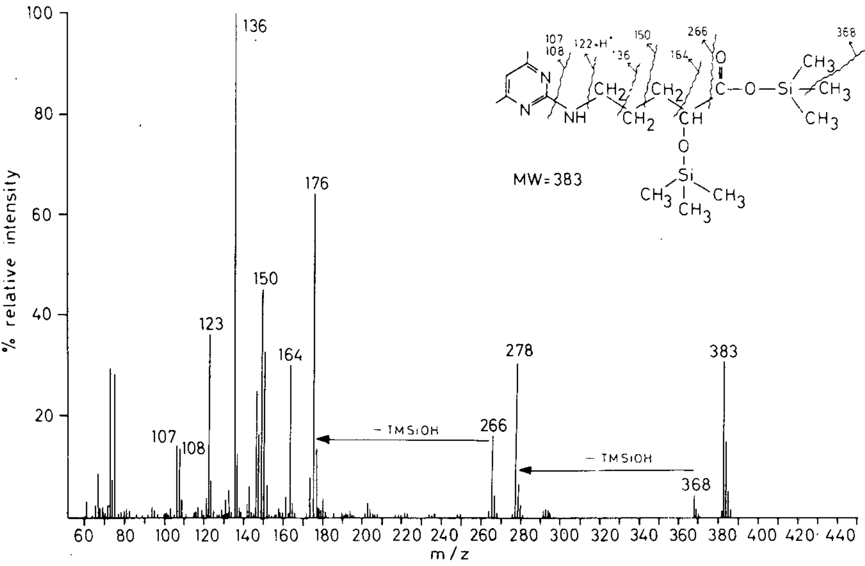

Fig. 5. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 12 (silylated dimethylpyrimidyl derivative of argininic acid).

Fig. 6 shows the mass spectrum of the silylated dimethylpyrimidyl derivative of peak 18. The molecular ion $M^{+\bullet}$ at m/z = 281 corresponds to the silylated dimethylpyrimidyl derivative of γ-guanidinobutyric acid. The typical fragment ions are at m/z = 266, 192, 164, 150, 136, 123 and 107—108, the origin of which is shown in Fig. 6.

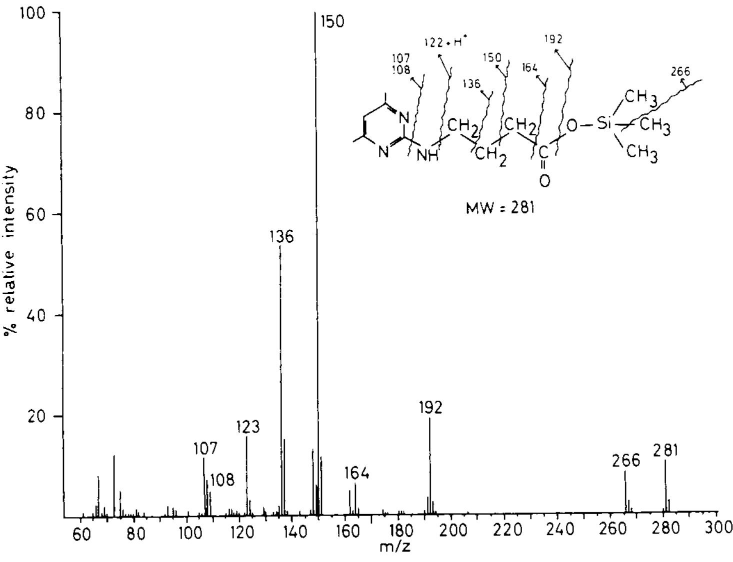

Fig. 6. Mass spectrum of the silylated dimethylpyrimidyl derivative of peak 18 (silylated dimethylpyrimidyl derivative of γ-guanidinobutyric acid).

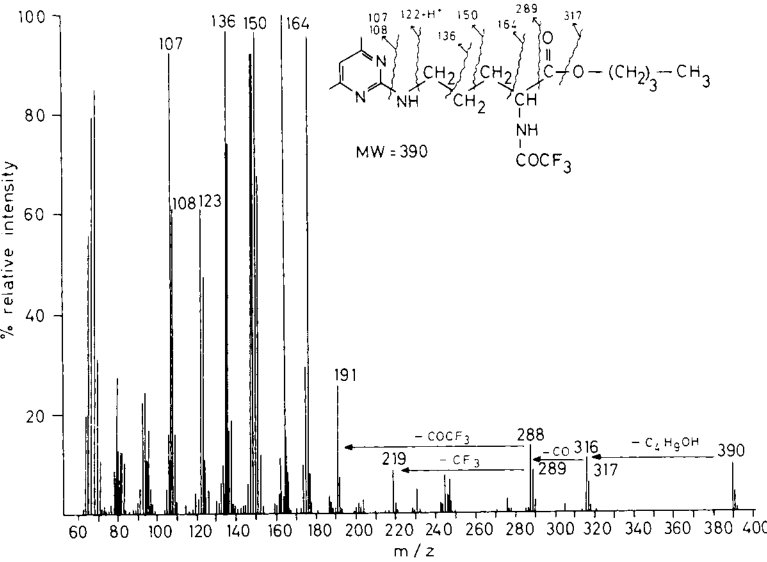

Fig. 7. Mass spectrum of the trifluoroacetylated dimethylpyrimidyl derivative of peak 19 (trifluoroacetylated dimethylpyrimidyl derivative of arginine).

Fig. 7 shows the mass spectrum of the trifluoroacetylated dimethylpyrimidyl derivative of peak 19. The molecular ion $M^{+\cdot}$ at m/z = 390 corresponds to the trifluoroacetylated dimethylpyrimidyl derivative of arginine. The typical fragment ions are at m/z = 316, 288, 219, 191, 164, 150, 136, 123 and 107—108, the origin of which is shown in Fig. 7.

DISCUSSION

The neurological complaints of hyperargininaemia patients raise the question of whether the neurological symptoms are caused by hyperammonaemia, by high arginine levels or by high levels of guanidino compounds. Perhaps all of these anomalies could influence the clinical picture.

Little is known about the neurotoxicity of guanidino compounds in man, although γ-guanidinobutyric acid [11], taurocyamine [12] and methylguanidine [13] have epileptogenic effects on rabbits and cats when administered intracisternally. When more knowledge is acquired about the neurotoxicity of the guanidino compounds, it might then be said that the guanidino compounds are more beneficial than harmful to the patients. In patients with hyperargininaemia the urea cycle is blocked at the last step. If arginine were catabolized by enzymes other than arginase, nitrogen excretion could occur as guanidino compounds instead of urea. Guanidinoacetic acid can be formed by a transamidination reaction: the amidino group of arginine is transferred to glycine [14]. Guanidinoacetic acid is an intermediate in the biosynthesis of creatine and creatinine. γ-Guanidinobutyric acid can also be formed by transamidination of arginine to γ-aminobutyric acid [15]. N-α-Acetylarginine could be formed by acetylation of arginine, just like the formation of N-α-acetylglycine [16]. Deamination of arginine, followed by hydrogenation, could give argininic acid. α-Keto-δ-guanidinovaleric acid could be formed by transamination or deamination of arginine. The other monosubstituted guanidino compounds, in minor concentrations, are also important as nitrogen waste products, and we shall therefore try to identify them all.

We have given here liquid chromatographic data for the mono-substituted guanidino compounds in the urine of three sisters with hyperargininaemia. TLC and GC—MS data for the isolated urinary guanidino compounds have confirmed the identities of guanidinoacetic acid, N-α-acetylarginine, argininic acid, γ-guanidinobutyric acid, arginine and α-keto-δ-guanidinovaleric acid.

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CHROMBIO. 868

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF α-KETO ACIDS

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of eight kinds of α-keto acids. These acids were derivatized with *o*-phenylenediamine into 2-quinoxalinol derivatives, which were extracted into chloroform. The quinoxalinol derivatives were separated by reversed-phase high-performance liquid chromatography using a 250 mm × 2.1 mm I.D. column packed with LiChrosorb RP-8 (5 μm). This method could be satisfactorily applied to urine samples without any prepurification.

INTRODUCTION

Certain α-keto acids, metabolites of amino acids, carboxylic acids or sugars, show a change in concentration in urine or plasma not only in cases of some inborn errors of metabolism such as phenylketonuria, maple syrup urine disease and deficiencies of the pyruvate dehydrogenase complex, but also when there is some change in the balance of the biological system. Therefore, a reliable method for the determination of various kinds of α-keto acids may give some important diagnostic indications.

Several methods [1—6] have been reported for the determination of α-keto acids in biological samples. A colorimetric method [7] using 2,4-dinitrophenylhydrazine has been most widely used. The separation of these 2,4-dinitrophenylhydrazones has been also attempted with some success using paper chromatography [8], gas—liquid chromatography [9] and high-performance liquid chromatography (HPLC) [10, 11]. However, these chromatographic determinations are complicated since the derivative of each α-keto acid can exist as *syn—anti* isomers; moreover, the reaction is not specific for α-keto acids.

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Hinsberg [12] reported originally that α-keto acids react with *o*-phenylenediamine to form stable quinoxalinols in aqueous acidic media. These derivatives were separated by means of thin-layer and paper chromatographic systems [13] and O-trimethylsilyl derivatives were separated by means of gas—liquid chromatography [14—19].

Liao et al. [20] applied this derivatization to the HPLC determination of pyruvic and α-ketoglutaric acids in human urine, because no further derivatization is necessary and the quinoxalinol has high molar absorptivity in the ultraviolet region. We also reported an HPLC determination of phenylpyruvic acid using this kind of reaction [21].

The purpose of this present investigation was to develop an HPLC method utilizing quinoxalinol formation for the determination of pyruvic acid (PA), α-ketobutyric acid (KBA), *p*-hydroxyphenylpyruvic acid (PHPPA), α-ketovaleric acid (KVA), α-ketoisovaleric acid (KIVA), α-ketoisocaproic acid (KICA), phenylpyruvic acid (PPA) and α-keto-β-methylvaleric acid (KMVA), which might be related to some metabolic disorders.

EXPERIMENTAL

Apparatus

A Tri Rotar I high-performance liquid chromatograph equipped with a Model GP-A30 solvent programmer, a Uvidec 100 UV detector and a Model RC-225 strip chart recorder (Japan Spectroscopic Co., Tokyo, Japan) was used in these studies. The HPLC separation was carried out with a 250 mm × 2.1 mm I.D. stainless-steel column packed with LiChrosorb RP-8 (5 μm) using a balanced density slurry packing method. The column was covered with a column jacket and its temperature was kept constant by circulating water from a constant-temperature bath through the jacket. HPLC operating conditions are given in Fig. 1.

Reagents

Sodium pyruvate, sodium α-ketobutyrate, sodium α-ketovalerate, sodium α-ketoisovalerate, sodium α-ketoisocaproate, sodium α-keto-β-methylvalerate, phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid were purchased from Sigma (St. Louis, MO, U.S.A.). *o*-Phenylenediamine sulfate was purchased from Kanto Chemicals (Tokyo, Japan) and used after recrystallization from a mixture (1:1) of 1% aqueous sulfuric acid and ethanol. Flavone was purchased from Tokyo Organic Chemicals (Tokyo, Japan). The other reagents and solvents used were reagent grade.

Preparation of the quinoxalinol derivatives

The 2-quinoxalinol derivatives were prepared via interaction of α-keto acid (0.2 mmol) and *o*-phenylenediamine sulfate (0.25 mmol) in 2 *N* aqueous hydrochloric acid (5 ml) at about 80°C for 2 h. The reaction mixture was diluted with distilled water (20 ml) and the product was extracted with chloroform (20 ml). The organic layer was dried over sodium sulfate and evaporated to dryness. The product was recrystallized from a mixture of distilled water and methanol. The quinoxalinol derivatives prepared are listed in Table I together with melting points and UV spectrophotometric data.

Procedure for the determination of α-keto acids in human urine

To 200 μl of human urine, appropriately diluted with distilled water, were added 2 ml of 2 *N* aqueous hydrochloric acid containing 2 mg of *o*-phenylene-diamine sulfate and 5 μl of 2-mercaptoethanol. The mixture was warmed in a water-bath at about 80°C for 2 h, then cooled and diluted with 8 ml of saturated aqueous sodium sulfate solution. The quinoxalinol derivatives were extracted into 10 ml of chloroform containing 50 nmoles of flavone. The organic phase was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in 3 drops of N,N-dimethylformamide and the solution was diluted with 5 drops of water. About 50 μl of the resulting solution were subjected to HPLC under the conditions shown in Fig. 1.

Calibration curves were prepared in a similar manner.

RESULTS AND DISCUSSION

Generally an α-keto acid does not have sufficient absorption in the UV region for sensitive monitoring with a spectrophotometric detector for HPLC. The quinoxalinol formation was used to provide sensitive determination of α-keto acids. Table I shows the melting points and the spectrophotometric data of the quinoxalinol derivatives. For all the derivatives, λ_{max} was observed at about 230, 280, 325 and 340 nm. Molar absorptivity was greatest at about 230 nm, but 340 nm was chosen as the monitoring wavelength because it seemed that a relatively high selectivity could be obtained using a longer wavelength.

TABLE I

MELTING POINTS AND SPECTROPHOTOMETRIC DATA OF QUINOXALINOL DERIVATIVES

R	m.p. (°C)	Spectrophotometric data [$\epsilon \times 10^{-4}$ (λ_{max}, nm)]			
CH_3	252–253	2.06(229)	0.56(279)	0.64(326)	0.67(338)
CH_2CH_3	198–199	2.15(229)	0.63(279)	0.71(326)	0.72(338)
p-CH_2-C_6H_4-OH	250–251	2.70(227)	0.74(280)	0.77(332)	0.78(342)
$CH_2CH_2CH_3$	188–189	2.13(229)	0.61(279)	0.69(326)	0.72(338)
$CH(CH_3)_2$	234–235	2.06(229)	0.59(270)	0.69(326)	0.71(338)
$CH_2CH(CH_3)_2$	192–193	2.32(229)	0.67(279)	0.74(326)	0.77(338)
CH_2-C_6H_5	205–206	2.59(230)	0.83(282)	0.86(326)	0.87(342)
$CH(CH_3)CH_2CH_3$	186–187	2.12(229)	0.62(279)	0.71(326)	0.73(338)

Quinoxalinol formation from α-keto acid is considered to result in a marked increase in its affinity for non-polar solvents, and various polar compounds present in biological samples. A reversed-phase chromatographic separation, therefore, offered a convenient method for the determination of α-keto acids in such samples. A LiChrosorb RP-8 column was used for this purpose. Table II shows the correlation between the capacity factors of the quinoxalinol derivatives and the acetonitrile percentage in the mobile phase. These data show that

TABLE II

CAPACITY FACTORS OF THE QUINOXALINOL DERIVATIVES DERIVED FROM α-KETO ACIDS

The values in parentheses are the percentage capacity factors compared with that at 15% acetonitrile in the mobile phase.

α-Keto acid	Acetonitrile in mobile phase				
	15%	20%	25%	30%	35%
PA	2.3(100)	1.2(52)	0.7(30)	0.5(22)	0.3(13)
KBA	7.3(100)	3.8(52)	2.5(34)	1.6(22)	0.8(11)
PHPPA	12.1(100)	5.1(42)	2.9(24)	1.6(13)	0.7(6)
KVA	16.9(100)	8.2(49)	5.1(30)	3.0(18)	1.5(9)
KIVA	25.1(100)	12.2(49)	7.5(30)	4.2(17)	2.1(8)
KICA	35.6(100)	16.2(46)	9.6(27)	5.2(15)	2.6(7)
PPA	56.4(100)	23.9(42)	13.5(24)	6.8(12)	3.1(5)
KMVA	56.4(100)	25.4(45)	14.6(26)	7.7(14)	3.7(7)

the capacity factors of the quinoxalinol derivatives which contain an aromatic ring in the substituent group at the 3-position change more sensitively than those of the others. The phenomenon is assumed to be the result of the difference in strength of the π-electron—π-electron interaction between the acetonitrile in the mobile phase and the quinoxalinol derivatives. And the lower concentration of acetonitrile in the mobile phase is more favorable for the separation of 3-ethyl-2-quinoxalinol and 3-(*p*-hydroxybenzyl)-2-quinoxalinol, while the higher concentration is more favorable for the separation of 3-benzyl-2-quinoxalinol and 3-(1-methylpropyl)-2-quinoxalinol in the acetonitrile concentration range investigated. Therefore, a gradient elution technique was adapted for the HPLC separation of the quinoxalinol derivatives. Fig. 1 shows a typical chromatogram obtained from a standard mixture of quinoxalinol derivatives and flavone (internal standard). The broken line in Fig. 1 shows a gradient curve of acetonitrile in the mobile phase measured at the top of the column. Good separation is obtained in 25 min.

The quinoxalinol derivative is formed when the α-keto acid reacts with *o*-phenylenediamine. On the basis of experiments concerning the reaction conditions that may affect the yields of the quinoxalinol derivatives, those described in the procedure were adopted as recommended reaction conditions. The addition of mercaptoethanol did not affect the reaction in the case of standard solutions of α-keto acids, but it depressed the appearance of interfering peaks in the chromatogram for the determination of α-keto acids in human urine sample. Table III shows the yields of the quinoxalinol derivatives, which were calculated by comparing the peak height ratios to those of a standard mixture of the authentic quinoxalinol derivatives and flavone after correction for the percentage extraction.

The extraction conditions of the quinoxalinol derivatives were then studied. The quinoxalinol derivatives could be extracted from the aqueous layer with chloroform and ethyl acetate; chloroform was chosen as the extracting solvent as it separated from the aqueous phase as a lower layer, which was convenient

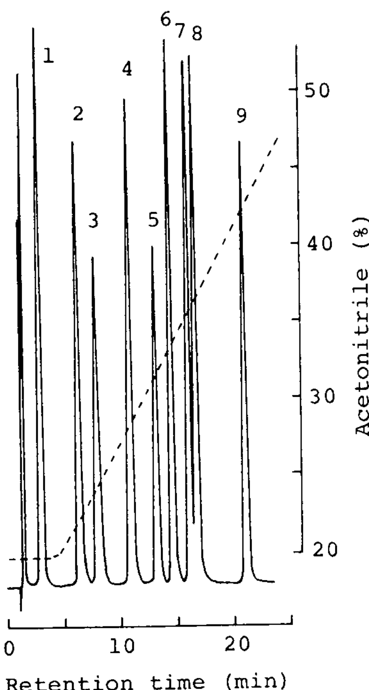

Fig. 1. High-performance liquid chromatogram of a standard mixture of the quinoxalinol derivatives of α-keto acids. The dashed line indicates the gradient curve. Operating conditions: column, 250 mm × 2.1 mm I.D. LiChrosorb RP-8 (5 μm); column temperature, 50°C; mobile phase, aqueous acetonitrile solution; the gradient was prepared using a Model GP-A30 solvent programmer (convex 1, 64 min); flow-rate, 0.6 ml/min; detector, UV spectrophotometer (340 nm). Peaks: 1 = PPA, 2 = KBA, 3 = HPPA, 4 = KVA, 5 = KIVA, 6 = KICA, 7 = PPA, 8 = KMVA, 9 = flavone (IS).

TABLE III

YIELDS OF THE QUINOXALINOL DERIVATIVES DERIVED FROM α-KETO ACIDS UNDER ANALYTICAL CONDITIONS

α-Keto acid	Yield (%)	α-Keto acid	Yield (%)
PA	100	KIVA	54
KBA	76	KICA	75
HPPA	70	PPA	75
KVA	81	KMVA	74

for the separation procedure. The relationship between the extractability of the quinoxalinol derivatives with chloroform and the hydrochloric acid concentration in the aqueous layer is shown in Fig. 2. The extractability of the quinoxalinol derivatives that have a relatively low hydrophobic substituent in 3-position, such as the methyl, ethyl or *p*-hydroxybenzyl group, decreases with the increase in hydrochloric acid concentration in the aqueous phase. Table IV shows the percentage extraction for the quinoxalinol derivatives in the presence of various kinds of salts. The addition of sodium sulfate gives satisfactory

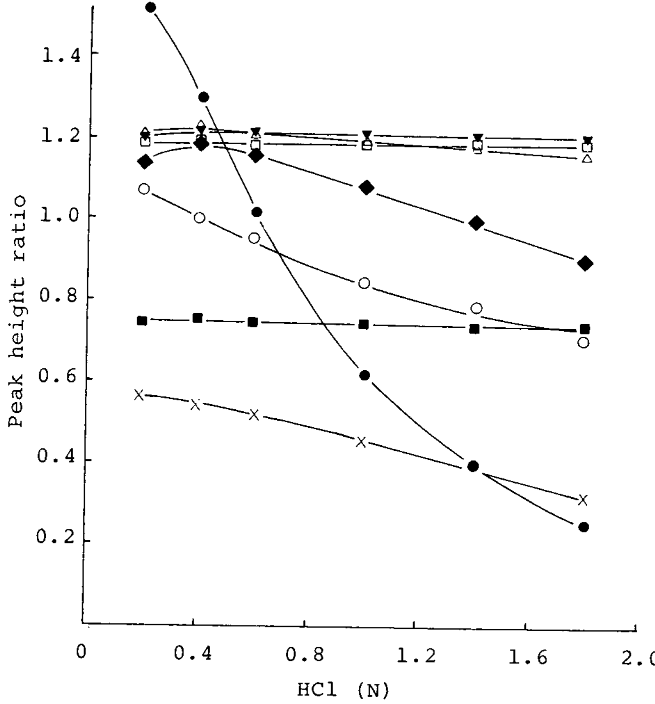

Fig. 2. Relationship between the extractability of the quinoxalinol derivatives and hydrochloric acid concentration in the aqueous layer. The quinoxalinol derivatives of the α-keto acids were extracted from 10 ml of the aqueous phase with 10 ml of chloroform. (●), PA; (○), KBA; (×), HPPA; (◆), KVA; (■), KIVA; (△), KICA; (□), PPA; (▼), KMVA.

TABLE IV

PERCENTAGE EXTRACTION OF THE QUINOXALINOL DERIVATIVES OF α-KETO ACIDS IN THE PRESENCE OF VARIOUS SALTS

α-Keto acid	Percentage extraction				
	—	KCl	NaCl	NH_4Cl	Na_2SO_4
PA	81.9	68.5	50.9	45.9	98.9
KBA	100.6	106.3	89.9	89.4	99.2
PHPPA	64.2	78.9	80.4	65.7	90.8
KVA	98.7	99.8	98.0	97.9	101.3
KIVA	98.7	100.5	98.0	101.2	98.5
KICA	101.5	101.2	102.0	101.5	100.2
PPA	99.2	100.4	98.7	100.3	98.1
KMVA	99.2	100.1	95.6	100.1	99.5

TABLE V

RECOVERY OF α-KETO ACIDS FROM HUMAN URINE

All values are expressed in nmol except where indicated.

Sample No.	PA	KBA	PHPPA	KVA	KIVA	KICA	PPA	PMVA
1	14.8	15.0	16.0	15.0	15.4	14.7	15.2	15.2
2	15.1	15.1	13.9	14.9	15.2	14.7	15.3	15.2
3	15.8	15.7	13.3	15.3	16.6	15.2	14.7	15.5
4	15.6	15.3	16.4	15.2	16.2	14.9	15.5	15.6
5	15.3	14.5	16.4	14.4	15.7	14.5	15.3	15.5
6	15.2	14.5	15.0	14.7	15.3	14.5	14.7	14.9
7	15.3	15.5	15.3	14.8	15.0	14.5	14.8	15.1
8	16.1	15.1	16.3	15.0	15.6	14.8	14.9	15.3
9	15.0	15.1	17.5	14.3	16.0	14.7	14.8	15.4
10	16.3	15.4	15.1	15.1	15.2	14.8	14.3	15.0
Average	15.5	15.1	15.5	14.9	15.6	14.7	15.0	15.3
C.V. (%)	3.1	2.6	8.4	2.2	3.4	1.4	2.4	1.0
Added	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Recovery (%)	103	101	103	99	104	98	100	102

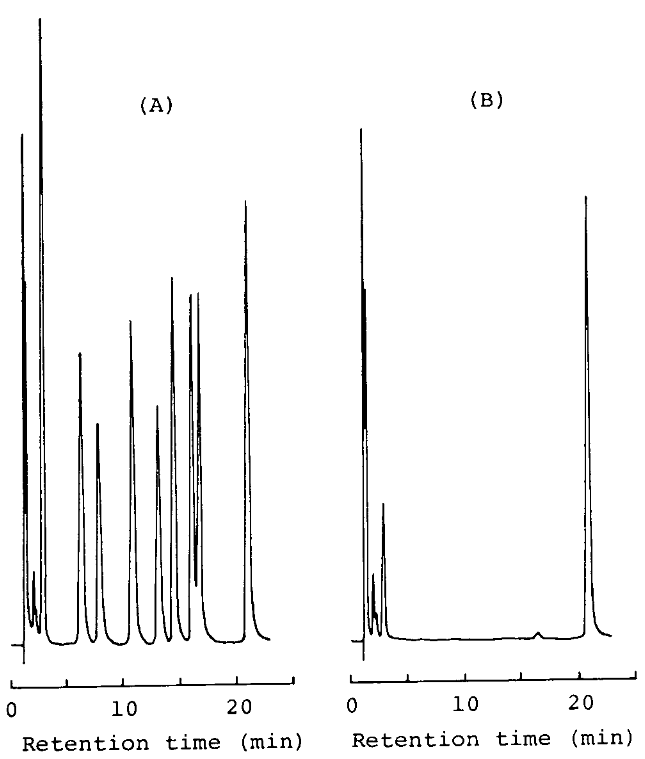

Fig. 3. High-performance liquid chromatograms obtained in the recovery experiments. (A) Spiked urine sample. (B) Urine sample.

results. The extraction was therefore carried out after dilution of the reaction mixture (2 ml) with saturated sodium sulfate solution (8 ml). The internal standard flavone was also almost completely extracted under these conditions.

The calibration graphs obtained using the recommended procedure were rectilinear for at least 0—20 nmoles of α-keto acids.

Table V shows the percentage recoveries and the coefficients of variation obtained for ten replicate measurements made on an identical ten-times diluted sample of human urine (200 μl) to which 15 nmoles of each α-keto acid were added. Fig. 3 shows the chromatograms obtained in the recovery experiments. No interfering peaks were observed. Then, the determination of α-keto acids in normal human urine was carried out. The pyruvic acid/creatinine ratios for normal samples were almost in accord with published values [20]. The other α-keto monocarboxylic acids were not found in normal urine samples.

We are now developing a further method for determining α-keto dicarboxylic acids that might be related to some metabolic disorders.

CONCLUSION

A method for the HPLC determination of eight α-keto acids in human urine has been developed. This method is sensitive, selective and reproducible.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ESTRADIOL-17β AND METABOLITES IN BIOLOGICAL MEDIA

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SUMMARY

A method was developed to resolve radiolabeled estradiol-17β and its various metabolites in biological fluids and tissues. After a rapid initial clean-up step, samples were analyzed with the sequential use of reversed-phase and normal-phase high-performance liquid chromatographic systems. Approximately 25 conjugated and non-conjugated standards could be resolved by the combined use of six systems. Radiolabeled parent compound and metabolites from biological samples were separated and tentatively identified by comparing their retention times to those of known standards. The method was found to be reproducible and quantitative for the majority of the estrogens and their conjugates, and semiquantitative for some of the more polar and di-conjugated estrogens.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the analysis of estrogens has become widespread [1—9]. The reasons for the popularity of HPLC over classical open-column and paper chromatographic techniques include excellent resolving power, short analysis time, good reproducibility and small sample size requirements [3, 5, 7, 9]. Gas chromatographic (GC) methods with resolving power equal to HPLC have been reported [10—16], but are generally inadequate for preparative work. In addition, since many of the estrogen conjugates have high molecular weights, low vapor pressure, and are relatively polar and in some instances, chemically unstable, the general utility of GC techniques would appear to be compromised.

The majority of the reported HPLC methodologies has been developed to resolve non-conjugated estrogens [1—7]. Analysis of estrogen conjugates

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generally required that the steroid nucleus first be liberated by enzyme hydrolysis or solvolysis [1, 3, 5]. Although selective cleavage of estrogen conjugates is considered possible [17—20], this approach precludes the simultaneous gathering of information concerning the relative abundance of individual conjugates and non-conjugates. In view of the growing evidence that specific estrogen conjugates are associated with particular physiological states (e.g., pregnancy) or toxicities (e.g., cholestasis) [21], methods providing a complete estrogen metabolic profile are needed.

Several reports have appeared in the literature describing HPLC methodologies for the resolution of estrogen conjugates but generally have centered around only a few of the many possible metabolites without regard for the accompanying non-conjugated estrogens [22] or have not demonstrated the utility of the methodology for biological media [23].

The present paper describes a series of HPLC systems designed to qualitatively and quantitatively resolve radiolabeled conjugated and non-conjugated estrogens. In addition, the effectiveness of the technique was assessed by its application to biological samples collected from a subhuman primate administered radiolabeled estradiol-17β.

EXPERIMENTAL

Instrumentation

The HPLC systems were composed of Waters Assoc. (Milford, MA, U.S.A.) equipment: Model 6000A pumps, Model U6K injectors, Model 660 solvent programmers and Model 440 dual-channel UV detectors equipped with 280-nm UV filters. Commercial pre-packed reversed-phase columns were used: LiChrosorb RP-18 (5 μm), 250 × 10 mm, and LiChrosorb C_2 (10 μm), 250 × 3.2 mm from Altex Scientific (Berkeley, CA, U.S.A.); LiChrosorb RP-18 (5 μm), 250 mm × 9 mm from Chrompack U.S.A. (Whittier, CA, U.S.A.); Chromegabond Diol (10 μm), 300 × 4.6 mm from ES Industries (Marlton, NJ, U.S.A.) and two (in series) μBondapak C_{18} (10 μm), 300 × 3.9 mm columns from Waters Assoc. Chromatograms were recorded on Fisher Recordall Series 5000 dual-pen recorders. Radioactive fractions were collected on a Micro-Fractionator Model FC-80K (Gilson Medical Electronics, Middleton, WI, U.S.A.) or an Isco Model 328 Golden Retriever (Instrumentation Specialties Company, Lincoln, NE, U.S.A.). The scintillation fluid was Scintisol from Isolab (Akron, OH, U.S.A.). Scintillation counting was performed on a Tracor Analytic Mark III 6881 Liquid Scintillation System (Tracor Analytical, Atlanta, GA, U.S.A.).

Estrogen standards

The estrogens and estrogen conjugates investigated in this study are listed in Table I. The compounds were purchased as sodium or potassium salts or as free acids and were used without further purification. Stock solutions of these estrogens and estrogen conjugates were maintained in 100% methanol. Prior to use, the desired amount of standard was pipetted into a 5.0-ml centrifuge tube and reduced to near dryness in a stream of nitrogen on an N-Evap (Organomation Assoc., Worcester, MA, U.S.A.). The residue was then dissolved in 100—200 μl of the required solvent system.

TABLE I

ESTROGENS AND ESTROGEN CONJUGATES

Trivial name	Abbreviation	Source*
Estrone	E_1	RP
Estradiol-17β	E_2	RP
Estriol	E_3	S
Estrone 3-methyl ether	E_1-3-Met	RP
Estradiol 3-methyl ether	E_2-3-Met	RP
Estriol 3-methyl ether	E_3-3-Met	RP
2-Methoxyestrone	2-MeO-E_1	RP
2-Methoxyestradiol	2-MeO-E_2	RP
2-Hydroxyestrone	2-OH-E_1	SWFRE
2-Hydroxyestradiol	2-OH-E_2	SWFRE
2-Hydroxyestriol	2-OH-E_3	RP
6α-Hydroxyestradiol	6α-OH-E_2	RP
16-Epiestriol	16-Epi-E_3	S
15α-Hydroxyestriol	15α-OH-E_3	Ster
Estrone 3-glucuronide	E_1-3G	S
Estrone 3-sulfate	E_1-3SO_4	RP
Estradiol 3-glucuronide	E_2-3G	S
Estradiol 17β-D-glucuronide	E_2-17G	S
Estradiol 3-sulfate	E_2-3SO_4	RP
Estradiol 17β-sulfate	E_2-17SO_4	RP
Estradiol 3,17-disulfate	E_2-3,17SO_4	RP
Estradiol 3-sulfate,17β-D-glucuronide	E_2-3SO_4.17G	S
Estriol 3-glucuronide	E_3-3G	S
Estriol 16α-glucuronide	E_3-16G	S
Estriol 17β-glucuronide	E_3-17G	S
Estriol 3-sulfate	E_3-3SO_4	RP
Estriol 17-sulfate	E_3-17SO_4	RP
17α-Estradiol 17β-D-glycoside	αE_2-17Glyc	SWFRE

*S = Sigma, St. Louis, MO, U.S.A.; RP = Research Plus Steroid Laboratories, Denville, NJ, U.S.A.; Ster = Steraloids, Wilton, NH, U.S.A.; SWFRE = Southwest Foundation for Research and Education, Custom Synthesis, San Antonio, TX, U.S.A.

To determine the recovery of each estrogen standard after chromatography, standards were chromatographed and the eluted peak was collected, reduced in volume, and rechromatographed. Peak height or peak area as determined by UV absorbancy at 280 nm was compared between the first and second chromatograms.

HPLC systems

The estrogen compounds of interest varied widely as to their polarity, therefore, several HPLC systems were necessary to resolve these agents. Two gradient elution systems and three isocratic systems were developed (Fig. 1).

System A consisted of a LiChrosorb RP-18 (5 μm) 250 × 10 mm (A_1) or a 250 × 9 mm (A_2) reversed-phase column protected by an in-line guard column (25 × 6 mm) packed with Bondapak C_{18}-Corasil. The 50-min convex gradient program (No. 5 on the Model 660 solvent programmer) began with 10% methanol in 0.01 *M* ammonium acetate buffer (pH 6.9) and concluded

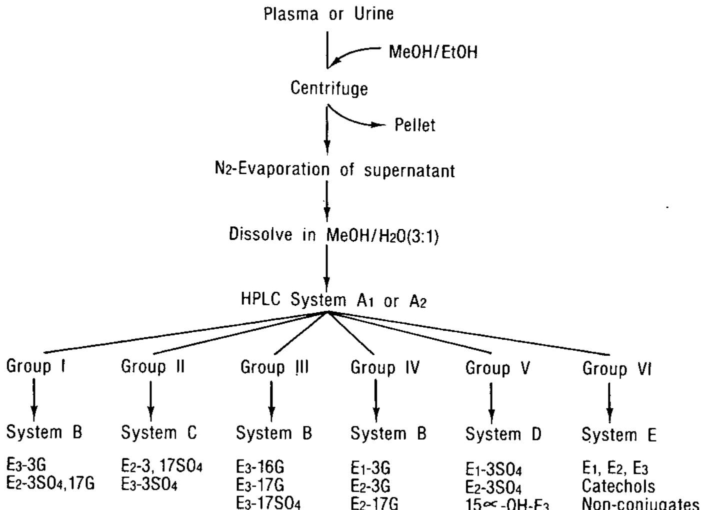

Fig. 1. Flow diagram of the urine and plasma preparation for separation of estrogens and estrogen metabolites into groups by various HPLC systems.

with 100% methanol. The flow-rate was 2.0 ml/min and 1.0-ml fractions were collected.

System B consisted of two μBondapak C_{18} (10 μm) 300 × 3.9 mm reversed-phase columns in series. The solvent contained 45% methanol in 0.01 *M* ammonium acetate buffer adjusted to pH 3.97 with glacial acetic acid. The isocratic system had a flow-rate of 1.0 ml/min and 0.5-ml fractions were collected.

System C was similar to system B except 35% methanol was used and the pH was adjusted to 7.74 with ammonium hydroxide.

System D consisted of a LiChrosorb C_2 (10 μm) 250 × 3.2 mm reversed-phase column. The solvent contained 25% methanol in 0.01 *M* ammonium acetate buffer adjusted to pH 7.56 with ammonium hydroxide. The flow-rate was 1.0 ml/min and 0.5-ml fractions were collected.

System E consisted of a Chromegabond Diol (10 μm) 300 × 4.6 mm normal-phase column. The 100-min linear gradient program (No. 6 on the Model 660 solvent programmer) began with 100% hexane and concluded with hexane—isopropanol (80:20). The flow-rate was 1.5 ml/min and 0.75-ml fractions were collected.

All of the solvents and chemicals used to prepare the solvent systems were reagent grade. The methanol and hexane were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); the isopropanol was from Fisher (Pittsburgh, PA, U.S.A.) and the ammonium acetate from Mallinckrodt (St. Louis, MO, U.S.A.). Deionized water was filtered through a Millipore system and all solvents were degassed prior to use.

Biological sample preparation

In order to assess the utility of the developed HPLC methodology for the analysis of biological samples, an in vivo experiment was performed. Under ketamine (Parke-Davis, Detroit, MI, U.S.A.) anesthesia (10 mg/kg, intra-

muscular) a pregnant 130-day gestational age rhesus monkey (*Macaca mulatta*) was prepared with a femoral artery catheter (No. 5 French) and with a foley catheter (No. 10 French). A HPLC-purified dose of [6,7-^{3}H]estradiol-17β (42.0 Ci/mmole, Amersham, Arlington Heights, IL, U.S.A.) was administered in a 20% ethanol—saline solution via the radial vein. Maternal blood and urine, and fetal blood and tissue samples were collected. The blood samples were immediately centrifuged. The resulting plasma samples and the urine and tissue samples were then stored at −60°C until analysis.

Proteins and salts were removed from urine samples by precipitation with two volumes of methanol—ethanol (1:1). After centrifugation, the precipitate was washed twice with equal volumes of methanol—ethanol (1:1) and the supernatants combined. The supernatant pool was then reduced to near dryness on the N-Evap and subsequently dissolved in 100 μl of methanol—water (3:1) in final preparation for HPLC analysis (Fig. 1).

Plasma samples (0.3—0.5 ml) were made up to 1.0-ml volume by addition of Millipore-filtered water. They were then centrifuged at 8730 *g* in a Beckman micro-fuge to remove the buffy coat. The supernatant was then treated with methanol—ethanol (1:1) and prepared for HPLC analysis in the same manner as the urine.

The procedure employed for tissue extraction and HPLC analysis has been published [9]. Briefly, samples were weighed and placed in ice-packed Erlenmeyer flasks. After adding 5 ml of cold distilled water per g of tissue, each sample was homogenized for 1 min. Five volumes of a mixture of methanol—dimethoxymethane (DMM) (1:1) were used to extract the unbound radioactivity and to precipitate macromolecular components. This mixture was shaken for 18 h and then centrifuged. The supernatant was removed and the pellet washed three times with methanol—DMM (1:1) which was then added to the supernatant. The supernatant was reduced to 1—2 ml volume on a rotary evaporator at 40°C. The sample was washed from the evaporator flask with methanol and filtered through a 0.6-μm BDWP Millipore filter. The filtrate was concentrated to less than 10 ml and passed through a C_{18} Sep-Pak (Waters Assoc.) followed by 5 ml methanol. An aliquot was taken for radioactivity determination and the volume was reduced under a stream of nitrogen to near dryness. The sample was then brought to 225 μl in methanol—water (3:2) in preparation for HPLC.

RESULTS

Estrogen standards

Due to the wide range of polarities, several chromatographic systems were developed to adequately resolve the compounds of interest. The LiChrosorb RP-18 Gradient System A_2 separated the compounds into six arbitrarily defined groups, I—VI, as shown in Figs. 1 and 2. Group 1 includes the most polar compounds E_3-3G and E_2-3SO_4,17G. Group II includes E_2-3,17SO_4 and E_3-3SO_4. Group III consists of E_3-16G and E_3-17G, which have proven inseparable in our laboratory, and E_3-17SO_4. E_1-3G, E_2-3G, and E_2-17G constitute Group IV and E_1-3SO_4 and E_2-3SO_4 are in Group V with 15α-OH-E_3. The least polar compounds consisting mainly of non-conjugates make up the large Group VI.

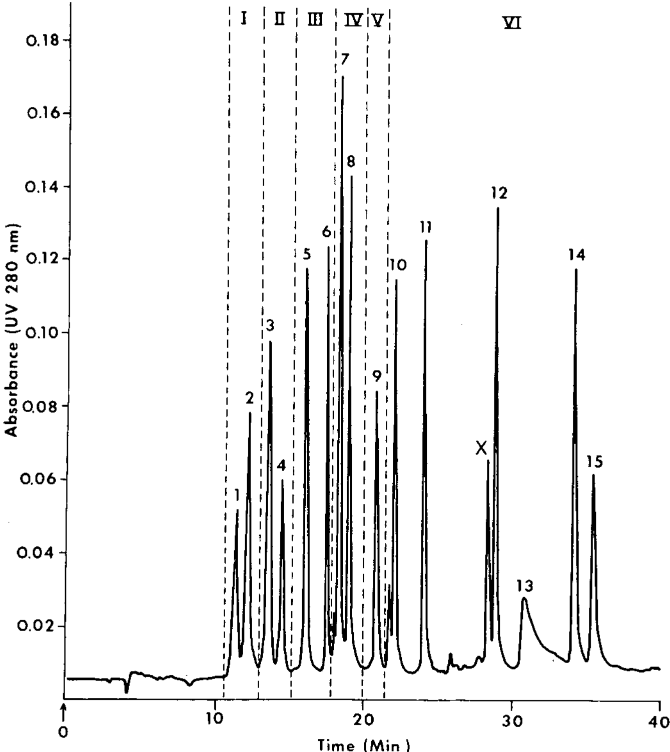

Fig. 2. Chromatogram of estrogen and metabolite standards. System A_2: LiChrosorb RP-18, 5 μm, 250 × 9 mm, reversed-phase column; mobile phase, 10% methanol in 0.01 *M* ammonium acetate, pH 6.9, to 100% methanol, convex gradient program (No. 5) in 50 min; flow-rate, 2.0 ml/min at 2500 p.s.i.; chart speed = 1 cm/2 min; standards: (1) E_3-3G, 20 μg; (2) E_2-$3SO_4$,17G, 200 μg; (3) E_2-3,$17SO_4$, 80 μg; (4) E_3-$3SO_4$, 100 μg; (5) E_3-16G, 20 μg; (6) E_3-$17SO_4$, 10 μg; (7) E_1-3G, 40 μg; (8) E_2-17G, 20 μg; (9) E_1-$3SO_4$, 100 μg; (10) 6α-OH-E_2, 10 μg; (11) E_3, 10 μg; (x) unknown degradation product; (12) αE_2-17Glyc, 20 μg; (13) 2-OH-E_2, 15 μg; (14) E_2, 10 μg, E_1, 10 μg; (15) 2-MeO-E_2, 5 μg.

The eluted fractions comprising the groups thus separated were reduced in volume under nitrogen to near dryness and re-dissolved in 100—200 μl of the appropriate solvent to be further chromatographed. The HPLC System B was found to be effective in further separating Group I standards, E_3-3G from E_2-$3SO_4$,17G (Fig. 3A). Group II standards, E_2-3,$17SO_4$ and E_3-$3SO_4$, were resolved by System C as shown in Fig. 3B. System B provided excellent resolution of Group IV standards (Fig. 3C). Group V was resolved by System D (Fig. 3D). This system also separates E_1-$3SO_4$ and E_2-$17SO_4$. However, E_2-$3SO_4$ and E_2-$17SO_4$ could not be resolved. System E successfully separates the parent compound, E_2, from its primary metabolites E_1 and E_3 and the catechol estrogens (Fig. 4). The conjugate, E_2-17Glyc was also resolved by this normal-phase system. Table II summarizes all the retention times of the estrogen standards in the various HPLC systems. The HPLC system which provides the best resolution of the respective estrogen compounds in biological media is indicated with an asterisk.

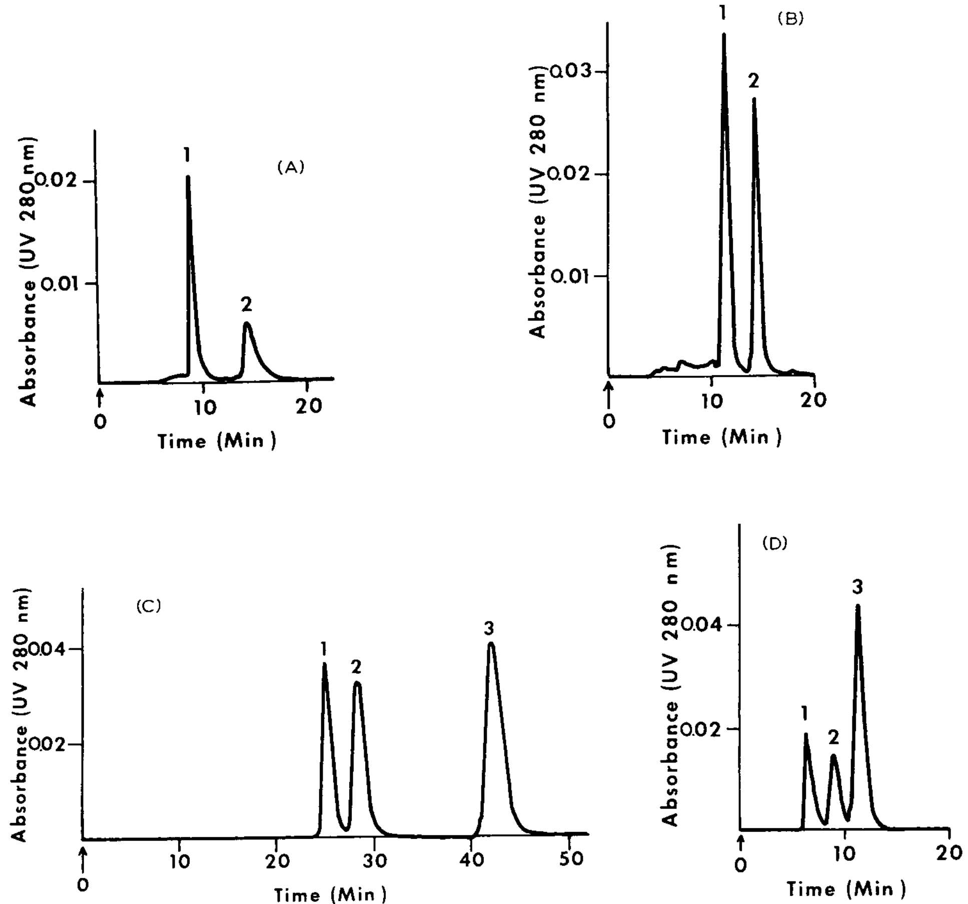

Fig. 3. Separation of Group I, II, IV and V metabolites. A, Group I. System B: two μBondapak C_{18}, 10 μm, 300 × 3.9 mm reversed-phase columns; mobile phase, 45% methanol in 0.01 *M* ammonium acetate buffer, pH 3.97; flow-rate, 1.0 ml/min at 2750 p.s.i.; chart speed = 1 in./10 min; standards: (1) E_3-3G, 15 μg; (2) E_2-3SO_4,17G, 60 μg. B, Group II. System C: same as Fig. 3A but methanol at 35% concentration and pH 7.74; standards: (1) E_2-17SO_4, 40 μg; (2) E_3-3SO_4, 60 μg. C, Group IV. System B: same as Fig. 3A; standards: (1) E_1-3G, 30 μg; (2) E_2-3G, 30 μg; (3) E_2-17G, 30 μg. D, Group V. System D: LiChrosorb C_2, 10 μm, 250 × 3.2 mm reversed-phase column; mobile phase 25% methanol in 0.01 *M* ammonium acetate, pH 7.56; flow-rate, 1.0 ml/min at 2000 p.s.i.; chart speed = 1 in./10 min; standards: (1) E_1-3SO_4, 60 μg; (2) E_2-3SO_4, 60 μg; (3) 15α-OH-E_3, 10 μg.

To determine the recovery of each estrogen standard after chromatography, standards were chromatographed and the eluted peak was collected, reduced in volume, and rechromatographed. Peak height or peak area as determined by UV absorbancy at 280 nm was compared between the first and second chromatograms. The percent recovery of each standard for HPLC System A_2 and subsequent system of choice is listed in Table II. The recovery of standards on the reversed-phase System A_2 ranged between 87 and 111% for all compounds with the exception of the catechol estrogens 2-OH-E_1 (74%), 2-OH-E_2 (65%)

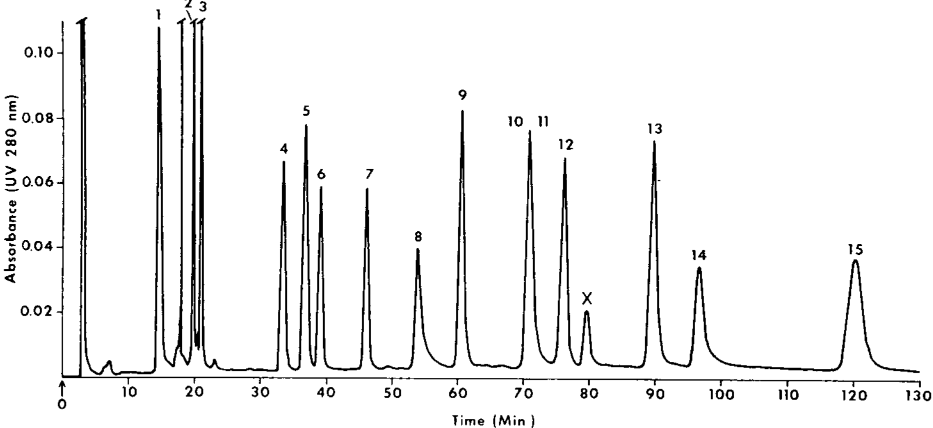

Fig. 4. HPLC profile of estrogen and metabolite standards. System E: Chromegabond Diol, 10 μm, 300 × 4.6 mm normal-phase column; mobile phase, 100% hexane to hexane—isopropanol (80:20), linear gradient program (No. 6) set for 100 min; flow-rate, 1.5 ml/min at 300 p.s.i.; chart speed = 1 in./10 min; standards: (1) E_1-3-Met, 30 μg; (2) E_2-3-Met, 10 μg; (3) 2-MeO-E_1, 10 μg; (4) E_1, 10 μg; (5) 2-MeO-E_2, 10 μg; (6) E_3-3-Met, 10 μg; (7) E_2, 10 μg; (8) 2-OH-E_1, 20 μg; (9) 16-Epi-E_3, 30 μg; (10) 2-OH-E_2, 15 μg; (11) E_3, 10 μg; (12) 6α-OH-E_2, 10 μg; (x) unknown degradation product; (13) 15α-OH-E_3, 25 μg; (14) 2-OH-E_3, 30 μg; (15) αE_2-17Glyc, 30 μg. The unlabeled UV spike between standard (1) and (2) is the isopropanol solvent front.

and 2-OH-E_3 (71%). These 2-hydroxylated estrogens also exhibited lower recovery on the normal-phase Chromegabond Diol column (System E). It would appear that the juxtaposition of the two OH-groups in the A-ring is responsible for the poor recovery since the structurally related metabolic precursors E_1, E_2 and E_3 or metabolic products (2-methoxy compounds) showed good recovery (87—96%) in HPLC System A_2 (Table II). In general, the sulfoconjugates exhibited slightly less recovery than other conjugates in the reversed-phase System D (59—87%) but not in the initial system, A_2. In all cases, the retention times of the estrogen standards were the same for the first and second chromatograms.

Biological samples

In order to demonstrate the utility of the HPLC methodology, radiolabeled E_2 was administered to a rhesus monkey and samples of urine, blood and tissue were collected. These samples were prepared for analysis as described in Biological Sample Preparation and then co-chromatographed with appropriate estrogen standards.

Fig. 5 is a typical chromatogram of a sample of monkey urine from System A_1. The majority of the polar endogenous material and pigments absorbing at 280 nm are eluted before the radioactivity. Then followed a large amount of endogenous UV-absorbing material co-migrating with the radioactivity of the polar Groups I and II and the intermediate Group III. The UV profiles of the crystalline standards eluting in these three groups were partially screened by the high UV-background. The percent recovery of seven conjugates based on three replicate analyses of a 1-h urine sample is seen in Table III. Approximate-

TABLE II

RETENTION TIME IN MINUTES OF THE ESTROGEN STANDARDS IN THE VARIOUS HPLC SYSTEMS

Values in parentheses are the percent recovery of the standards after rechromatography in the designated HPLC system.

Estrogen	HPLC system					
	A_1	A_2	B	C	D	E
E_3-3G	14	11(88)	8(100)*	9	2	
E_2-3SO_4,17G	15	12(105)	14(91)*	7	2	
E_2-3,17SO_4	16	13(108)	9	11(88)*	2	
E_3-3SO_4	16	14(90)	9	15(84)*	2	
E_3-16G	19	16(96)	16(98)*	17	4	
E_3-17G	19	16(96)	16(100)	17	4	
E_3-17SO_4	19	17(96)	16	20(83)*	5	
E_1-3G	21	18(102)	25(100)*	22	4	
E_2-3G	21	18(98)	28(90)*	26	4	
E_2-17G	22	19(104)	42(95)*	32	6	
15α-OH-E_3	24	20(92)	—		12	90(79)*
2-OH-E_3	24	20(71)*	—		—	97(39)
E_2-17SO_4	—	20(95)	26		9(87)*	—
E_1-3SO_4	24	21(99)*	24		7(70)*	—
E_2-3SO_4	24	21(88)*	26		9(59)	—
6α-OH-E_2	25	22(111)				76(81)*
E_3	27	24(96)*				71
16-Epi-E_3	31	28(95)				61(81)*
αE_2-17Glyc	—	28(97)*				120
2-OH-E_2	34	30(65)				71(89)*
2-OH-E_1	34	30(74)*				54(54)
E_1	36	34(94)				34(85)*
E_2	37	34(88)				46(80)*
2-MeO-E_2	39	38(87)				37(83)*
2-MeO-E_1	—	38(88)				21(90)*
E_1-3-Met						14(85)*
E_2-3-Met						20(83)*
E_3-3-Met						39(84)*

*HPLC system of choice for resolution of estrogens in biological samples.

ly 62% of the radioactivity in the sample co-migrated with known estrogen standards while the other 38% was not identifiable. The largest portion of urinary radioactivity co-chromatographed with E_1-3G, 27.63% ± 1.76 (mean ± S.E.) as seen in Fig. 6. Another 17% migrated with the standards E_2-3G, E_2-17G and E_1-3SO_4 (Table III). The standard error of the mean and coefficient of variation (C.V.) for these four estrogen conjugates is quite small indicating a reproducible assay. Three more polar conjugates were also found to co-migrate with significant amounts of radioactivity (17.3%). As indicated by the larger C.V., these polar conjugates were more difficult to reproducibly quantify. The total recovery of radioactivity after complete analysis was >92%.

A representative HPLC chromatogram of fetal liver tissue as resolved by

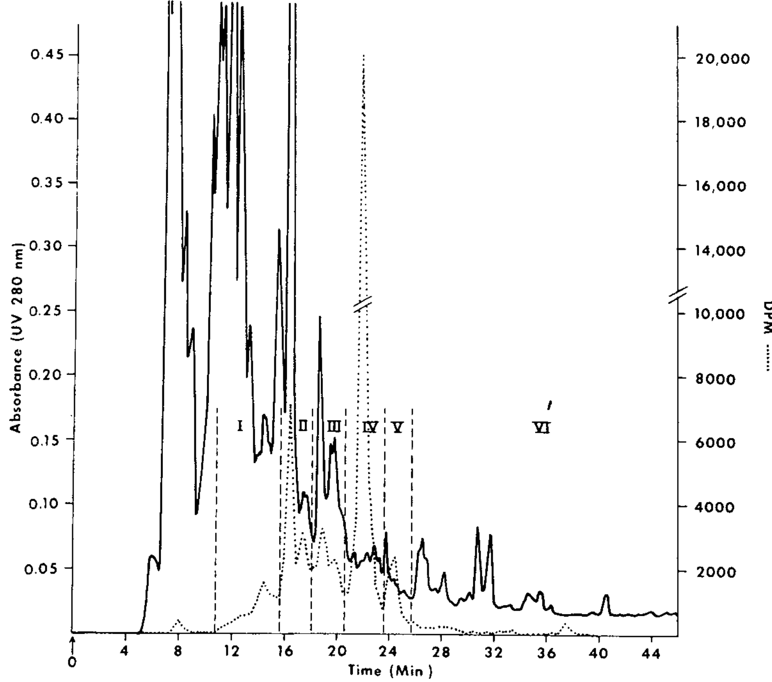

Fig. 5. A typical chromatogram of a sample of monkey urine containing tritium-labeled metabolites of [^{3}H]estradiol-17β. System A_1: same conditions as Fig. 2. Standard, E_3-16G, 25 μg; retention time, 19 min. The solid line is the UV absorbancy at 280 nm. The dotted line represents the distribution of tritium radioactivity in dpm.

TABLE III

PERCENT RECOVERY OF ESTROGEN CONJUGATES FROM URINE

Conjugate	Trial no.			Mean	S.E.	C.V.*
	1	2	3			
E_3-3G	3.2	5.6	5.1	4.63	0.73	27
E_2-3,17SO_4	4.1	5.0	8.5	5.87	1.34	39
E_3-3SO_4	4.1	10.3	6.0	6.80	1.84	47
E_1-3G	26.4	31.3	25.4	27.63	1.76	11
E_2-3G	6.2	7.5	6.1	6.60	0.45	12
E_2-17G	6.1	7.1	5.5	6.23	0.47	13
E_1-3SO_4	4.4	4.5	3.3	4.07	0.39	17
Unknown	29.1	24.5	37.2	30.27	3.72	21
Total	83.6	95.6	97.1	92.10	—	—

$$^{*}\text{C.V.} = \frac{\text{standard deviation}}{\text{mean}} \times 100.$$

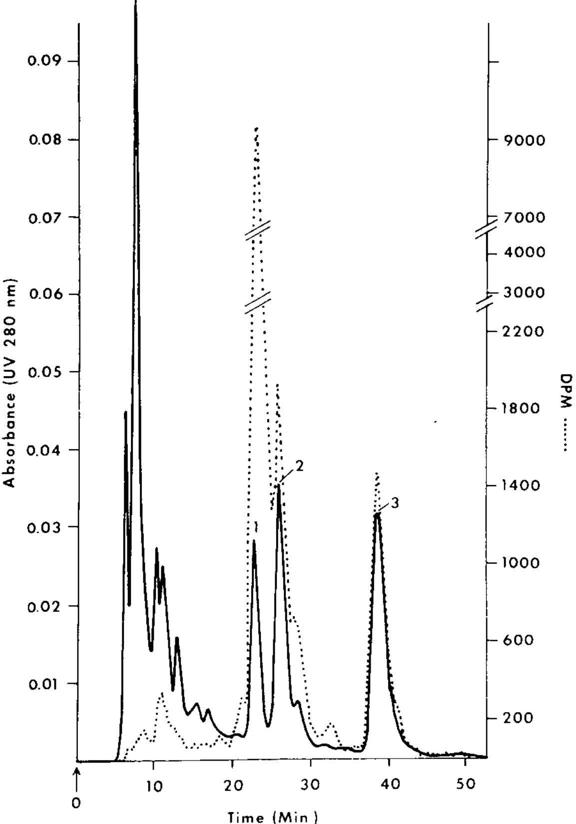

Fig. 6. Separation of metabolites upon rechromatography of Group IV fraction from monkey urine (HPLC System B). Chromatographic conditions same as in Fig. 3C; standards: (1) E_1-3G, 10 μg; (2) E_2-3G, 20 μg; (3) E_2-17G, 20 μg. The solid line is UV absorbancy at 280 nm. The dotted line represents the distribution of tritum radioactivity in dpm.

system A_1 is shown in Fig. 7. The majority of the radioactivity administered to the maternal monkey as [^{3}H] estradiol-17β was converted to a metabolite which co-migrated with E_1-3SO_4. Further, chromatography of this radioactivity in system D demonstrated that over 95% co-migrated again with E_1-3SO_4 and not E_2-3SO_4 or 15α-OH-E_3 (data not shown). Fetal plasma samples showed similar results following HPLC analysis (Fig. 8). In either the tissue or plasma, the endogenous UV-absorbing material near the front of the chromatogram did not interfere with the identification of estrogen standards. Total recovery of radioactivity from the plasma and tissue samples was always greater than 80%.

DISCUSSION

The HPLC methodology herein presented provides a means to qualitatively and quantitatively describe the metabolic profile of the naturally occurring estrogen, estradiol-17β. With the aid of radiolabeled isotopes, the described method may be used to determine the pharmacokinetics of estradiol and its

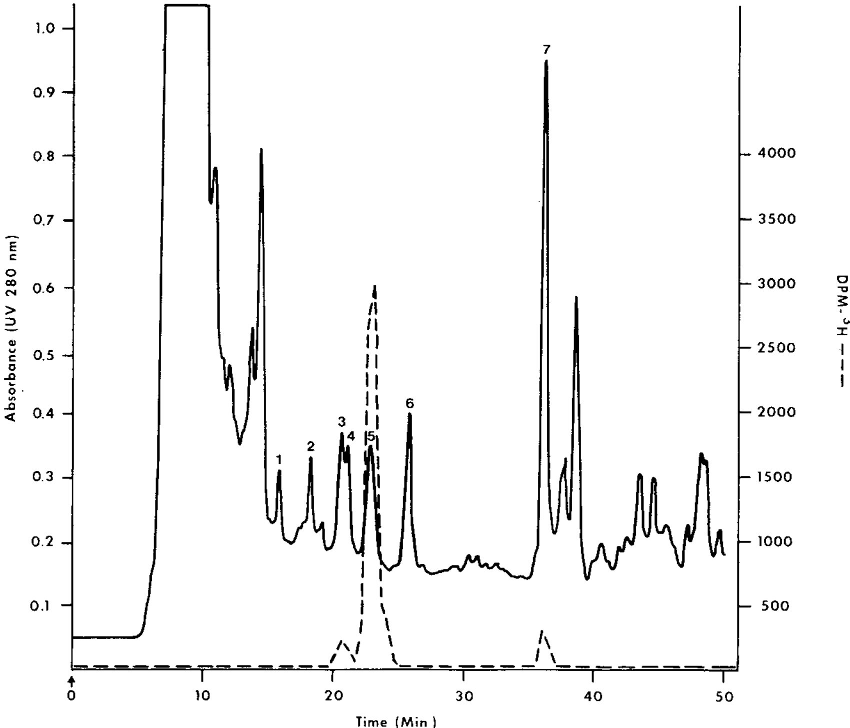

Fig. 7. A typical chromatogram of fetal monkey liver tissue containing tritium-labeled metabolites of [^{3}H]estradiol-17β. System A_1: same conditions as Fig. 2; standards: (1) E_2-3,17SO_4; (2) E_3-16G; (3) E_1-3G; (4) E_2-17G; (5) E_1-3SO_4; (6) E_3; (7) E_2. The solid line is UV absorbancy at 280 nm. The dashed line represents the distribution of tritium radioactivity in dpm.

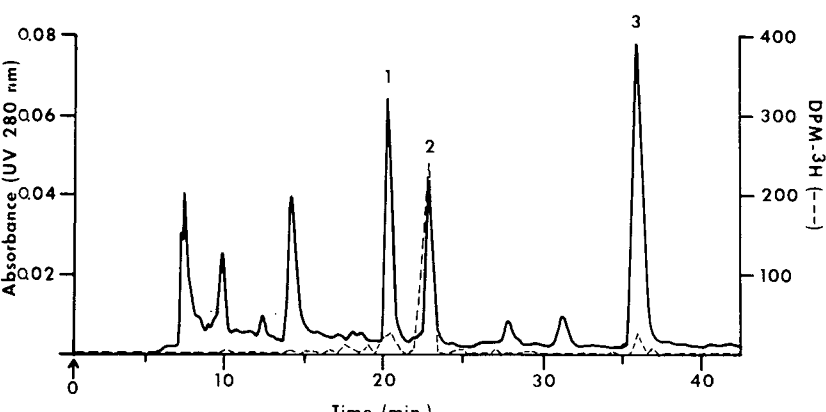

Fig. 8. A typical chromatogram of a fetal plasma sample containing tritium-labeled metabolites of [^{3}H]estradiol-17β. System A_1: same conditions as Fig. 2; standards: (1) E_1-3G; (2) E_1-3SO_4; (3) E_2. The solid line is UV absorbancy at 280 nm. The dashed line represents the distribution of tritium radioactivity in dpm.

metabolites in the plasma, tissue and excretory products of experimental animal models. A variety of methods are available in the literature concerning estrogen analysis [1—3, 5, 22, 24], but to our knowledge, this report is the first to exemplify the utility of a HPLC method for biological samples which provides for the profiling of estrogen metabolites including conjugates and non-conjugates simultaneously. By eliminating the need for solvent extraction and hydrolysis steps, the time required for quantitation of metabolites has been reduced as compared to previous techniques [25].

In the present report, quantification of the various estrogen metabolites is based on radioactivity. A disadvantage of this technique is that the use of radioisotopes is generally restricted to animal studies and can only rarely be applied to human experiments. On the other hand, an advantage of radioisotope studies is that the sensitivity of the method is determined by the specific activity of the radioisotope in question. In the case of estradiol-17β, an isotope is available commercially which has a specific activity of over 100 Ci/mmole. Therefore, based on the ability to quantify 200 dpm of radiolabeled estrogen, (signal-to-noise ratio of 8:1) the sensitivity of the present radioisotope method is greater than 0.5 pg per 0.5 ml biological fluid (1.0 ppt).

Because of the range of polarities of the many possible estradiol metabolites, a number of HPLC systems and columns were found necessary for the complete resolution of the 25—30 standards. It was essential that the initial HPLC system applied to resolve the various estrogens be capable of separating the entire polarity range of possible metabolites from highly polar diconjugates to low polarity methylated non-conjugated metabolites. A reversed-phase gradient system was found suitable to accomplish this separation. As expected, the relative retention times of standards were the same between the two commercially available columns which contained the same stationary phase (LiChrosorb RP-18) but differed in column diameter (A_1, 250 × 10 mm; A_2, 250 × 9 mm). As was observed in our studies with the HPLC analysis of diethylstilbestrol [9], the ammonium acetate buffer was found to decrease solute tailing.

In order to resolve the several closely related estrogen conjugates in each of Groups I—V, two different reversed-phase packings, three different methanol – ammonium acetate solvents and three different pH adjustments were necessary. As expected, it was observed that the polar conjugates were more readily separated with the use of a more polar solvent mixture (i.e., larger water – methanol ratio).

The single normal-phase system (E) adequately resolved the 14 non-conjugates and one glycoside conjugate studied. Williams and Goldzieher [26] were the first to report the use of a Chromegabond Diol column for the separation of estrogens (i.e., ethynylated estrogens). More recently, this normal-phase column has been used to resolve a variety of estrogens including 17α-ethynyl-estradiol and diethylstilbestrol [25].

The recovery of the non-catechol estrogen standards following rechromatography ranged between 87 and 111% in the initial HPLC System A_2. This high recovery indicates that the collection, evaporation and chromatography steps of the procedure do not result in alteration or loss of estrogen standard. The catechol estrogens, however, exhibited a lesser recovery (65—74%) perhaps

relecting their susceptibility to oxidation. In an attempt to increase the recovery of the labile catechol estrogens, an inert gas, argon, was bubbled through the HPLC solvents and utilized in all evaporatory steps of the method. The percent recovery of E_1 and 2-hydroxy-E_1 and E_3 was compared with and without the use of argon in HPLC system A_2. The argon gas treatment failed to increase the recovery of the catechol estrogens (unpublished observation). An earlier report in the literature also described reversed-phase and normal-phase HPLC separation of the 2-OH-E_1 and 2-OH-E_2 [6]. However, no estimation of the recoveries of these catechol estrogens was provided for comparison.

As noted in the results, the sulfoconjugates resolved in HPLC System D exhibited lesser recovery than in HPLC System A_2. An explanation of this difference may reside in the fact that the reversed-phase column packing for System D was C-2 and not the more common C-18. The shorter side-chains on the silicon base of the C-2 column may allow for more ionic interactions and thereby reduce the stability and recovery of these relatively unstable conjugates, E_2-17SO_4, E_1-3SO_4 and E_2-3SO_4.

The requirement of more than one chromatographic system to separate the various E_2 metabolites was also observed by Van der Wal and Huber [23]. It is important to note however, that up to 14 metabolites, both free and conjugated, can be resolved with the initial LiChrosorb RP-18 HPLC system (A_1 or A_2). We have found that the significant, plasma-borne conjugates of E_2 are relatively few in number as compared to the urinary products and may be adequately defined by this single chromatographic system (Fig. 8).

A problem area of the described methodology revolves around three of the least polar estrogen standards; E_1-3-Met, E_2-3-Met and E_3-3-Met. These standards are well resolved by HPLC System E and exhibit good recovery (83—90%). However, they are so non-polar that their recovery from the methanol-based reversed-phase HPLC System A_2 is impeded. If these estrogen derivatives are expected to be present in a biological sample, then they may be first extracted with a non-polar solvent such as chloroform or benzene and then chromatographed on HPLC System E.

Based on the relative retention times of the estrogens on the LiChrosorb RP-18 column, it would appear that classical solvent partitioning methods would not discriminate conjugates from polar non-conjugated estrogens. For example, both 2-OH-E_3 and 15α-OH-E_3 exhibited the same retention time as E_1-3SO_4 in System A_1 and A_2, while αE_2-17Glyc had a greater retention time than several non-conjugates including 2-OH-E_3, 15α-OH-E_3, 6α-OH-E_2 and E_3 in these reversed-phase systems.

The reproducibility and recovery of the method was tested by comparing multiple HPLC separations of a single urine sample collected from an animal dosed with radiolabeled E_2. Total recovery of radioactivity from the procedural analysis was over 92% while approximately 62% of this co-migrated with known standards. The coefficient of variation ranged from 11 to 17% for the most abundant and least polar metabolites and up to 47% for the more polar metabolites. This trend, i.e., that the more polar conjugates exhibited greater variability than the others, may in part be produced by the large amount of interfering, endogenous material which co-migrates with these polar compounds.

In conclusion, the described sample preparation and HPLC methodologies are capable of the rapid and efficient resolution of free and conjugated metabolites of radiolabeled estradiol-17β from biological tissues and fluids.

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ANALYSIS OF PREDNISONE, PREDNISOLONE AND THEIR 20β-HYDROXYLATED METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic technique primarily developed for use on samples from kidney perfusion studies is presented for simultaneous determination of prednisone, prednisolone and their 20β-hydroxylated metabolites. The technique employs 6β-hydroxycortisol as the internal standard. Samples are extracted with ethyl acetate, washed with sodium hydroxide and water and injected onto a silica gel column with UV detection at 254 nm. Inter- and intraday variability of the assay was determined at two concentrations of each steroid and was less than 10%. Assay steroid recovery ranged from 54.1% for prednisone to 63.2% for 20β-hydroxyprednisone. Sensitivity is 4—10 ng/ml for the steroids measured. The chromatographic conditions may be modified to permit quantitation of these steroids from plasma samples. This method may alternatively be used for quantitation of 6β-hydroxycortisol, an endogenous indicator of enzyme induction. A perfusate concentration—time profile is presented from a kidney perfusion study using prednisolone.

INTRODUCTION

Perfusion studies examining the renal disposition of prednisone and prednisolone have demonstrated extensive metabolism of these compounds by the rat kidney [1, 2]. In a similar experiment exploying cortisol, a high degree of metabolism to cortisone as well as production of the 20β-hydroxylated derivatives of cortisol and cortisone was demonstrated using radioactive cortisol and thin-layer chromatography [3]. Given the similarities in structure between the prednisone prednisolone and cortisone—cortisol steroid pairs, the 20β-hydroxy derivatives of prednisone and prednisolone may be major metabolites resulting from the renal biotransformation of these substrates by the kidney

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[3]. To quantitatively examine the renal metabolism of prednisone and prednisolone in the isolated perfused kidney, a sensitive and specific assay for measurement of these steroids was needed. This report describes a high-performance liquid chromatographic (HPLC) technique for determinations of prednisone, prednisolone, and their 20β-hydroxylated metabolites in perfusate and urine samples. Modification of the chromatographic conditions to permit quantitation of these compounds in plasma is also described.

EXPERIMENTAL

Materials

The HPLC system utilized in this procedure consisted of a Waters Model 6000A solvent delivery system, U6K universal loop injector and a Model 440 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.). The UV absorbance of all steroids was measured at 254 nm. A Zorbax™ Sil (DuPont Labs., Wilmington, DE, U.S.A.) column (25 cm × 4.6 mm I.D., 5–6 μm particle size) equipped with a 70 × 6 mm stainless-steel Whatman precolumn was used to separate the compounds. The precolumn was packed with HC-Pellosil (37–53 μm particles) (Whatman, Clifton, NJ, U.S.A.). The ethyl acetate used in the extraction procedure and the heptane and methylene chloride used in the mobile phase were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The pure ethyl alcohol (U.S.A.) employed in the mobile phase was obtained from U.S. Industrial Chemicals (New York, NY, U.S.A.). The glacial acetic acid, also used in the mobile phase was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Prednisone and prednisolone were purchased from Sigma (St. Louis, MO, U.S.A.). The 1,4-pregnadiene-17α,20β,21-triol-3,11-dione (20β-hydroxyprednisone) was a gift from Schering Corporation (Bloomfield, NJ, U.S.A.). The 1,4-pregnadiene-11β,17α,20β,21-tetrol-3-one (20β-hydroxyprednisolone) was a gift from the Steroid Reference Collection (Westfield College, London, Great Britain) and 6β-hydroxycortisol was a gift from Lederle Laboratories (American Cyanamide, Pearl River, NY, U.S.A.).

Standard preparation

Stock solutions of prednisone, prednisolone, 20β-hydroxyprednisone and 20β-hydroxyprednisolone prepared in an acetonitrile–methanol mixture (1:1) were added to perfusate and water to produce standard concentrations ranging from 50–1500 ng/ml.

Extraction procedure

Samples of perfusate or urine (1 ml) were added to glass culture tubes. The internal standard, 6β-hydroxycortisol (ca. 600 ng) was added to each of the samples. Each tube was vortexed to mix the internal standard and sample. To this mixture, 20 ml of ethyl acetate was added. The tubes were shaken for 20 min. The aqueous layer was removed and the organic phase was washed with 1 ml of 0.1 *N* sodium hydroxide and centrifuged. After removal of the aqueous layer the organic phase was washed with 1 ml of distilled, deionized water. After centrifugation and aspiration of the aqueous phase, 1 g of anhydrous sodium sulfate was added to dry the organic phase, which was sub-

sequently evaporated to dryness at 45°C under a continuous nitrogen stream.

Chromatography

Approximately 200 μl of mobile phase was used to reconstitute the residues just prior to injection. The mobile phase consisted of a heptane—methylene chloride—ethanol—acetic acid (60.8:29.5:8.1:1.6) mixture and was pumped through the column at a solvent speed of 3 ml/min.

Steroid recovery

The assay recovery of each steroid was assessed at 100 ng/ml and 1000 ng/ml in the following manner. Five perfusate samples (1 ml) containing each steroid were extracted and injected. Five injections of the same amount of steroid (e.g. 100 or 1000 ng) in mobile phase were directly injected. The peak heights of the steroids in both sets of samples were measured. The assay recovery of each steroid was computed using the following equation:

$$\% \text{ Recovery} = \frac{\text{Peak height extract}}{\text{Mean peak height direct injection}} \times 100$$

Perfusion

A kidney perfusion study was performed to assess if the 20β-hydroxy metabolites of prednisone and prednisolone were major renal metabolites of these steroids. A rabbit kidney was perfused with prednisolone at an initial perfusate concentration of approximately 900 ng/ml for a period of 90 min. Perfusate samples were obtained at selected times during the experiment and were assayed for prednisone, prednisolone, 20β-hydroxyprednisone and 20β-hydroxyprednisolone.

RESULTS

The chromatograms resulting from the injection of blank perfusate as well as perfusate spiked with prednisone, prednisolone, 20β-hydroxyprednisone, 6β-hydroxycortisol and 20β-hydroxyprednisolone are presented in Fig. 1. Fig. 1b illustrates the response to steroid concentrations of approximately 250 ng/ml and to 625 ng of internal standard.

The steroid recoveries at two concentrations and sensitivity limits for each of the steroids are presented in Table I. The mean assay recovery ranged from 54.1% for prednisone to 63.2% for 20β-hydroxyprednisone; the approximate sensitivity limits of the assay range from 4—10 ng/ml (Table I). A typical calibration plot resulting from the injection of perfusate standards containing the steroids at selected concentrations in the 0—1500 ng/ml range is illustrated in Fig. 2. Detector response was linear in this range for each of the steroids with the greatest response observed for prednisone judging from the slopes of the calibration plot.

The intraday and interday variabilities of the assay for each of the steroids are presented for low and high concentrations in Table II. Both variability studies resulted in coefficients of variation of less than 10% for each steroid examined.

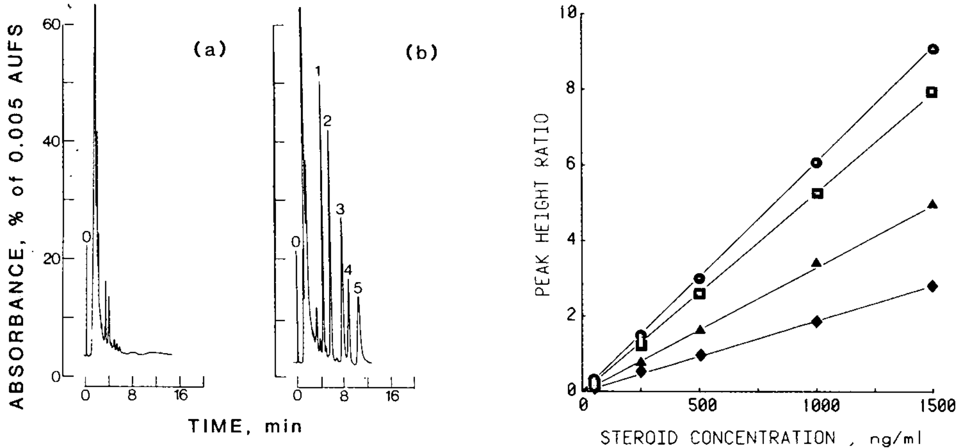

Fig. 1. Chromatograms of (a) blank perfusate extract; (b) perfusate extract spiked with 250 ng of (1) prednisone, (2) prednisolone, (3) 20β-hydroxyprednisone and (5) 20β-hydroxyprednisolone. 6β-Hydroxycortisol (4), 625 ng, is the internal standard. The symbol (○) designates the injection point.

Fig. 2. Calibration curve for the determination of prednisone (●), prednisolone (■), 20β-hydroxyprednisone (▲) and 20β-hydroxyprednisolone (♦) in a 1-ml perfusate extract.

TABLE I

STEROID RECOVERIES AT TWO CONCENTRATIONS AND SENSITIVITY LIMITS OF THE ASSAY

Steroid	Recovery (%)			Sensitivity limit* (ng/ml)
	100 ng/ml**	1000 ng/ml**	Mean	
Prednisone	50.8 (5.0)	57.4 (9.2)	54.1	10
Prednisolone	53.0 (5.0)	70.3 (5.8)	61.6	10
20β-Hydroxyprednisone	62.1 (5.2)	64.4 (4.6)	63.2	4.0
6β-Hydroxycortisol	54.7 (2.6)	63.5 (4.2)	59.1	—
20β-Hydroxyprednisolone	58.9 (4.7)	56.1 (6.5)	57.5	5.0

*Based on a signal-to-noise ratio of 2.5.
**Mean (± S.D.).

Although the assay was primarily developed for use on perfusate and urine samples obtained from kidney perfusion experiments, the assay may be adapted for use on plasma samples through alteration of the mobile phase. Fig. 3 presents chromatograms resulting from the injection of blank plasma (Fig. 3a) and plasma spiked with each steroid (Fig. 3b) to a concentration of approximately 250 ng/ml. The mobile phase employed during injection of the plasma extracts was heptane—methylene chloride—ethanol—acetic acid (71.4: 20.4:6.9:1.3) at a solvent speed of 2 ml/min. This modification of the chromatographic conditions results in good separation of all steroids from each other and from interferences present in the plasma samples. The interference between prednisone and prednisolone in Fig. 3b is probably endogenous

TABLE II

INTRADAY AND INTERDAY COEFFICIENTS OF VARIABILITY

All variability statistics are based on ten measurements.

Steroid	Intraday variability coefficient of variation (%)		Interday variability coefficient of variation (%)	
	50 ng/ml	1000 ng/ml	50 ng/ml	1000 ng/ml
Prednisone	8.6	5.7	8.1	7.8
Prednisolone	5.0	3.1	4.4	4.4
20β-Hydroxy-prednisone	6.0	5.9	8.4	4.4
20β-Hydroxy-prednisolone	9.8	6.2	8.2	6.4

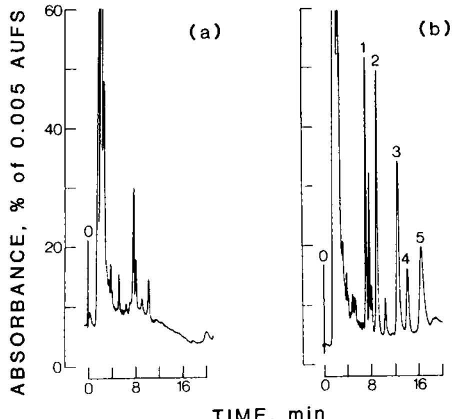

Fig. 3. Chromatograms of (a) blank plasma extract; (b) plasma extract spiked with 250 ng of (1) prednisone, (2) prednisolone, (3) 20β-hydroxyprednisone and (5) 20β-hydroxyprednisolone. 6β-Hydroxycortisol (4), 625 ng, is the internal standard. The symbol (○) designates the injection point.

cortisol which is eluted between prednisone and prednisolone employing this solvent system. Since 6β-hydroxycortisol is an endogenous by-product of cortisol metabolism, it is not suitable for use as an internal standard for samples procured from in vivo experimentation. An alternative internal standard for these biological samples is triamcinolone which possesses a retention time under these chromatographic conditions of 15.8 min.

The results of the kidney perfusion study are presented in Fig. 4. Metabolism of prednisolone to prednisone by the kidney occurred and was accompanied by further metabolism of these compounds to their 20β-hydroxylated metabolites. Peak concentrations of 20β-hydroxyprednisone and 20β-hydroxyprednisolone during the perfusion were 127 and 118 ng/ml, respectively.

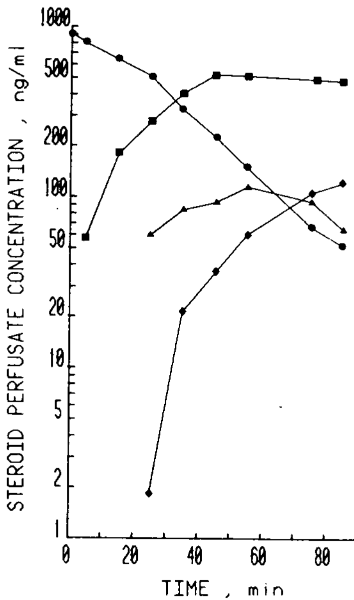

Fig. 4. Perfusate concentrations of prednisolone (●), prednisone (■), 20β-hydroxyprednisolone (▲) and 20β-hydroxyprednisone (◆) as a function of time during perfusion of a rabbit kidney with prednisolone.

DISCUSSION

This HPLC technique provides a sensitive and reproducible means of determining concentrations of prednisone and prednisolone as well as their 20β-hydroxylated derivatives in perfusate and urine. The method will also detect the glucocorticoids and their metabolites listed in Table III. Furthermore, this technique may be modified to permit plasma determinations of these steroids, allowing simultaneous examination of the disposition of prednisone and prednisolone as well as the formation and disposition of these metabolites in man.

TABLE III

RETENTION TIMES OF SELECTED CORTICOSTEROIDS AND THEIR METABOLITES

Corticosteroid	Retention time (min)
Methylprednisone	4.05
17α, 20α, 21-Trihydroxy-1,4-pregnadiene-3,11-dione	4.17
Cortisone	4.17
Dexamethasone	4.17
Cortisol	4.25
Methylprednisolone	5.92
Triamcinolone	7.75
6α-Hydroxycortisol	8.55

The analytical capability for detecting these compounds will enable precise quantitation of the metabolic disposition of prednisone and prednisolone in perfused organ systems, which is the initial step in understanding the non-linear pharmacokinetics of these compounds in vivo [4]. The results of the renal perfusion study (Fig. 4) confirm the metabolism of prednisolone to prednisone and the formation of the 20β-hydroxylated derivatives of both of these compounds by the kidney. The pharmacokinetics of these compounds will be reported more fully in subsequent reports.

There are several HPLC methods available for steroid analysis [5—8]. None of these methods quantitate the parent compounds and the steroid metabolites with the sensitivity and rapidity of the proposed procedure.

An alternative use for this technique arises from its ability to detect 6β-hydroxycortisol, an endogenous metabolite of cortisol. The excretion rate of this compound has been used to reflect enzyme induction or inhibition in man [9]. Through the use of either prednisone, prednisolone or their 20β-hydroxylated metabolites as an internal standard, 6β-hydroxycortisol determinations are possible. Thus this technique can be used to evaluate drug metabolizing enzyme activity in man.

ACKNOWLEDGEMENT

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QUANTITATION OF PREGNENOLONE AND 17-HYDROXYPREGNENOLONE IN HUMAN SERUM BY AUTOMATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT RADIOIMMUNOASSAY

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SUMMARY

A method for the determination of pregnenolone and 17-hydroxypregnenolone in human serum is described which uses high-performance liquid chromatography as a prepurification step followed by radioimmunological quantitation. As to specificity and practicability, the present technique is superior to previously reported methods. Chromatographic assessment of unspecific pregnenolone and 17-hydroxypregnenolone immunoreactivities arising in the ether extracts of normal serum samples clearly emphasizes the necessity of efficient chromatographic isolation of the steroids prior to immunoassay, if specific estimation is to be made. Normal values and physiological changes of serum pregnenolone and 17-hydroxypregnenolone accord well with the data already published in literature.

INTRODUCTION

Pregnenolone and 17-hydroxypregnenolone are precursors of gonadal and adrenal steroid hormones. Their estimation in serum is of special importance for the chemical diagnosis of the different forms of the adrenogenital syndrome [1] and is of a complementary nature in the diagnosis of other adrenal disorders [2]. Due to insufficient specificity of the corresponding antisera, the immunological methods described hitherto require cumbersome chromatographic purification preceding immunological quantitation [3—7]. On the other hand, estimation solely by chromatography with photometric detection is scarcely feasible, particularly as these steroids lack UV-absorbing configurations in the molecule.

The present paper describes an automatic high-performance liquid chromatographic (HPLC) procedure with subsequent quantitation of the purified steroid fractions by radioimmunoassay (RIA).

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EXPERIMENTAL

Materials and reagents

Extrelut® was purchased from Merck (Darmstadt, G.F.R.); plastic syringes (20 ml) used as extraction columns were from Pharmaseal Laboratories (Glendale, CA, U.S.A.). Solvents, reagents and other accessories used for the RIA were as previously described [8].

Radioactive steroids: [7-^{3}H]pregnenolone (17 Ci/mmol) and 17-OH-[7-^{3}H]-pregnenolone (14 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.).

Non-radioactive steroids: progesterone, 17-OH-progesterone, androstendione, 11-deoxycorticosterone, 11-deoxycortisol, 18-OH-11-deoxycorticosterone, corticosterone, aldosterone, cortisone, cortisol, testosterone, dehydroepiandrosterone and estriol were from Merck; pregnenolone and 17-OH-pregnenolone were from Steraloids (Pawling, NJ, U.S.A.).

Antisera: antisera against pregnenolone and 17-OH-pregnenolone were raised in rabbits immunized with the corresponding 3-hemisuccinate bovine serum albumin conjugates synthesized according to the procedure described by Erlanger et al. [9]. The technique of immunization followed the protocol outlined by Vaitukaitis et al. [10]. The final dilutions of antisera used in this method were 1:60,000 for pregnenolone and 1:70,000 for 17-OH-pregnenolone. Cross-reactivities of the antisera with related steroids (Table I) were studied by the method of Abraham [11].

TABLE I

SPECIFICITY OF ANTISERA USED

Steroid	Cross-reactivity (%) of steroids with antisera against	
	pregnenolone	17-OH-pregnenolone
Progesterone	45	0.02
17-OH-Progesterone	0.03	16.4
11-Deoxycorticosterone	1.3	0.02
11-Deoxycortisol	<0.02	0.44
Corticosterone	<0.02	<0.01
Aldosterone	<0.02	<0.01
Cortisol	<0.02	<0.01
Cortisone	<0.02	<0.01
Pregnenolone	100	0.19
17-OH-Pregnenolone	11.0	100
Testosterone	<0.02	<0.01
Androstendione	<0.02	<0.01
Dehydroepiandrosterone	<0.02	<0.01
Estriol	<0.02	<0.01

Instrumentation

A Hewlett-Packard high-performance liquid chromatograph (Model 1084 B), equipped with a fixed-wavelength UV detector at 254 nm, two solvent and two pump systems, a variable-volume injector and a plot/print terminal were used for chromatography. All operating parameters were regulated and controlled by

microprocessors. For the chromatography of steroids applied in the present method, a stepwise gradient elution technique was used. Automatic injection of up to 60 samples was provided by an autosampler. Automatic collection of individual fractions was achieved by a fraction collector (LKB, UltroRac 7000) triggered by the microprocessors of the chromatograph itself.

^{3}H-Radioactivity was measured in a liquid scintillation spectrometer (Packard Instruments, Model 2480).

An IBM 1800 computer was used for computing of assay data.

Samples: blood was drawn between 8 and 9 a.m. except when evening samples were required for studies of diurnal rhythm. After clotting and centrifugation, serum was stored at −20°C until analysis.

Procedures

The protocol of the total assay procedure is outlined in Table II.

TABLE II

FLOW SHEET OF THE TOTAL ASSAY

I. Extraction
- 1 ml serum + [^{3}H]pregnenolone and 17-OH[^{3}H]pregnenolone (each dissolved in 500 µl of water)
- Transfer to Extrelut column
- Elute with 20 ml of carbon tetrachloride
- Evaporate
- Redissolve in 150 µl of *n*-hexane—isopropanol (95 : 5)

II. High-performance liquid chromatography
- Transfer to autosampler
- Automatic injection and chromatography
- Automatic collection of individual fractions
- Evaporate individual fractions
- Redissolve in 180 µl of ethanol—water (50 : 50)
- Count 50 µl for recovery

III. Radioimmunoassay
- 50 µl of sample (duplicate) or standard (1600—6.25 pg) (triplicate) + 800 µl of γ-globulin buffer containing ^{3}H-labelled steroid and antiserum
- Incubation for at least 2 h at 4 °C
- Add 100 µl of charcoal suspension
- Shake, centrifuge and decant
- Count supernatant for ^{3}H-radioactivity
- Calculate results

Extraction. The extraction procedure followed the technique recently described [12]. In brief, tracer amounts of [^{3}H]pregnenolone and 17-OH-[^{3}H]-pregnenolone, each dissolved in 500 µl of distilled water, were added to 1 ml of serum. After equilibration for about 30 min, the mixture was transferred on to an Extrelut column and eluted with 20 ml of carbon tetrachloride. Concomitantly with elution, the organic phase was evaporated under a stream of air in a 30°C water-bath. The residue was redissolved in 150 µl of a mixture of *n*-hexane—isopropanol (95:5).

Automatic high-performance liquid chromatography. For HPLC, a column of polar-bonded phase packing (DIOL®, Knauer, Berlin, G.F.R.), *n*-hexane—isopropanol as mobile phase and gradient elution was used [13]. Further conditions of HPLC were as follows: flow-rate 1.3 ml/min, column pressure 62 bar, temperature of solvents and oven 40°C, attenuation $64 \cdot 10^{-4}$ a.u./cm, slope sense 0.5, column length 25 cm, diameter of column 4.6 mm, diameter of particles 5 μm, solvent A *n*-hexane, solvent B *n*-hexane—isopropanol (85:15). The gradient was run from 15% B to 100% B within 41 min. The organic solution (150 μl) of the serum extracts (see above) was transferred to microvials, then positioned in the autosampler and automatically subjected to HPLC. The specific fractions containing pregnenolone and 17-OH-pregnenolone were automatically collected by time-triggering of the chromatograph dictated by the retention times of the steroids. Before each batch, these retention times were established by a calibrating run of a ^{3}H-labelled steroid mixture containing about 10 nCi of each steroid. In this run, ^{3}H-radioactivity was measured in 1-min fractions eluted by HPLC.

Radioimmunoassay. The organic fractions were evaporated under a stream of air and redissolved in 180 μl of ethanol—water (50:50). A 50-μl aliquot of the solution was measured for recovery, and 50 μl aliquots in duplicates were subjected to RIA. Evaluation of RIA data was done by a computer program using the "spline approximation" technique as standard curve model [14].

RESULTS

Extraction

The percentage recoveries of [^{3}H]pregnenolone and 17-OH-[^{3}H]pregnenolone added to a serum sample were found to be more than 95% after carbon tetrachloride extraction.

High-performance liquid chromatography

Fig. 1a demonstrates the chromatogram with UV detection of a steroid

TABLE III

CHROMATOGRAPHIC PARAMETERS OF THE HPLC SYSTEM APPLIED TO THE SEPARATION OF ADRENAL STEROIDS

Steroid	Retention time (min)	Capacity ratio	Resolution
Progesterone	7.23	1.78	
Androstendione	9.88	2.80	1.27
Pregnenolone	12.80	3.90	1.40
Deoxycorticosterone	18.06	5.94	2.52
Testosterone	19.50	6.50	0.70
17-OH-Progesterone	22.64	7.70	1.50
17-OH-Pregnenolone	29.20	10.23	3.16
11-Deoxycortisol	33.07	11.71	1.85
18-OH-Deoxycorticosterone	33.73	11.97	0.32
Corticosterone	35.05	12.48	0.63
Aldosterone	38.97	13.98	1.87

mixture chromatographed by the HPLC system applied. The non-UV-absorbing steroids pregnenolone and 17-OH-pregnenolone were located by ^{3}H-radioactivity measurement in 1-min fractions. The chromatographic parameters, such as retention time, capacity ratio and resolution between adjacent steroids, are

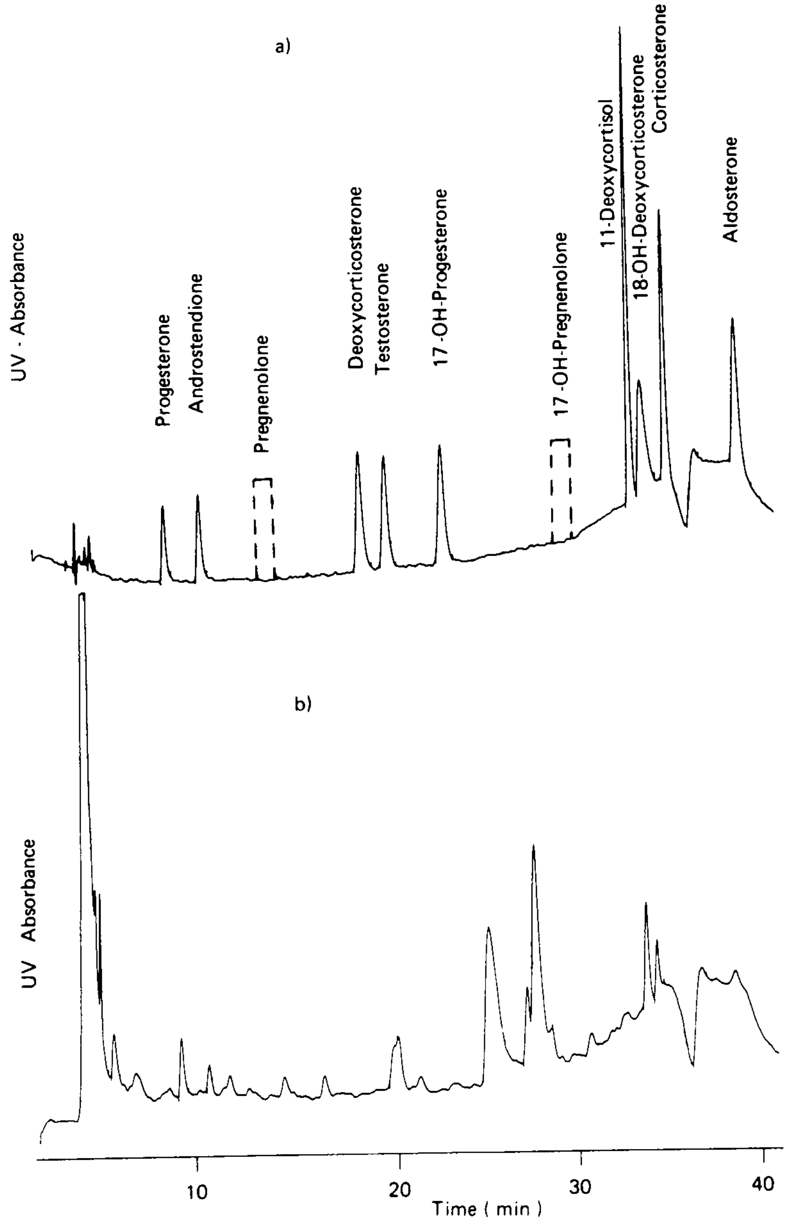

Fig. 1. Chromatogram of a mixture of steroid standards (a) and of the ether extract of a normal serum sample (b). HPLC parameters used are outlined in Methods. Amounts of steroids injected for chromatogram a were about 500 ng of each. The non-UV-absorbing steroids pregnenolone and 17-OH-pregnenolone were localized by chromatography of the tritiated steroids.

summarized in Table III. The reproducibility of retention times of UV-absorbing steroids was studied in 20 runs and found to be less than 0.6% (C.V.). The chromatogram with UV detection of a normal serum sample is shown in Fig. 1b, thus demonstrating that considerable amounts of non-specific, UV-absorbing compounds occur in the areas of the steroids of interest.

The percentage recovery of [^{3}H]pregnenolone and 17-OH-[^{3}H]pregnenolone after HPLC was found to be 62.5 ± 8.5% (S.D.) and 65.3 ± 14.4% (S.D.), respectively. Due to the considerable variation in recoveries, the individual losses of each sample were adjusted according to the radioactive count of 50 μl of the final HPLC fraction.

Analytical variables

Sensitivity. The detection limit of the total assay is mainly dictated by the affinity of the antisera used, i.e. the sensitivities of standard curves, the procedural losses and the blank levels. The sensitivity of a standard curve was defined as the mass of steroid necessary to suppress the ^{3}H-radioactivity bound to the antibody at zero conditions by two standard deviations of the radioactive count. The corresponding values were found to be 28.3 ± 16.3 pg (S.D.) for pregnenolone and 7.7 ± 3.8 pg (S.D.) for 17-OH-pregnenolone. Blank levels arising in water were found to be lower than the sensitivities of the standard curves. Thus, the total detection limits of the total assays, deriving from standard curve sensitivity and procedural losses, amounted to 0.69 nmol/l for pregnenolone and 0.21 nmol/l for 17-OH-pregnenolone.

Specificity. The cross-reactivities of the antisera used against known steroids of related structure are shown in Table I. In addition to this documentation of assay specificity, we studied unknown interferences potentially arising in serum. Thus, the pregnenolone and 17-OH-pregnenolone immunoreactivities were estimated in 1-min fractions eluted by HPLC of carbon tetrachloride extracts of normal serum samples. The profiles outlined in Fig. 2 demonstrate that — although assay specificity checked by cross-reactivity (Table I) seems quite satisfactory — there arise considerable unspecific immunoreactivities which are not attributable to steroids commonly studied for specificity.

Precision and accuracy. Within-batch and between-batch variability was studied in replicates of a normal serum sample. Within-batch variation (C.V.) was found to be 7.5% for pregnenolone and 5.7% for 17-OH-pregnenolone; between-batch variation was 12.5% and 8.7%, respectively. Accuracy was studied by recovery measurement of three different amounts of each steroid added to a charcoal-stripped serum sample. Amounts found ranged from 89.4% to 102.1% for pregnenolone and between 92.3% and 98.2% for 17-OH-pregnenolone.

Practicability. The automatic mode of HPLC, which eliminates the time-consuming handling of the manual sequential technique, renders the present method suitable for processing large batches of samples within a reasonable time, particularly if the facility of overnight operation is utilized.

Normal values

Pregnenolone and 17-OH-pregnenolone concentrations were estimated from 28 normal males, aged between 22 and 42 years. The mean concentration as

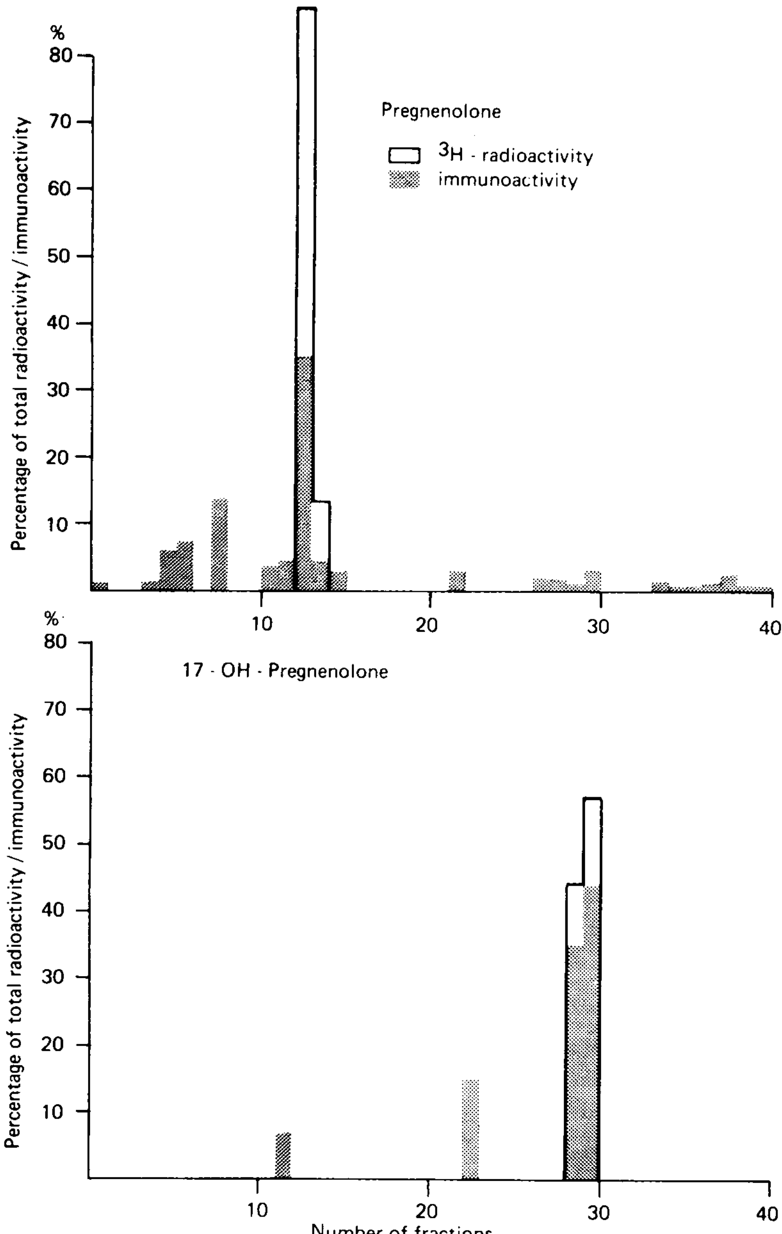

Fig. 2. Profiles of immunoreactivities (hatched areas) and of the specific ^{3}H-radioactivities (open areas) as measured in fractions eluted by the HPLC system described. Volume of each fraction was 1.3 ml. ^{3}H-Radioactivities added to the serum samples were 2 nCi.

well as the upper and lower limits established according to a logarithmic distribution are listed in Table IV.

Physiological validation

Serum concentrations of pregnenolone and 17-OH-pregnenolone were as-

sessed before and after adrenal stimulation and suppression, respectively. Diurnal variation was monitored by steroid estimation at 8 a.m. and 8 p.m. The corresponding data evaluated in four subjects are listed in Table V.

TABLE IV

SERUM CONCENTRATIONS OF PREGNENOLONE AND 17-OH-PREGNENOLONE IN NORMAL MALES

Values were evaluated according to a logarithmic distribution.

	Lower limit (nmol/l)	Median (nmol/l)	Upper limit (nmol/l)
Pregnenolone	2.34	3.34	4.75
17-OH-Pregnenolone	4.25	5.52	7.16

TABLE V

PHYSIOLOGICAL CHANGES OF SERUM PREGNENOLONE AND 17-OH-PREGNENOLONE

	Pregnenolone (mean ± S.D., nmol/l)	17-OH-Pregnenolone (mean ± S.D., nmol/l)
Adrenal stimulation with 250 μg Synacthen® i.m.		
before	1.28 ± 0.36	2.31 ± 1.78
30 min after	2.38 ± 0.34	10.95 ± 1.52
Adrenal suppression with 2 mg of dexamethasone at midnight		
8 a.m. before	2.24 ± 0.9	2.80 ± 1.4
8 a.m. after	1.71 ± 0.27	0.41 ± 0.13
Diurnal variation		
8 a.m.	4.37 ± 1.02	9.7 ± 3.08
8 p.m.	2.4 ± 1.49	2.05 ± 0.95

DISCUSSION

The present data on pregnenolone and 17-OH-pregnenolone immunoreactivities arising in the ether extract from normal serum samples (Fig. 2) clearly demonstrate that assessment of crude organic extracts would overestimate specific steroid concentration due to considerable amounts of unspecific immunoreactivities. Furthermore, it is well documented by these data that exclusive monitoring of cross-reactivity of antisera against some steroids related to that of interest is not sufficient for validation of assay specificity at all and that only efficient chromatographic prepurification would provide specific steroid estimation by immunoassay.

In comparison to other chromatographic techniques, the present HPLC system, which is related to that featured previously [13], provides, besides the chromatographic qualities outlined elsewhere [15], the great advantage that it is well suited for automation. Thus, running the HPLC system automatically and overnight, the tedious and time-consuming operations well known from the

chromatographic techniques applied hitherto [4—6], are almost completely eliminated.

The validation of assay parameters and of physiological changes in serum pregnenolone and 17-OH-pregnenolone accord well with the data published in the literature [3—6].

ACKNOWLEDGEMENT

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CATALYTIC DETECTION PRINCIPLE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: DETERMINATION OF ENANTIOMERIC IODINATED THYRONINES IN BLOOD SERUM

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SUMMARY

A method for the trace determination of iodinated thyronines with differentiation of the optical isomers by high performance liquid chromatography (HPLC) is described. The detection is effected by means of a catalytic principle based on the iodide-catalysed reaction of chloramine-T and N,N'-tetramethyldiaminodiphenylmethane, producing a coloured complex that can be measured spectrophotometrically at 600 nm. Owing to the selectivity of the catalytic reaction, iodine-containing compounds can be easily determined in a complex matrix such as blood plasma. The sensitivity is sufficient for the detection of plasma levels of iodinated thyronines. The limit of detection for thyroxine is in the sub-nanogram range. The enantiomers of thyronines can be separated on commercial reversed phases after pre-column synthesis of diastereomers. For this derivatization the reagent *tert.*-butyloxycarbonyl-L-leucine-N-hydroxysuccinimide ester is used. The coupling of the stereospecific HPLC separation with the catalytic detector offers the possibility of determining both D- and L-thyroxine in human plasma.

INTRODUCTION

The determination of iodinated thyronines in plasma is of great importance. The human thyroid gland produces the L-isomer of thyroxine and the D-isomer is being produced synthetically. D-Thyroxine was shown to reduce serum cholesterol levels significantly, acting by increasing the rate of degradation and

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oxidation of cholesterol [1—5]. Additionally, a thyrotropine suppression caused by D-thyroxine has been described [6]. For these reasons D-thyroxine is of pharmacological importance.

As the concentrations of iodinated thyronines in plasma are very low, only trace methods can be applied for their determination. Radioimmunoassay [7, 8], fluorescence immunoassay [9], enzyme immunoassay [10] and competitive protein binding assay [11] are most frequently used. A selective determination of enantiomeric thyronines is not possible with these techniques. The cross-reaction of L-thyroxine antibodies with D-thyroxine for a radioimmunoassay was found to be up to 100% [6].

Chromatographic separations of thyroid hormones can be carried out using gel chromatography [12, 13], ion-exchange chromatography [14, 15], gas chromatography (GC) [16—18] and high-performance liquid chromatography (HPLC) [19—21]. Separation of enantiomeric amino acids is possible by both GC and HPLC. Recently the determination of D,L-triiodothyronine and D,L-thyroxine by derivatization liquid chromatography was described [22, 23]. The D,L-thyronines are derivatized with *tert.*-butyloxy-L-leucine-N-hydroxysuccinimide ester and the resulting diastereomeric peptides are separated by ion-pair chromatography on reversed-phase columns. This method was applied successfully to the control of the purity of pharmaceuticals and its application to human plasma seemed to be promising.

For the analysis of plasma samples by HPLC a prior separation from the serum proteins is necessary. A simple method is precipitation with organic solvents such as tetrahydrofuran (THF). At the same time, extraction of the iodinated thyronines is achieved. The THF extract is used for the subsequent derivatization.

UV detection after HPLC separation is not sensitive enough for plasma levels of thyroid hormones. A possibility for improving the sensitivity is to use UV or fluorescence derivatization [24]. Owing to the presence of many amino acids in plasma, a suitable detection principle for iodinated thyronines must be both sensitive and highly selective. Both requirements can be met by making use of the catalytic effect of iodine. Knapp and Spitzy [25] described a modified form of the catalytic Sandell—Kolthoff reaction for the trace determination of thyroid hormones. This reaction was coupled to a liquid chromatograph by Nachtmann et al., and a sub-nanogram detection limit for thyroxine was achieved [26]. Owing to the corrosive reagents necessary for this technique, its routine application causes problems. Feigl and Jungreis [27] described the catalytic effect of iodide on the reaction of N,N′-tetramethyldiaminodiphenylmethane (tetrabase) with chloramine-T. The high sensitivity of the reaction was applied to the titrimetric and colorimetric determination of iodine [27—30]. The reaction was used as the basis for the development of a new catalytic detector for HPLC.

By combining the separation efficiency of modern HPLC with the catalytic reaction detector, new possibilities for the determination of iodinated thyronines in human plasma are offered.

EXPERIMENTAL

Reagents

All solvents were of analytical-reagent grade (Merck, Darmstadt, G.F.R.). Triiodothyronine (T_3) and thyroxine (T_4) were produced by Sanabo (Kundl, Austria). L-[3',5'-^{125}I]Thyroxine was purchased from the Radiochemical Centre (Amersham, Great Britain). The derivatization reagent *tert.*-butyloxy-L-leucine-N-hydroxysuccinimide ester (BOC-L-Leu-SU) was purchased from Fluka (Buchs, Switzerland), chloramine-T from Merck and N,N'-tetramethyldiamino-diphenylmethane (tetrabase) from Merck-Schuchardt (Darmstadt, G.F.R.). N,N'-Tetramethyldiaminodiphenylmethane was recrystallized in ethanol; all other reagents were used without further purification.

Apparatus

For HPLC a Waters Type 1000A pump in combination with a WISP 710A injection system was used. Stainless-steel columns (150 × 3.2 mm I.D.) packed with Nucleosil 5 C-18, particle size 5 μm (Macherey, Nagel & Co., Düren, G.F.R.) were employed. The separations were carried out isocratically at room temperature, thermostating being unnecessary. The detection system consisted of a Technicon AutoAnalyzer II proportioning pump with flow-rated Solvaflex pump tubes and a Zeiss PM 2A spectrophotometer. The cell volume was 70 μl. Peak integration and calculation of data were performed with a Hewlett-Packard 3380A integrator and a Waters 730 data module.

Procedure

Determination of total thyroxine in plasma. Ethanol (200 μl) containing an internal standard (30 μg of triiodothyronine per 100 ml) is pipetted into a vial, 100 μl of plasma are added, the vial is tightly closed, shaken well for 2 min and centrifuged at 2700 *g* for 3 min. An aliquot of the clear supernatant liquid is injected into the liquid chromatograph.

Stereoselective determination of D- *and* L-*thyroxine in plasma.* A 0.5-ml volume of plasma is pipetted into a vial containing 1 ml of THF and the vial is tightly closed, shaken well for 2 min and centrifugated at 2700 *g* for 2 min. A 1-ml volume of the supernatant liquid is transferred into another tube, 50 μl of sodium hydrogen carbonate solution (1 *M*) and 50 μl of the derivatization solution (20 mg of BOC-L-Leu-SU per 1 ml of THF) are added. The solvents are removed by evaporation and 100 μl of trifluoroacetic acid are pipetted on to the dry residue. After 15 min at room temperature, 0.5 ml of sodium hydrogen carbonate solution (1 *M*) is added and the mixture is centrifuged at 4500 *g* for 10 min. The clear solution is removed and the precipitate is treated with 200 μl of ethanol and 100 μl of 0.5 *M* sodium hydroxide solution containing the internal standard (1 μg/ml of triiodothyronine). The insoluble part of the precipitate is removed by centrifugation and an aliquot of the clear solution is injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Sample preparation

As plasma cannot be injected directly into an HPLC column, the thyroid hormones have to be extracted from the matrix. One possibility is to use short ion-exchange columns, which have already been applied successfully for this purpose [31]. A simpler and faster alternative involves precipitation of the plasma proteins with an organic solvent. In this step the iodinated thyronines are extracted at the same time. After centrifugation the clear extract can be used directly for further analysis.

For the optimization of this technique, plasma samples spiked with $[^{125}I]\,T_4$ were used. In this way the extraction yields could be determined radiometrically. The extraction yield using ethanol as solvent was 63.4 ± 2.7%, using acetone 68.4 ± 3.2% and using THF 86.2 ± 3.5%. For each solvent twelve tests were performed, adding 200 μl to 100 μl of serum and measuring the clear extract after centrifugation. For the HPLC determination of total T_4 this extract was used.

After several injections, blocking of the columns can occur due to the co-extraction of many other non-polar compounds into the solvent. These problems can be minimized by using ethanol for the extraction.

For differentiation of the enantiomers of T_4 the plasma extract is derivatized. Diastereometric dipeptides are formed from D,L-amino acids with the reagent BOC-L-Leu-SU [22, 32]. The reaction is carried out in mixtures of THF and sodium hydrogen carbonate solution [32] or methanol and sodium hydrogen carbonate solution [22, 33]. Optimization studies with $[^{125}I]\,T_4$ showed that the reaction is nearly quantitative in THF solutions. Mild reaction conditions are necessary owing to the chemical lability of iodinated thyronines. Most important is the purity of the reaction and extraction medium THF, which must be free from peroxides. The commercial analytical-reagent grade material, stabilized with 2,6-di-*tert*.-butyl-4-methylphenol, can be used without further purification. A 0.5-ml volume of serum is added to 1 ml of THF, the mixture is shaken for 2 min and the precipitate is centrifuged at 2700 *g*. For the derivatization 1 ml of the extract is necessary.

Human plasma contains many amino acids that react with BOC-L-Leu-SU, and therefore the amount of reagent had to be investigated empirically. A 50-μl volume of sodium hydrogen carbonate solution (1 *M*) and various amounts of the reagent solution (20 mg of BOC-L-Leu-SU per 1 ml of THF) were added to 1 ml of the THF extract and the solvents removed by evaporation. After splitting off the BOC groups the solutions were analysed by HPLC. Fig. 1 shows the peak height of the L-Leu derivative of L-T_4 as a function of the amount of reagent. Under the conditions described, 1 mg of reagent is sufficient for the quantitative derivatization of 400 ng of L-T_4 per 1 ml of plasma. The sodium hydrogen carbonate solution must be added in order to obtain the optimal pH value for the reaction. It is important to keep the temperature below 30°C during the evaporation, otherwise artifacts are formed [22]. The time necessary for the evaporation (about 30 min) is sufficient for the reaction equilibrium to be attained.

The selectivity of the liquid chromatographic separation is greatly improved by splitting off the BOC groups from the derivatives [22, 33]. The optimal reagent proved to be trifluoroacetic acid (TFA), which splits off the BOC groups at room temperature without causing decomposition of the thyronine derivatives. A 100-μl volume of TFA is added to the dry sample. The reaction is quantitative after 15 min at room temperature (20—22°C). The TFA must be removed prior to chromatography. Therefore, the peptides are precipitated by addition of 0.5 ml of sodium hydrogen carbonate solution (1 *M*) and separated by centrifugation. In order to remove non-polar substances that are insoluble in the mobile phase of the HPLC system, the liquid is aspirated off and 200 μl of ethanol and 100 μl of 0.5 *M* sodium hydroxide solution are added to the precipitate. After centrifugation, the clear extract is injected into the HPLC column. This sample clean-up results in effective purification of the sample. Several hundred injections into the same column are possible without a decrease in the separation efficiency.

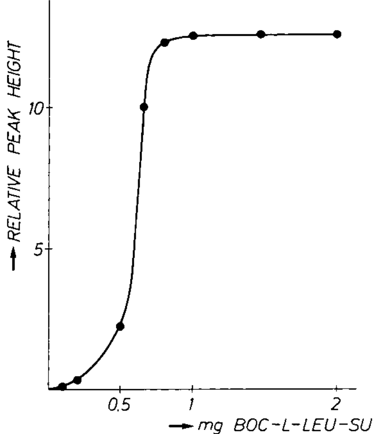

Fig. 1. Relative peak height of the L-Leu derivative of L-T_4 (400 ng/ml serum) as a function of the reagent concentration (20 mg BOC-L-Leu-SU/ml THF). External standard: L-T_4 (6 ng per injection, without derivatization).

The catalytic reaction detector

The reaction detector is based on the catalytic effect of iodide on the oxidation of N,N′-tetramethyldiaminodiphenylmethane (tetrabase). According to Feigl and Jungreis [27], the reaction is described by three equations (Fig. 2). As shown in Fig. 2, hypochlorite is liberated during the hydrolysis of chloramine-T (A). The hypochlorite is able to oxidize iodide ions to give elemental iodine (B). Iodine oxidizes the tetrabase and is itself reduced to iodide again (C). The oxidized form of the tetrabase can be determined spectrophotometrically at 600 nm.

In order to realize an on-line detection system for HPLC the reaction conditions had to be optimized with respect to a continuous flow system. Chloramine-T was dissolved in dilute acetic acid because water is needed for the

(A) $$[CH_3-C_6H_4-SO_2NCl]^- + H_2O \rightleftharpoons CH_3-C_6H_4-SO_2NH_2 + ClO^-$$

(B) $$ClO^- + 2\,I^- + 2H^+ \longrightarrow Cl^- + H_2O + 2\,I^\circ$$

(C) $$(CH_3)_2N-C_6H_4-CH_2-C_6H_4-\overset{+}{N}H(CH_3)_2 + 2\,I^\circ \longrightarrow$$
$$\longrightarrow (CH_3)_2N-C_6H_4-CH=C_6H_4=\overset{+}{N}(CH_3)_2 + 2\,I^- + 2H^+$$

Fig. 2. Catalytic reaction of iodide with chloramine-T and N,N'-tetramethyldiaminodiphenylmethane (tetrabase).

hydrolysis and protons are necessary for the oxidation of iodide. Tetrabase is poorly soluble in water, and ethanol was chosen as a solvent.

The schematic arrangement of the detector is shown in Fig. 3. The reagents are pumped by means of a Technicon peristaltic pump and the appropriate flow-rated pump tubes to the mobile phase. The peak broadening is minimized by air segmentation (0.1 ml/min). As a result of optimization studies the best sensitivity of the reaction was found for a concentration of 300 mg of chloramine-T per litre of 0.15% acetic acid and 2 g of tetrabase per litre of ethanol. The chloramine-T solution is pumped at a flow-rate of 2 ml/min and the tetrabase solution at a flow-rate of 0.23 ml/min. The reaction is performed at 20—22°C; thermostating is not necessary.

The separation of iodinated amino acids and the corresponding L-leucine derivatives is achieved by ion-pair chromatography on reversed phases [22]. The optimal mobile phase for a C-18 column is a mixture of methanol and water (67:33) with the addition of 0.05% of methanesulphonic acid. The alcohol in the mobile phase must not be replaced by solvents with other

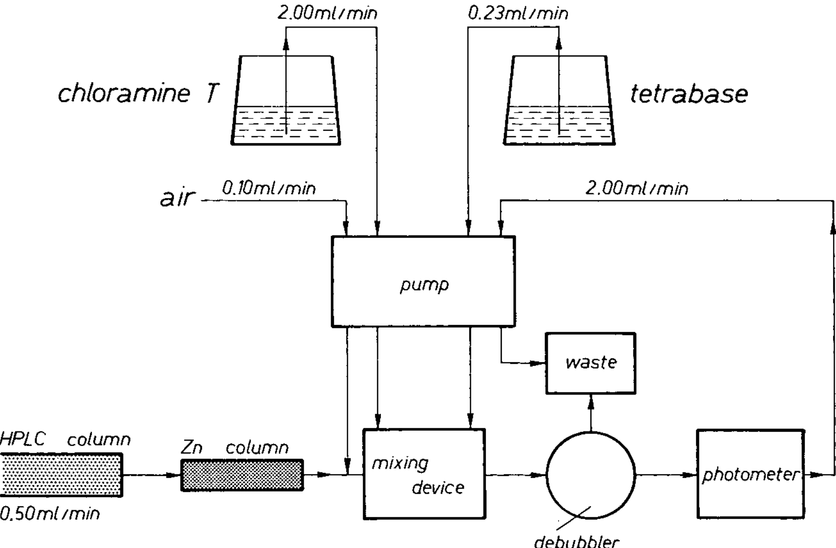

Fig. 3. Configuration of the catalytic reaction detector.

functional groups such as acetonitrile, as they are not compatible with the catalytic detection system. Instead of methanesulphonic acid, phosphate buffers or phosphate—citrate buffers can be used [22].

Iodine bound covalently in iodinated thyronines shows only a low catalytic activity. Therefore, the effluent of the HPLC columns is passed through a small glass column (50 × 1 mm I.D.) packed with zinc powder (75—150 μm, purity 99.999%; Goodfellow Metals, Cambridge, Great Britain). The column is closed with glass-wool at both ends. By pumping the acidic mobile phase through this column, hydrogen is produced. This hydrogen in statu nascendi splits off the iodine from the phenyl groups of the iodinated thyronines. The resulting iodide ions show high catalytic activity. As zinc is dissolved in this reaction, the zinc column must be renewed daily.

A homogeneous mixture of the reagents with the mobile phase is of great importance. An incomplete mixture of the aqueous and organic solvents causes high baseline noise in the spectrophotometer. Therefore, some mixing units were tested and the signal-to-noise ratio was measured under constant conditions. The mixing systems tested are illustrated in Fig. 4. Types A, B and C were constructed as described by Nachtmann et al. [26]; type E was a mixing device as published recently by Kobayashi and Imai [34].

Table I shows the signal-to-noise ratio and the peak half-width for a signal obtained after injection of 1 ng of T_4 under the chromatographic conditions given in Fig. 5A. As the signal height was nearly constant, the signal-to-noise ratio was measured as the index of mixing efficiency and the half of the peak width was used as the index of the peak broadening. Type F, a newly constructed mixing chamber, gave the best efficiency. The cylindrical magnetic stirrer has a fin at its upper side. This mixing device was used for all further investigations.

The pumping of the reagents by a peristaltic pump would give rise to periodic oscillations of the detector baseline. Therefore, as depicted in Fig. 3, the

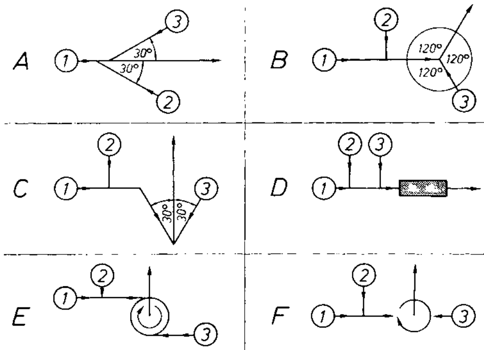

Fig. 4. Mixing devices for HPLC column effluent and reagents. A, mixing against flow direction, 1 mm I.D. bores; B, 120° mixing device, 1 mm I.D. bores; C, 30° mixing device, 1 mm I.D. bores; D, column reactor filled with glass beads (60 mesh), 50 × 2 mm I.D.; E, rotating flow mixing device, 5 × 4 mm I.D.; F, dynamic mixing chamber with magnetic stirring bar, 12 × 8 mm I.D. (cylindrical stirring bar, 11.5 × 7.7 mm). 1, Air-segmented chloramine-T solution; 2, HPLC column effluent; 3, tetrabase solution.

TABLE I

EFFECTS OF DIFFERENT MIXING DEVICES ON THE SIGNAL-TO-NOISE RATIO AND ON PEAK BROADENING AT CONSTANT ATTENUATION AND CONSTANT FLOW-RATE

Test substance: T_4. For chromatographic parameters, see Fig. 5A.

Type of mixing device (see Fig. 4)	Signal-to-noise ratio	Peak half-width (s)
A	1.5	26
B	2.8	25
C	2.9	26
D	3.0	30
E	2.9	29
F	24.0	28

solvents are sucked through the detector cell with a constant flow. By this technique the pulsations are compensated very effectively. Air bubbles and the excess of solvent are separated by means of a debubbler.

Analyses of plasma samples

For the determination of total T_4 50 μl of the ethanolic plasma extract and for the stereospecific determination of D- and L-T_4 30 μl of the derivatized extract are injected into the HPLC system. An improvement in the precision of the analysis was achieved by internal standardization. A suitable internal standard was found to be T_3, which is eluted before T_4 under the conditions described. Interferences from the T_3 level present in human plasma do not occur because the natural T_3 level is two or three orders of magnitude smaller than the concentration of the internal standard. For the determination of the total T_4 content the internal standard is dissolved in the ethanol used for the extraction of the plasma samples. The distribution coefficients for T_3 and T_4 are identical under the conditions used. In the stereospecific determination of D- and L-T_4 the internal standard is dissolved in the sodium hydroxide that is added to the samples after the removal of TFA. Addition before derivatization is not possible owing to the different solubilities of T_3 and T_4 in the precipitation steps.

Typical chromatograms are shown in Fig. 5. The separations take place rapidly. In peak 1 (A, B) the halide anions iodide, bromide and chloride are eluted. The sensitivity for bromide and chloride is smaller than that for iodide: the factor for bromide is 200 and that for chloride is 500. The simultaneous determination of free iodide in plasma with the method described is not successful because the large excess of chloride and bromide in normal serum [35] interferes with the signal of iodide.

In the biologically relevant concentration range of 0—310 nmol/l in serum linear calibration graphs are obtained for the L- and D-isomers and the correlation coefficients found were 0.985—0.999. With higher concentrations, dilution of the sample or reduction of the injection volume is necessary. Table II gives the relative standard deviations for the determination of total T_4 and the deter-

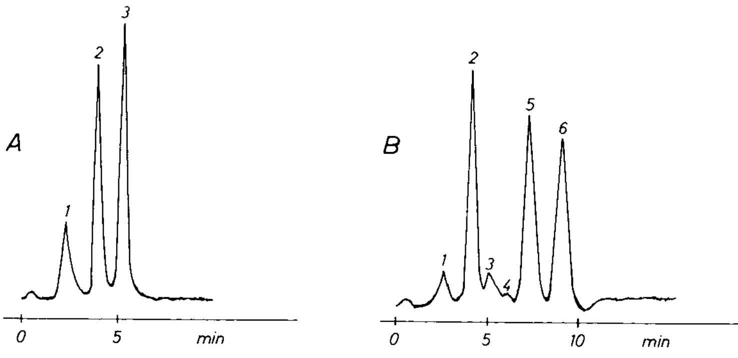

Fig. 5. Chromatographic determination of iodinated thyronines with catalytic detection. Column, Nucleosil 5 C-18 (150 × 3.2 mm I.D.); mobile phase, methanol—water (67 : 33) plus 0.05% of methanesulphonic acid; flow-rate, 0.5 ml/min at 1100 p.s.i.; detection wavelength, 600 nm. (A) Determination of total T_4. Injection volume, 50 µl of ethanolic serum extract. 1, Free halide ions; 2, 10 ng of T_3 (internal standard); 3, 8 ng of T_4. (B) Stereospecific determination of D- and L-T_4 in serum after derivatization. Injection volume, 30 µl. 1, Free halide ions; 2, 10 ng of T_3 (internal standard); 3 and 4, not identified; 5, L-Leu-L-T_4 (corresponding to 7 ng of L-T_4); 6, L-Leu-D-T_4 (corresponding to 7 ng of D-T_4).

TABLE II

RELATIVE STANDARD DEVIATION (S_{rel}) FOR THE DETERMINATION OF D,L-T_4 IN SERUM USING INTERNAL STANDARDIZATION

Concentration of T_4 (nmol/l serum)	S_{rel} (%) (n = 6)		
	Total T_4	L-T_4	D-T_4
38.6	5.3	9.5	9.8
77.2	4.8	7.4	6.9
154.4	4.2	6.8	7.5
308.8	5.1	7.1	6.4

mination of D- and L-T_4. The standards were prepared by spiking a T_3- and T_4-free plasma, which was prepared as follows: 40 ml of human plasma were pipetted on top of a glass column (300 × 25 mm I.D.) filled with Dowex 1-X2 (50—100 mesh) and the same anion exchanger (100—200 mesh) (1 : 1 mixture). After incubation overnight the elution was carried out with 40 ml of an isotonic sodium chloride solution. The first 5 ml were discarded, the remainder of the solution being plasma free of thyroid hormones. For regeneration the column was washed with 500 ml of water followed by 500 ml of 70% acetic acid and 500 ml of water.

The quantitative analysis of plasma samples is performed by comparison of the sample values with a calibration graph with use of the internal standard. The detection limit for D,L-T_4, taking a signal-to-noise ratio of 3 : 1, is 125 pg absolute or approximately 3 nmol/l in plasma.

The method described is being used for a pharmacokinetic study of D-T_4 in collaboration with the Department of Internal Medicine, University of Graz,

Austria. This method is the only one known so far that permits the stereoselective determination of D- and L-T_4 at plasma levels. The sensitivity and precision are sufficient for clinical studies. Of course, the catalytic reaction detector can also be used for the trace determination of other iodine-containing compounds.

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CHROMBIO. 875

SIMPLE MICRO-METHOD FOR THE ISOLATION OF GANGLIOSIDES BY REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

A simple and convenient technique has been developed for the isolation of gangliosides from small amounts of tissues or cells. A ganglioside fraction obtained by chromatography of the total lipid extract of DEAE-Sephadex was subjected to alkaline hydrolysis and salts and other non-lipid contaminants were removed by reversed-phase chromatography on a C_{18} Sep-Pak cartridge. The purified gangliosides were then obtained by chromatography on a small Iatrobeads or Unisil column. This procedure yields a quantitative recovery of gangliosides that are free of contaminants which interfere with thin-layer chromatographic analysis. The procedure was used for the quantitative isolation of gangliosides from human brain white matter and human erythrocytes.

INTRODUCTION

Current techniques for the separation of salts, sugars and other low-molecular-weight contaminants from gangliosides do not yield a consistently good recovery. Reversed-phase chromatography on octadecyl-coated silica gel has been used to separate molecular species of glycolipids [1, 2]. Williams and McCluer [3] recently used reversed-phase chromatography on C_{18} Sep-Pak cartridges to isolate gangliosides from Folch upper-phase fractions, but gangliosides isolated by this procedure contain contaminants that interfere with thin-layer chromatographic (TLC) analysis. In this report we describe a simple procedure for the quantitative isolation of pure gangliosides. This method combines the method of Ledeen et al. [4] with reversed-phase chromatography on a C_{18} Sep-Pak cartridge.

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EXPERIMENTAL

C_{18} Sep-Pak cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). DEAE-Sephadex A-25 was purchased from Pharmacia (Piscataway, NJ, U.S.A.). DEAE-silica-gel (total capacity 150 μmol/g dry weight; pore diameter 200 Å; 120—200 mesh) was prepared as described previously [5, 6] and was also available commercially from Bio-Supports (Clifton, NJ, U.S.A.). Unisil (200—325 mesh) and Iatrobeads (6RS-8060) were procured from Clarkson Chemical Company (Williamsport, PA, U.S.A.) and Iatron Laboratory (Tokyo, Japan), respectively.

Human brain was obtained at autopsy from a patient without neurological disease and samples of human blood were obtained in acid—citrate—dextrose anticoagulant. The purified gangliosides used as standard components have been described previously [7, 8]. Tritiated beef brain ganglioside mixture was prepared by mild periodate oxidation followed by reduction with $[^3H]NaBH_4$ (specific activity 282 mCi/mmol; obtained from New England Nuclear, Boston, MA, U.S.A.) according to the procedure described by Veh et al. [9]. The specific activity of the tritiated beef brain ganglioside mixture was $2 \cdot 10^4$ cpm/μg sialic acid [10]. TLC—autoradiography revealed that all ganglioside species were labeled and unchanged in migratory rate. N-$[^{14}C]$Acetylneuraminic acid (specific activity 256 mCi/mmol) and $[^{14}C]$glucose (specific activity 59 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). N-Acetylneuraminic acid was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.) and glucose from Fisher Scientific (Fairlawn, NJ, U.S.A.).

The ganglioside yield from each preparation was determined by gas—liquid chromatographic analysis of the lipid-bound sialic acid [11]. TLC was carried out on pre-coated silica gel 60 plates (E. Merck, Darmstadt, G.F.R.) with chloroform—methanol—water (55:45:10) containing 0.02% (w/v) of $CaCl_2 \cdot 2H_2O$ or chloroform—methanol—2.5 N ammonia solution (60:40:9) as solvent systems. Gangliosides were revealed by spraying the plate with resorcinol reagent [12] and heating the covered plate at 100°C for 10 min.

Lipid extraction from human brain white matter and human erythrocytes

A portion of human brain white matter (30 g, wet weight) was extracted three times with 10 volumes of chloroform—methanol (1:1) at room temperature. The total lipid obtained after evaporation was dissolved in 1200 ml of chloroform—methanol—water (30:60:8). Aliquots of 5, 10, 20, 40, 60 and 80 ml, which are equivalent to 0.125, 0.25, 0.5, 1.0, 1.5 and 2.0 g of white matter, respectively, were diluted to 100 ml with chloroform—methanol—water (30:60:8) and used for isolation of gangliosides as described below.

A 400-ml volume of freshly drawn blood in acid—citrate—dextrose anticoagulant was washed with phosphate-buffered saline (pH 7.3) and the packed erythrocytes (ca. 200 ml) were extracted with chloroform—methanol as described previously [8]. The total lipid was dissolved in 800 ml of chloroform—methanol—water (30:60:8). Aliquots of this solution equivalent to 0.5, 1.0, 5.0, 10 and 20 ml of packed erythrocytes were diluted to 100 ml with chloro-

form—methanol—water (30:60:8) and used for the isolation of gangliosides as described below.

Isolation of gangliosides from human brain white matter and human erythrocytes using a C_{18} Sep-Pak cartridge

The diluted lipid extract (100 ml) was applied to a DEAE-Sephadex (2 g, acetate form, dry weight) or DEAE-silica gel (10 g, acetate form, dry weight). After washing with 100 ml of chloroform—methanol—water (30:60:8) and 50 ml of methanol to remove the uncharged and zwitterionic lipids, acidic lipids were eluted with 50 ml of 0.2 *M* sodium acetate in methanol. The methanol was removed in a rotary evaporator and the residue was treated with 15 ml of 0.1 *N* sodium hydroxide in methanol and incubated at 37°C for 2 h to destroy the alkali-labile acidic phospholipids. The solvent was evaporated to dryness without heat and the residue was dissolved in about 50 ml of ice-cold water and neutralized to pH 4.5 by dropwise addition of 0.5 *N* hydrochloric acid. The solution was diluted to 115 ml with water to adjust the salt concentration to 0.1 *M*; this solution was then passed through a C_{18} Sep-Pak cartridge fitted tightly to a 25-ml glass disposable pipet at a flow-rate of approximately 1 ml/min under a slight positive pressure. [The cartridge was pre-washed with 25 ml of chloroform—methanol (1:2), 25 ml of methanol and 50 ml of water before use.] The cartridge was washed with 25 ml of water and the gangliosides were then eluted with 5 ml of methanol followed by 25 ml of chloroform—methanol (1:2). The cartridge can be used again after washing with methanol and equilibration with water (washing with methanol is necessary to remove chloroform from the cartridge).

Iatrobeads or Unisil column chromatography

The gangliosides eluted from the C_{18} Sep-Pak column were dried, dissolved in 2 ml of chloroform—methanol (85:15) and then chromatographed on an Iatrobeads [13] or Unisil [14] column to remove sulfatides and other colored impurities. For a 2-g column, 40 ml of chloroform—methanol (85:15) were used to remove impurities and gangliosides were quantitatively eluted with 40 ml of chloroform—methanol (1:2).

Isolation of gangliosides from human brain white matter and human erythrocytes using a dialysis method

The gangliosides from human brain white matter and human erythrocytes were isolated according to the procedure described previously [5, 14]. The method included DEAE-Sephadex column chromatography, treatment with base, dialysis against cold water and finally Unisil column chromatography.

Recovery of gangliosides from C_{18} Sep-Pak cartridge

Tritiated beef brain ganglioside mixture (1 μg of sialic acid, $2 \cdot 10^4$ cpm) was added to 100 ml of diluted lipid extract solution of human brain white matter (0.125—2.0 g wet weight) or human erythrocytes (0.5—20 ml of packed erythrocytes). The gangliosides were then isolated as described above and the amount of gangliosides recovered were measured.

Separation of gangliosides from glucose and sialic acid

[^{14}C] Glucose was dissolved in 25 ml of 0.1 *N* sodium chloride solution and allowed to pass through the C_{18} Sep-Pak cartridges as above. The cartridge was washed with 25 ml of water and the combined eluates (fraction 1) were counted to measure free glucose. The cartridge was then washed with methanol and chloroform—methanol as described above (fraction 2) and the percentage recovery of glucose was measured. Similarly, in separate experiments, N-[^{14}C]-acetylneuraminic acid was subjected to the separation procedure.

In another series of experiments, [^{14}C] glucose and N-[^{14}C] acetylneuraminic acid were separately mixed with tritiated beef brain ganglioside (1 μg of sialic acid, $5.3 \cdot 10^4$ cpm) and subjected to the same separation procedure as above. The percentage recoveries of glucose and N-acetylneuraminic acid in fraction 1 were 98% and 94%, respectively. Gangliosides were recovered from fraction 2 in almost quantitative yields (93—96% recovery; data not shown).

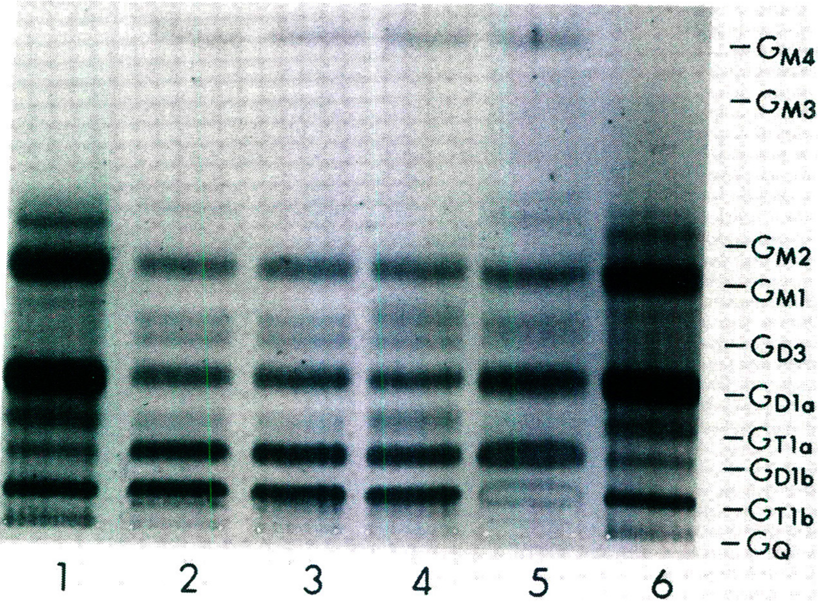

Fig. 1. Thin-layer chromatogram of gangliosides from normal human white matter: (1) and (6), standard beef brain ganglioside mixture; (2), white matter gangliosides by dialysis method; (3) and (4), white matter gangliosides by Sep-Pak method (overnight retention). Each of the lanes 2—5 contained 7 μg of sialic acid. Solvent system, chloroform—methanol—water (55:45:10) with 0.02% (w/v) of $CaCl_2 \cdot 2H_2O$. Plate, Merck pre-coated silica gel 60. All bands were purple after spraying by resorcinol reagent [12]. The ganglioside nomenclature of Svennerholm [15] is depicted: G_{M_4} = [NeuAc(α2-3)]Gal-Cer, G_{M_3} = [NeuAc(α2-3)]Gal (β1-4)Glc-Cer, G_{M_2} = GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{M_1} = Gal(β1-3) GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{D_3} = [NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4) Glc-Cer, $G_{D_{1a}}$ = [NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer $G_{D_{1b}}$ = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, $G_{T_{1a}}$ = [NeuAc (α2-8)NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer, $G_{T_{1b}}$ = [NeuAc (α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{Q_1} = [NeuAc (α2-8)NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, where Gal = D-galactose, Glc = D-glucose, GalNAc = N-acetyl-D-galactosamine, NeuAc = N acetylneuraminic acid, Cer (ceramide) = N-acylsphingosine.

RESULTS

The recovery of the lipid-bound sialic acid from human brain white matter and normal human erythrocytes by the C_{18} Sep-Pak method and the dialysis method are presented in Table I. The values were similar and the yields agreed

TABLE I

LIPID-BOUND SIALIC ACID OF HUMAN BRAIN WHITE MATTER AND HUMAN ERYTHROCYTES

The values were determined by gas—liquid chromatography [11] and are expressed as mean ± standard deviation (n = 5).

Sialic acid	Sep-Pak method	Dialysis method
Sialic acid per gram wet weight of human brain white matter (mg)	296.8 ± 0.98	299 ± 1.50
Sialic acid per 100 ml (mg)	752.2 ± 1.25	751 ± 2.20

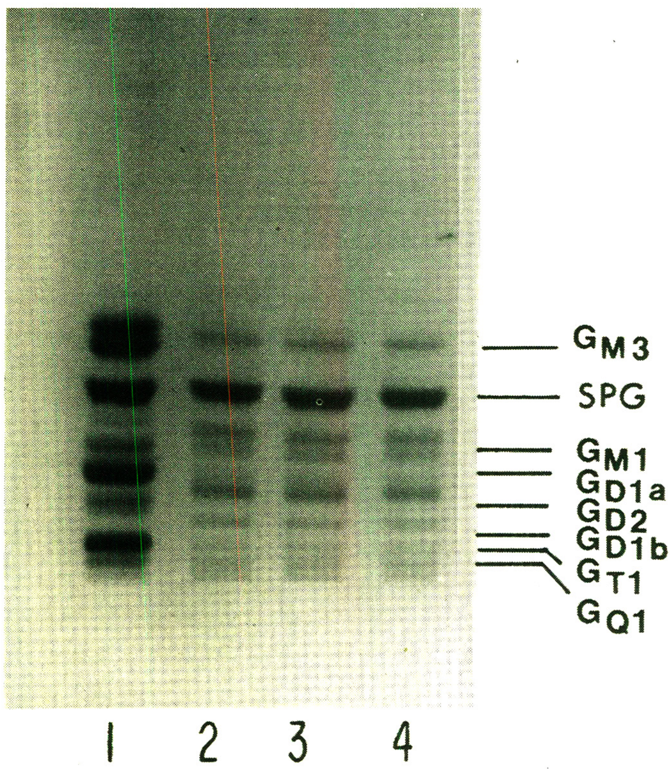

Fig. 2. Thin-layer chromatogram of gangliosides from normal adult human erythrocytes: (1), standard mixture of beef brain ganglioside, sialosylparagloboside (SPG) and G_{M3}; (2), human erythrocyte gangliosides by dialysis method; (3) and (4), human erythrocyte gangliosides by Sep-Pak method. Solvent system, chloroform—methanol—2.5 N ammonia solution (60:40:9). Each of the lanes 2—4 contained 8 μg of sialic acid. SPG = [NeuAc(α2-3)]Gal(β1-4)-GlcNAc(β1-3)Gal(β1-4)Glc-Cer, G_{D_2} = GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)-Glc-Cer, where GlcNAc = N-acetyl-D-glucosamine. $G_{T_1} = G_{T_{1a}} + G_{T_{1b}}$; $G_{Q_1} = G_Q$.

well with previous data [5]. The TLC patterns of gangliosides from human brain white matter and human erythrocytes isolated by both methods were also identical, as shown in Figs. 1 and 2, respectively. It should be pointed out that the plates did not give any positive band for amine and phospholipid on spraying with ninhydrin and phosphorus spray reagent [7].

The recovery of gangliosides from C_{18} Sep-Pak cartridges, ascertained by adding known amounts of the tritiated beef brain ganglioside mixture to the total lipid extracts from human brain white matter and human erythrocytes, is presented in Table II. For human brain white matter the recovery of gangliosides was nearly quantitative between 0.125 and 1.5 g wet weight. Similarly, an excellent recovery of gangliosides was obtained from 0.5 to 20 ml of packed human erythrocytes (Table II).

TABLE II

RECOVERY OF GANGLIOSIDES FROM SEP-PAK CARTRIDGES

Human brain white matter sample (g)*	Unlabeled ganglioside sialic acid recovered (μg)**	Recovery of added [³H]ganglioside (%)***
0.125	37.0	98
0.25	74.3	97
0.50	148.0	100
1.0	298.2	95
1.5	448.2	96
2.0	530.8	92
Packed human erythrocytes sample (ml)*	**Unlabeled ganglioside sialic acid recovered (μg)****	**Recovery of added [³H]gangliosides (%)*****
0.5	3.6	99
1.0	7.4	98
5.0	36.5	100
10.0	74.0	95
20.0	144.2	96

*Known amounts of tritiated beef brain ganglioside mixture (1 μg of sialic acid, $2 \cdot 10^4$ cpm) were added to each sample before passing through Sep-Pak cartridges, to check the recovery of gangliosides.

**Determined by gas–liquid chromatography [11].

***The percentage recovery of ganglioside was determined from the recovery of tritium in fraction 2 (see Experimental for details). The values given here are the means of two separate experiments.

The results also showed that free sugars and free sialic acid are not absorbed by C_{18} Sep-Pak cartridges. As an illustration, [^{14}C]glucose and N-[^{14}C]acetylneuraminic acid were separately passed through C_{18} Sep-Pak cartridges. [^{14}C]-Glucose and N-[^{14}C]acetylneuraminic acid were recovered in fraction 1 (Table III).

We also observed from the double-labeling experiments with mixtures of [^{14}C]glucose or N-[^{14}C]acetylneuraminic acid and tritiated beef brain ganglio-

TABLE III

SEPARATION OF GLUCOSE AND SIALIC ACID FROM GANGLIOSIDES ON A SEP-PAK CARTRIDGE

Triplicate samples were utilized in each experiment and the results are means ± standard deviations.

Sample	Amount applied (cpm)*	Fraction 1**		Fraction 2**	
		cpm recovered	recovery (%)	cpm recovered	recovery (%)
[^{14}C]Glucose	$1.14 \cdot 10^5$	$1.11 \cdot 10^5$ ± 380	98	500 ± 200	0.4
N-[^{14}C]Acetylneuraminic acid	$5.9 \cdot 10^4$	$5.64 \cdot 10^4$ ± 300	96	260 ± 100	0.4

*Column blanks were processed to determine the cpm applied to the Sep-Pak cartridge.
**Fractions 1 and 2 represent the unabsorbed and absorbed fractions, respectively.

side that the recoveries of glucose and N-acetylneuraminic acid in fraction 1 were over 94% and gangliosides were isolated in fraction 2 in almost quantitative yield.

In addition, we have shown that during Iatrobeads or Unisil column chromatography, which is needed as a final purification step, the recovery of gangliosides was over 93%. This recovery was ascertained by adding known amounts of tritiated beef brain ganglioside to unlabeled human brain white matter and human erythrocyte ganglioside mixtures prepared by the dialysis method described above. The amounts of gangliosides recovered in the chloroform—methanol (1 : 2) eluates were 94.2 ± 3.9% (mean ± standard deviation, n = 9) and 93.2 ± 4.0% (n = 9) for white matter and erythrocytes, respectively.

DISCUSSION

A major problem in the isolation of small amounts of gangliosides has been the removal of salts and other non-lipid contaminants. Dialysis is the procedure most commonly used for this purpose, but at concentrations below 150 μg/ml of sialic acid there is considerable loss of gangliosides [16]. Other methods, such as gel filtration on Sephadex G-50 [13] or precipitation by trichloroacetic acid—phosphotungstic acid [17], are cumbersome and do not provide good recoveries when small amounts of gangliosides are used. The reversed-phase chromatographic procedure reported here provides a simple and efficient means for separating this type of impurity from gangliosides. It should be emphasized that the ganglioside solution should be passed through the Sep-Pak cartridge at a flow-rate of 1—2 ml/min because if the gangliosides remain on the column for long periods of time, the polysialo-gangliosides may form inner esters. The sample shown in lane 5 in Fig. 1 was passed through the cartridge over a period of 15 h. The G_{T1a} and G_{T1b} bands appear to be low in this sample, but treatment of this fraction with base hydrolyzed the inner esters and the chromatographic pattern became similar to those depicted in lanes 2—4.

While this work was in progress, a similar chromatographic procedure using a Sep-Pak cartridge was published by Williams and McCluer [3]. Their method

uses a Folch partition procedure in which most gangliosides are found in the aqueous phase but some G_{M3} and G_{M2} remain in the organic phase and the more polar neutral glycolipids are also found in the aqueous phase. The series of procedures used in our work provide a highly efficient micro-method for the total purification of small amounts of gangliosides and it should have wide applicability.

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SIMPLE SELECTED ION MONITORING METHOD FOR DETERMINATION OF FOSFOMYCIN IN BLOOD AND URINE

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SUMMARY

A rapid, sensitive and specific selected ion monitoring method is described for the determination of fosfomycin in plasma and urine. The extraction of the drug from serum involves deproteinization with ethanol (1 ml per 0.25 ml of serum), evaporation of an aliquot of supernatant and derivatization with a silylating mixture consisting of bistrimethylsilylacetamide—dichloromethane (1:1) + 5% of trimethylchlorosilane. The analysis of fosfomycin in urine requires the dilution of samples and their derivatization only. The results were compared with those obtained by analysing the same samples using a microbiological method.

INTRODUCTION

Fosfomycin (*cis*-1,2-epoxyphosphonic acid) is a broad-spectrum antibiotic discovered in 1969 [1] and recently used in the therapy of different bacterial infections. It is a small molecule (molecular weight 138) with a molecular structure not related to that of any other antibiotic. The antimicrobial activity of fosfomycin consists in inhibiting bacterial cell-wall formation, without interfering with the organism [2].

Up to now, fosfomycin pharmacokinetics have been studied by measuring microbiologically its level in biological fluids. In fact the microbiological method, measuring drug activity in serum, does not require drug extraction, which is very difficult. Using the microbiological method, the fosfomycin concentration is determined by evaluating the inhibitory potency of the drug on bacterial growth. Many factors can interfere with this procedure, such as the inhomogeneity of the culture medium, the presence of ions that can inhibit bacterial growth and sterility of the working area, which necessitate continuous control, even during routine analyses. In contrast, a chemical method

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involving the use of an internal standard requires fewer precautions and less drastic experimental conditions.

In 1970, Shafer et al. [3] described a method for evaluating the purity of concentrated fosfomycin samples by gas chromatography (GC) of its bistrimethylsilyl derivative; they also reported the mass spectrum of this compound. The low levels of fosfomycin in biological fluids (a few micrograms per millilitre) do not make this method useful in clinical studies. This paper describes a simple selected ion monitoring (SIM) procedure for the determination of nanogram amounts of fosfomycin in small volumes of biological specimens, such as 0.25 ml of serum or 0.1 ml of urine. The analysis is based on the relative peak-area ratio of the bistrimethylsilyl derivatives of fosfomycin and propenylphosphonic acid, used as an internal standard.

EXPERIMENTAL

Fosfomycin (sodium salt) and propenylphosphonic acid (PPA) were synthesized in our laboratories.

Bistrimethylsilylacetamide (BSA), trimethylchlorosilane (TMCS), XE-60 stationary phase and Gas-Chrom Q were purchased from Supelchem (Milan, Italy). Dichloromethane and methanol (analytical reagent grade) were obtained from Carlo Erba (Milan, Italy).

Transparent polystyrene plates (235 × 235 mm) were purchased from AS Nunc-Dane (Roskilde, Denmark). DIFCO (Detroit, MI, U.S.A.) nutrient agar, nutrient broth and yeast extract were used. The test organism was *Proteus mirabilis* ATCC 21100.

Gas chromatography

A Hewlett-Packard Model 5710A gas chromatograph equipped with a flame ionization detector was used. Analyses were carried out using a 1.5 m × 2 mm I.D. silanized glass column packed with 3% XE-60 on Gas-Chrom Q (100—120 mesh). The oven temperature was 130°C, injector and detector temperatures 250°C and carrier gas (helium) flow-rate 30 ml/min. Under these conditons the retention times of PPA and fosfomycin were 2.5 and 4 min, respectively.

Gas chromatography—mass spectrometry (GC—MS)

A Hewlett-Packard GC—MS system, Model 5993B, equipped with a data system was used. The GC conditions were as described above. For mass spectrometry, the electron energy was 70 eV, the molecular separator was of the jet type and the separator temperature was 250°C.

Preparation of fosfomycin and PPA derivatives

A 1-mg amount of fosfomycin and 1 mg of PPA were dissolved in 1 ml of methanol and 100 µl of the solution were transferred into screw-capped vials and dried under nitrogen. The residue was treated with 100 µl of a mixture consisting of BSA—dichloromethane (1:1) + 5% of TMCS as a catalyst. After 10 min at 60°C the solution was analysed by GC—MS.

Animal treatment

Two male Beagle dogs weighing 12 and 9.7 kg, fasted overnight, were treated intramuscularly (i.m.) with 50 mg/kg of fosfomycin. Urine was collected 1, 3 and 6 h after the treatment in a graduated cylinder and then filtered over a HAWP 0.45-μm Millipore membrane. Blood was withdrawn 1, 3 and 6 h after treatment and collected in plastic tubes. Serum was prepared by incubating the blood at 37°C for 30 min, followed by centrifugation at 1500 *g* for 10 min.

Determination of fosfomycin in serum

A 250-μl volume of serum was transferred into centrifuge tubes and proteins were precipitated by adding 1 ml of methanol in which PPA was dissolved at a concentration of 2 μg/ml as an internal standard. After centrifugation at 20,000 *g* for 15 min at 0°C, 100 μl of supernatant were transferred into screw-capped vials and dried under nitrogen. A 20-μl volume of the silylating mixture was added to dry residue. At the end of the reaction 1—2 μl of the solution were injected into the GC—MS system.

Determination of fosfomycin in urine

Urine was diluted 10-fold with distilled water containing 20 μg/ml of PPA as internal standard. A 10-μl volume of this solution was transferred into screw-capped vials and dried under nitrogen. A 50-μl volume of the silylating mixture was added to the residue. At the end of the reaction 1 μl of the solution was injected into the GC—MS system.

Calibration graphs were prepared using known amounts of fosfomycin dissolved either in serum or in urine of untreated dogs.

Microbiological method

Fosfomycin concentrations in serum and urine were determined by the cup method [4] using 235 × 235 mm polystyrene plates covered with a 20-mm layer of nutrient agar containing 0.2% of yeast extract. The inoculum was 0.5% (v/v) test organism subculture in nutrient broth. Stainless-steel cylinders (8 mm I.D., 10 mm height) were placed on the agar layer and filled with assay samples diluted with Tris—hydrochloric acid buffer, pH 7.4, 0.05 *M*. The plates were incubated at 37°C overnight and the diameters of the zones of inhibition were measured.

RESULTS

Formation and characterization of fosfomycin and PPA derivatives

The reaction of fosfomycin and PPA with the silylating mixture under the conditions described produces two single compounds, which give rise to symmetrical GC peaks. The mass spectra, shown in Figs. 1 and 2, indicate that two trimethylsilyl groups are introduced, as described previously [3].

The peaks at *m/e* 211 (base peak), 226 and 267 were used to monitor fosfomycin and those at *m/e* 251 (base peak) and 266 to monitor the internal standard.

Fig. 3 shows a mass fragmentogram obtained by analysing a standard sample of serum containing 2.5 μg/ml of drug.

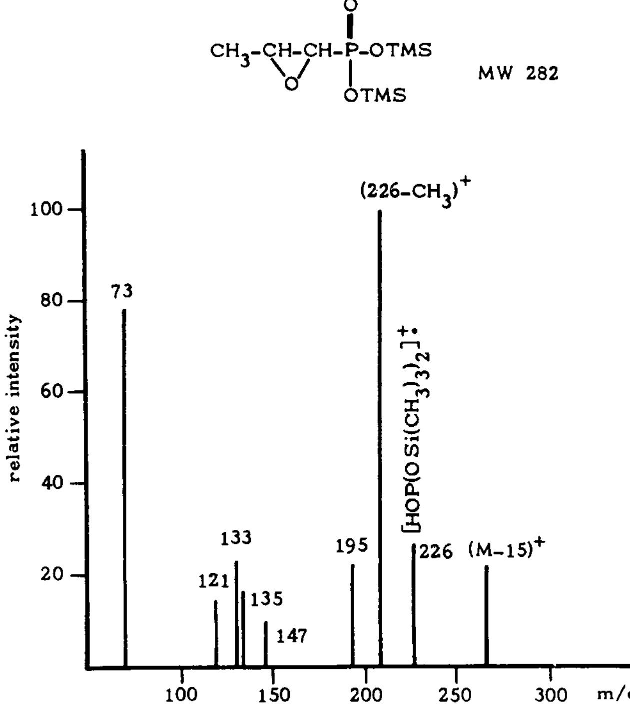

Fig. 1. Mass spectrum of fosfomycin bis-TMS.

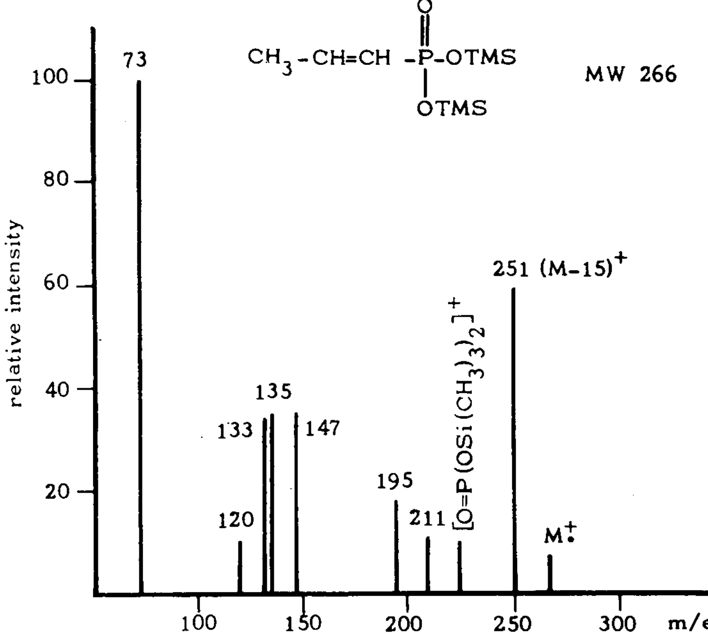

Fig. 2. Mass spectrum of propenylphosphonic acid bis-TMS.

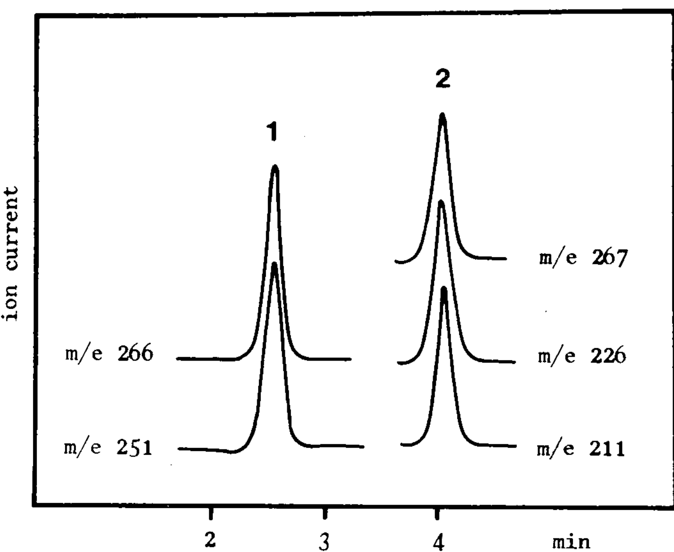

Fig. 3. Mass fragmentogram obtained from bis-TMS derivatives of propenylphosphonic acid (peak 1) and fosfomycin (peak 2).

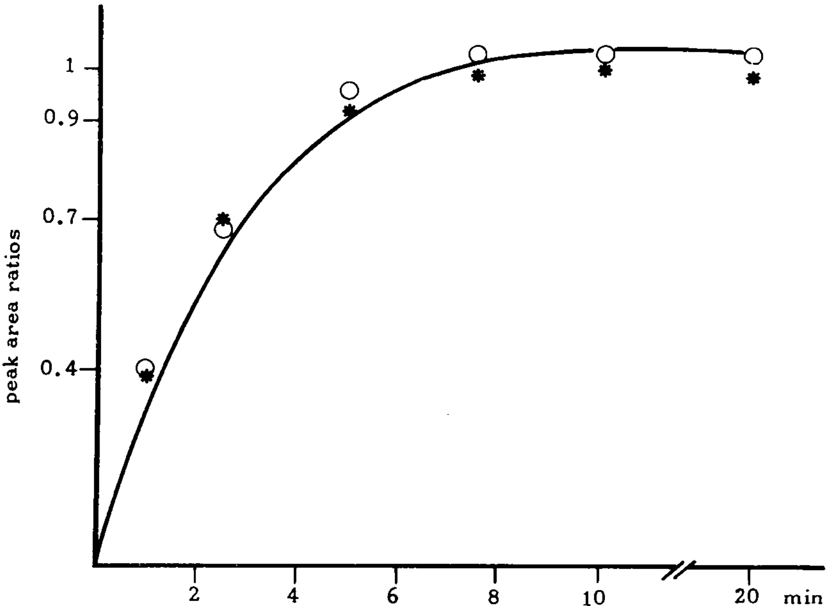

Fig. 4. GC response of fosfomycin (*) and PPA (○) with respect to *n*-pentadecane (internal standard). The samples contained the same amount of the three compounds reacted with the same amount of silylating mixture.

Fig. 4 shows the kinetics of derivatization of fosfomycin and PPA at 60°C; complete reaction is obtained in 10 min. The results are plotted as ratio of the peak areas of the two drugs versus time with a constant amount of n-$C_{15}H_{32}$ being added to all the samples.

Sensitivity and linearity

Under routine conditions a linear response in the range 0.25—50 μg/ml was obtained. A calibration graph was obtained by adding known amounts of fosfomycin to dog serum and analysing the samples by SIM.

Recovery of fosfomycin

Samples of serum obtained from the blood of dogs that had received 50 mg/kg of fosfomycin were divided into two groups. Methanol containing the internal standard only was added to one group, and methanol containing the internal standard and a known amount of fosfomycin was added to the other. The samples were analysed by SIM and the recovery was 95.5 ± 0.5% (10 experiments).

Identification of fosfomycin in biological fluids

Identification of fosfomycin in both serum and urine was based on the measurement of ion density ratio of the recorded selected ions at *m/e* 267, 226 and 211. Table I gives the ion density ratios obtained from the determination of fosfomycin in the serum of treated dogs (50 mg/kg i.m.), compared with those of standard samples.

TABLE I

ION DENSITY RATIOS OF FRAGMENTS OF *m/e* 211, 226 AND 267 OBTAINED ON DETECTING FOSFOMYCIN BY SELECTED ION MONITORING IN STANDARD AQUEOUS SOLUTIONS AND IN THE SERUM OF TREATED DOGS (50 mg/kg i.m.)

Sample	*m/e*			No. of determinations
	211	226	267	
Standard fosfomycin	100	26.2±0.2	22.5±0.1	10
Serum fosfomycin	100	26.0±0.2	22.1±0.2	18

Comparison between selected ion monitoring and microbiological methods

Two dogs were treated with 50 mg/kg i.m. of fosfomycin and blood and urine were collected after 1, 2 and 3 h. The samples were divided into two groups: one group was analysed by SIM and the other by a microbiological method on the same day. The same standard solution of fosfomycin was used to prepare calibration graphs.

Tables II and III show that the concentrations of fosfomycin found by SIM are very similar to those obtained by microbiological determination.

TABLE II

LEVELS OF FOSFOMYCIN IN SERUM OF TREATED DOGS DETERMINED BY SELECTED ION MONITORING (SIM) AND THE MICROBIOLOGICAL (MB) METHOD

Values are the averages of three determinations from two separate experiments. Samples were obtained from two dogs treated with 50 mg/kg i.m. of fosfomycin.

Dog No.	Time of sampling (h)	Fosfomycin (μg/ml)	
		SIM	MB
1	1	40.8±0.8	40.0±1.7
	3	13.2±0.2	12.3±0.5
	6	3.6±0.01	3.6±0.2
2	1	35.4±0.7	37.5±1.0
	3	10.5±0.1	8.6±0.2
	6	2.5±0.05	2.9±0.1

TABLE III

CONTENT OF FOSFOMYCIN IN URINE OF TREATED DOGS DETERMINED BY SELECTED ION MONITORING (SIM) AND THE MICROBIOLOGICAL (MB) METHOD

Values are the averages of three determinations from two separate experiments. Samples were obtained from two dogs treated with 50 mg/kg i.m. of fosfomycin.

Dog No.	Time of sampling (h)	Urinary elimination (ml)	Fosfomycin (μg/ml)		Recovery (%)		Total recovery (%)	
			SIM	MB	SIM	MB	SIM	MB
1	1	50	742±5	740±5	6.0	6.0	25.6	25.0
	3	21	4410±8	4438±15	15.1	15.2		
	6	110	255±2	215±3	3.8	3.8		
2	1	65	1234±3	1180±8	16.5	15.8	28.4	28.1
	3	35	321±1	310±3	2.3	2.2		
	6	113	410±1	430±5	9.6	10.0		

DISCUSSION

In 1970, Shafer et al. [3] used GC—MS to follow the isolation and purification of fosfomycin from natural products. The antibiotic was purified by preparative paper chromatography and concentrated samples were made to react with BSA for 5 min at 60°C before the GC—MS analysis. An attempt was also made to purify fosfomycin from urine by paper chromatography, but as extraneous substances present in urine influence the mobility of the antibiotic, the results were difficult to interpret [3]. Moreover, as the sensitivity of this procedure was low, a biological method had to be used to measure the fosfomycin level. In contrast, the procedure described here is reproducible and sensitive enough to detect fosfomycin in both the serum and urine of animals treated with pharmacological doses of the antibiotic. In addition, using a silylating mixture consisting of BSA—dichloromethane (1:1) + 5% of TMCS

we obtained a better reaction yield and a better chromatographic profile than that obtained by using only BSA.

The bistrimethylsilyl derivative of fosfomycin has excellent GC and, as its molecular weight is 282, i.e., double that of the original compound, its SIM properties are excellent.

Under the experimental conditions described, no interference was found in either serum or urine at the mass numbers used for the antibiotic detection; however, simultaneous monitoring of three characteristic fragments allows the univocal identification of fosfomycin.

The sensitivity of the SIM method is higher than that of the microbiological method. The microbiological procedure has a linear response in the range 0.5—10 μg/ml, whereas the range of the SIM method is 0.25—50 μg/ml. Another advantage of the SIM method is its simplicity and rapidity: preparation of samples requires 30 min, their evaporation 10 min and injection into the gas chromatograph can be performed 10 min after silylation. Moreover, the high sensitivity of the method may allow the study of intracellular fosfomycin levels, so that it should be possible to investigate its mechanism of action more deeply.

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CHROMBIO. 864

ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY FOR PRIMAQUINE IN BLOOD*

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SUMMARY

A sensitive gas chromatographic method for the quantitative determination of the antimalarial drug primaquine is described. The method involves derivatization with heptafluorobutyric anhydride to form the diheptafluorobutyramide derivative after a single extraction at alkaline pH. The derivatives are quantitated by electron-capture gas chromatography. Blood levels of primaquine as low as 8 ng/ml can be measured with good precision.

INTRODUCTION

The antimalarial drug primaquine, 8-(4-amino-1-methyl-butylamino)-6-methoxyquinoline is the drug of choice in the treatment of *Plasmodium vivax* malaria and is being increasingly used in the chemotherapy of chloroquine-resistant forms of malaria [1]. The pharmacokinetic profile of primaquine in animals and man is largely unknown due to the lack of sensitive and specific assay methods for the determination of primaquine in biological fluids. Recently Greaves et al. [2] have described a method for the analysis of primaquine in plasma and urine using gas chromatography—mass spectrometry (GC—MS). The lower limit for quantitation by this method is 50 ng/ml of plasma or urine and measurement of intraerythrocytic primaquine levels has not been successful with this method. This paper describes an analytical method for the measurement of primaquine at a concentration of 10 ng/ml and above in blood or plasma by selective extraction, derivatization using heptafluorobutyric (HFB) anhydride to form the diheptafluorobutyramide (diHFB) derivative and subsequent separation and quantitation using GC with electron-capture detection.

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EXPERIMENTAL

Solvents, standards and reagents

Reagent grade cyclohexane and benzene were obtained from Glaxo Laboratories (India) Ltd., Bombay, India; methylene chloride and methanol Uvasol® were from E. Merck, Bombay, India.

Cyclohexane and benzene were purified by washing with concentrated sulphuric acid until free from colour, followed by successive washings with water, dilute sodium hydroxide and water. The washed solvents were dried over calcium chloride and distilled using a 120-cm Vigreux column before use. Methylene chloride was also distilled using the Vigreux column and stored in the refrigerator. Heptafluorobutyryl chloride and heptafluorobutyric anhydride were obtained from Fluka (Buchs, Switzerland). Primaquine diphosphate was from Aldrich (Milwaukee, WI, U.S.A.) and the internal standard diphosphate was synthesised according to the previously reported procedure [3]. The column packing material, 3% SP-2401 on 80—100 mesh Supelcoport, was from Supelco (Bellefonte, PA, U.S.A.). Ultra high purity nitrogen (IOLAR-2) used as carrier gas was from Indian Oxygen Ltd. (Bombay, India).

All other chemicals used were of analytical reagent grade obtained locally.

Apparatus

The gas chromatograph was a Pye-Unicam Model 204 equipped with a 10 mCi ^{63}Ni electron-capture detector. Nitrogen was used as the carrier gas at a flow-rate of 46 ml/min. The column was operated at 235°C, the injector at 200°C and the detector at 300°C. Peak areas were measured by the height × width at half-height method. The samples were injected "on column" using the solvent-flush technique.

All the glassware used in processing the samples and the glass column were silanized to prevent adsorption by soaking overnight in a 2.5% solution of dimethyldichlorosilane in toluene followed by two rinses with distilled toluene and a final rinse with distilled methanol. They were dried at 90—100°C before use. Solvents and glassware used in the method were checked at random for contamination by carrying out blank runs through the entire method.

The glass column (2.1 m × 4 mm I.D.) was packed with 3% SP-2401 on 80—100 mesh Supelcoport. The packed column was conditioned without being connected to the detector for two to three days at 270°C using a carrier flow-rate of 40 ml/min. After this four to six injections of 10 μl of Silyl 8 (Pierce, Rockford, IL, U.S.A.) were given at 30-min intervals at a column temperature of 250°C. The conditioned column was kept at this temperature overnight and was primed with a mixture of primaquine diHFB and internal standard diHFB at a concentration of 1—2 ng/μl. After 15—20 priming injections, the column was connected to the detector and the response to injections of 0.4 ng of standard diHFB derivatives of primaquine and internal standard was checked. If the response was poor, priming and conditioning injections were repeated until the optimal response was obtained. In our experience it takes about eight to ten days to prepare a column that exhibits optimal sensitivity. After repeated use the column material may require topping-up, and this was easily accomplished by removing the top 2—3 cm of the packing and replacing it with fresh packing material.

Synthesis of mono- and diheptafluorobutyramide derivatives of primaquine and internal standard

The melting points reported here are uncorrected. Infrared (IR), ultraviolet (UV) and mass spectra were recorded, respectively, on a Perkin-Elmer Infracord, a Beckmann DK-2A and a Varian MAT CH-7. The general procedures used in the synthesis of the various derivatives are summarised in Fig. 1.

Fig. 1. Summary of reactions used in the synthesis of heptafluorobutyramides of primaquine and internal standard.

Synthesis of monoheptafluorobutyramides II and V

These derivatives were prepared under Schotten—Baumann reaction conditions as described below. The free bases (1 m*M*) of compounds I and IV were liberated from either the diphosphate or the dihydrochloride salts [3, 4] by the addition of 10 ml of 8% aqueous potassium hydroxide at 15°C, to a stirred suspension of the salt in 50 ml of benzene. Then 0.3 ml of heptafluorobutyryl chloride was added dropwise. A second 5-ml lot of 8% potassium hydroxide and 0.2 ml of heptafluorobutyryl chloride were added after a few minutes and the mixture at alkaline pH was stirred at 15°C for 30 min and then at room temperature for 60 min. The benzene layer was separated and the aqueous layer was extracted once with 25 ml of benzene. The pooled benzene layer was washed with water, dried over anhydrous sodium sulphate and filtered. The solvent was removed by rotary evaporation and the residue was purified by chromatography on a 50-g silica gel column equilibrated with hexane—acetone (9:1, v/v). Elution with the above solvent mixture and crystallisation of the gummy residue from hexane yielded the monoHFB derivatives as nice crystalline solids.

A. 8-(4′-heptafluorobutyrylamido-1′-methyl-butylamino)-6-methoxyquinoline or primaquine monoHFB (II). m.p. 88—90°C. Analysis: found C, 50.29; H, 4.77; N, 9.52. $C_{19}H_{20}F_7N_3O_2$ (mol. wt. 455) requires C, 50.11; H, 4.43; N, 9.23. IR: ν_{max}^{KBr} cm^{-1} 3410 (NH); 1700 (amide C = O). UV: λ_{max} 260 (CH_3OH), 287 (CH_3OH + HCl). MS: 455 (M^+), *m/e* 201, 202.

B. 8-(3′-heptafluorobutyrylamido-1′-methyl-propylamino)-6-methoxyquinoline or internal standard monoHFB (V). m.p. 73—75°C. Analysis: found C, 49.03; H, 4.38; N, 9.82. $C_{18}H_{18}F_7N_3O_2$ (mol. wt. 441) requires C, 48.98; H, 4.11; N, 9.52. IR: ν_{max}^{KBr} cm^{-1} 3400 (NH); 1700 (amide C = O). UV: λ_{max} 262 nm (CH_3OH). MS: 441 (M^+), *m/e* 201.

Synthesis of diheptafluorobutyramides III, VI and VIII

A solution of the free base, 1 m*M*, in 10 ml of dry benzene was treated with an excess 1.24 g of heptafluorobutyric anhydride. The mixture which turned an orange colour was refluxed for 90 min with stirring. The solvent was removed first on a rotary evaporator and the excess anhydride was then removed in vacuo. The residue was redissolved in 25 ml of benzene and washed with 10 ml of saturated sodium bicarbonate followed by water. The solvent was removed and the residue was chromatographed on a 40-g silica gel column. Elution with a mixture of cyclohexane—ethyl acetate (8:2) removed the faster-moving by-products. Further elution with cyclohexane—ethyl acetate (6:4), evaporation of these eluates and crystallisation of the residue from hexane—acetone yielded the diheptafluorobutyramides as nice yellow crystals. The above derivatives could also be obtained from the corresponding monoHFBs by heating with HFB anhydride at 100°C for 45 min.

C. 8-(1′,4′-bisheptafluorobutyrylamido-1′-methyl-butylamino)-6-methoxyquinoline or primaquine diHFB (III). m.p. 98—100°C; Analysis: found C, 42.78; H, 3.37; N, 7.28. $C_{23}H_{19}F_{14}N_3O_3$ (mol. wt. 651) requires C, 42.41; H, 2.94; N, 6.45. IR: ν_{max}^{KBr} cm^{-1} 3340 (NH); 1700 (amide C = O). UV: λ_{max} (CH_3OH) 258, 320, 400 nm. MS: 651 (M^+), *m/e* 482, 397, 201.

D. 8-(1′,3′-bisheptafluorobutyrylamido-1′-methyl-propylamino)-6-methoxyquinoline or internal standard diHFB (branched) (VI). m.p. 80—82°C. Analysis: found C, 42.23; H, 3.31; N, 6.95. $C_{22}H_{17}F_{14}N_3O_3$ (mol. wt. 637) requires C, 41.45; H, 2.69; N, 6.59. IR: ν_{max}^{KBr} cm^{-1} 3340 (NH); 1700 (amide C = O). UV: λ_{max} 258, 320, 395 nm. MS: 637 (M^+), *m/e* 468, 397, 201.

E. 8-(1,4′-bisheptafluorobutyrylamido-butylamino)-6-methoxyquinoline or internal standard diHFB (linear) (VIII). m.p. 107—108°C. Analysis: found C, 41.71; H, 2.95. $C_{22}H_{17}F_{14}N_3O_3$ (mol. wt. 637) requires C, 41.45; H, 2.69. UV: λ_{max} (CH_3OH) 265 (broad), 320 (shoulder), 333. MS: (M^+) 637, *m/e* 468, 467.

Method

Step 1. Primaquine diphosphate, 1.76 mg, and 1.8 mg of the internal standard diphosphate (branched) corresponding to 1 mg of free base were dissolved

separately in 10 ml of distilled water and the volume made up to 100 ml with methanol. Internal standard stock solution (100 μl) was diluted to 5 ml with methanol in a volumetric flask to give the working internal standard solution, 20 ng per 100 μl. Volumes of 50, 100, 150 and 200 μl of the primaquine diphosphate stock solution were each diluted to 5 ml with methanol to give solutions containing 10, 20, 30 and 40 ng primaquine in 100 μl. The stock solutions were kept refrigerated and dilutions from stock solutions were made fresh since the diluted solutions were found to be unstable.

Step 2. A 100-μl volume of the diluted internal standard and 100 μl of the primaquine solution containing 10, 20, 30 or 40 ng primaquine were pipetted into a glass-stoppered silanized tube. Then 1.0 ml of blood was added and the tube was gently swirled by hand. This was followed by the addition of 1 ml of 2.5 *N* sodium hydroxide. The tube was swirled and 3 ml of a mixture of cyclohexane—methylene chloride (4:1, v/v) were added. The stopper was sealed with a drop of water and the tube was mounted horizontally on a reciprocal shaker and shaken at full speed for exactly 2 min.

Step 3. The tube was removed from the shaker and centrifuged at 4000 *g* for 10—15 min. The organic layer was transferred to a 4-ml conical glass stoppered tube and concentrated under a gentle stream of nitrogen at 37°C to a volume of approximately 50 μl. The tube was vortexed once or twice to wash the residue down to the tip of the tube.

Step 4. Heptafluorobutyric anhydride (50 μl) was added and the stopper was quickly sealed with a drop of dry benzene. The stopper was sealed with a strip of parafilm and heated at 65—70°C in a water-bath for 30 min.

Step 5. After cooling to room temperature, 1.2 ml of saturated aqueous sodium bicarbonate were added and gently mixed. The tube was allowed to stand for 10—15 min with occasional mixing and the pH checked with indicator paper to ensure complete neutralization.

Step 6. Distilled benzene (200 μl) was added and the tube was mixed at full speed in a vortex mixer for 30 sec and centrifuged.

Step 7. A maximum aliquot of the upper benzene layer was transferred to another tube and a 2- or 3-μl aliquot was injected "on column" into the gas chromatograph in duplicate.

The calibration curve was obtained by extraction of blood spiked with varying amounts of primaquine (from 10 to 40 ng/ml) and a constant amount of internal standard (20 ng/ml of blood). The peak area ratios were calculated by dividing the area of the peak due to primaquine by the area of the peak due to the internal standard. Calibration curves were constructed by plotting peak area ratio as a function of primaquine concentration. Six to eight samples were analysed for each calibration point. This calibration curve was used subsequently to calculate unknown concentrations of primaquine in blood.

RESULTS AND DISCUSSION

Successful quantitative analysis of primaquine at the nanogram range was dependent on the proper selection of an internal standard and the choice of a suitable derivative for electron-capture GC. Several analogues of primaquine were examined before selecting compound II (cf. Fig. 1). This analogue was found to be ideally matched in its extraction, derivatization and GC resolution

behaviour when compared to primaquine. The amine function at 8 and 3′ or 4′ positions of the molecule (cf. Fig. 1, compounds I and IV) was the obvious site for derivatization.

Greaves et al. [2] had reported that attempts to form acyl derivatives with reagents like heptafluorobutyric anhydride and heptafluorobutyrylimidazole resulted in the formation of mixtures of mono- and diacylated products. Therefore, this reaction was investigated in detail to arrive at the optimal conditions for derivatization which would favour the formation of the diheptafluorobutyramides of both primaquine and the internal standard. The synthesis of pure, chemically characterised derivatives of both primaquine and the internal standard helped a great deal in the study of their GC behaviour and in establishing their sensitivity for detection using the electron-capture detector. Fig. 2 illustrates the separation of both mono- and diheptafluorobutyramides of primaquine and internal standard. The diheptafluorobutyramides are approximately 6–8 times more sensitive than the corresponding mono derivatives and possess excellent chromatographic properties. The introduction of fourteen fluorine atoms to both primaquine and the internal standard imparts excellent sensitivity for detection in the sub-nanogram range.

Several solvents and solvent mixtures were tried for the initial extraction of primaquine and the internal standard from blood. A single extraction using the solvent mixture cyclohexane–methylene chloride at a highly alkaline pH (pH

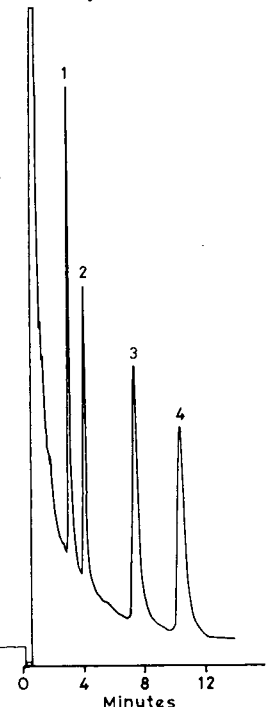

Fig. 2. Chromatogram of synthetic heptafluorobutyramide derivatives of primaquine and internal standard. Peaks: 1 = internal standard monoHFB; 2 = primaquine monoHFB; 3 = internal standard diHFB; 4 = primaquine diHFB.

> 13.0) was found to yield the least complex extract free of extraneous peaks in the primaquine and internal standard diheptafluorobutyramide regions following derivatization and chromatography. The recovery of nanogram quantities of primaquine and internal standard added to blood ranged between 60 and 70% in the extraction step.

The recovery was poor when 2.5 *N* NaOH was replaced by buffers in the alkalinization step. Concentration of the cyclohexane—methylene chloride extract to complete dryness under nitrogen led to severe losses of both primaquine and internal standard. Concentrating the extract to approximately 50 μl with intermittent vortexing to rinse the sides of the tubes resulted in good recoveries of both the compounds. The effect of time, temperature and the amount of heptafluorobutyric anhydride required for maximum yield of the diHFB derivatives was studied by GC using the synthetic derivatives as standards. Plateau conditions were reached in 30 min at 65°C using 50 μl of the anhydride. The overall recoveries of primaquine and the internal standard ranged between 30 and 50% as determined by GC using the standard compounds.

Typical gas chromatograms obtained with the method are shown in Fig. 3. The calibration curve constructed by adding known quantities of primaquine and a constant amount of internal standard to blood was linear from 10 to 40

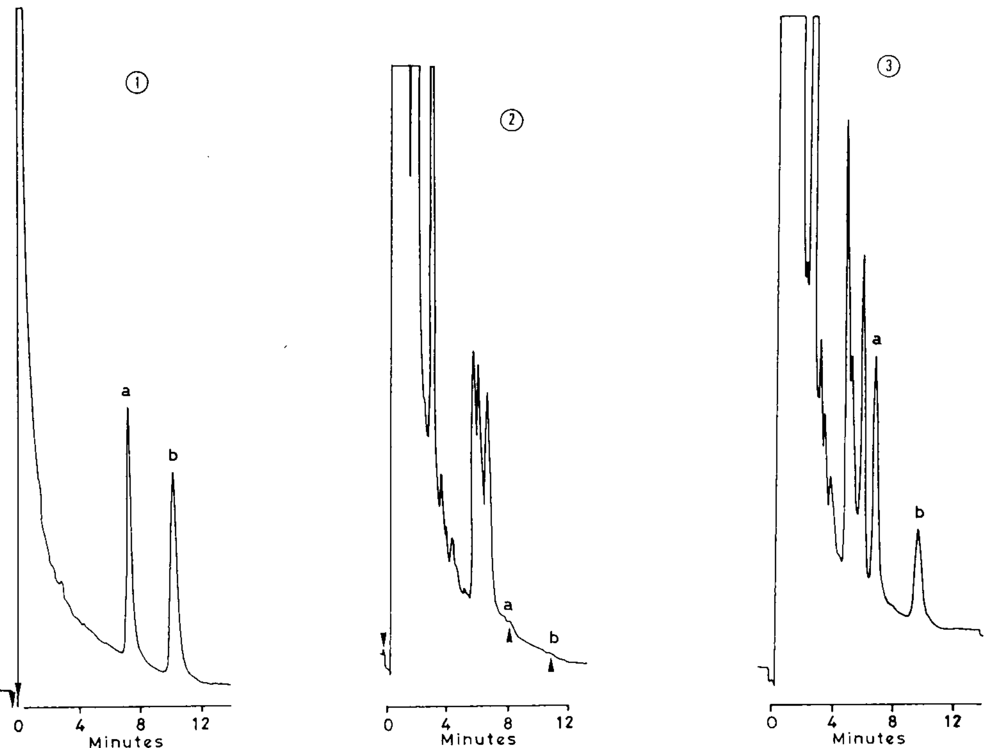

Fig. 3. Examples of chromatograms. (1) 0.2 ng each of internal standard diHFB (a) and primaquine diHFB (b). (2) Human blood blank sample (1 ml). (3) Human blood sample spiked with 8 ng of primaquine and 20 ng of internal standard.

ng/ml primaquine with a minimum detectable concentration of 8 ng/ml, based on a 1-ml sample volume. The equation for the calibration curve from blood was calculated and found to be $y = 0.0521x - 0.01$ with an excellent correlation ($\gamma = 0.999$). Table I shows the results obtained when the above method was applied to spiked blood samples. The results demonstrate good reproducibility of the method to a concentration of primaquine as low as 8 ng/ml of blood.

TABLE I

WITHIN-RUN PRECISION OF THE METHOD APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Amount added (ng/ml)	Amount found (ng/ml) (mean ± S.D., n = 4)	Precision/reproducibility (C.V. %)	Mean recovery (%)
8	9.16 ± 0.52	5.67	114.5
15	14.85 ± 0.83	5.6	99
25	25.5 ± 0.25	0.98	102
35	35.75 ± 0.74	2.1	102

The specificity and sensitivity of the described method for pharmacokinetic studies was ascertained by an in vivo experiment in dog. Fig. 4 illustrates the time course of whole-blood levels of primaquine for the first 15 h following the oral administration of an aqueous solution of primaquine diphosphate equal to 0.5 mg/kg body weight of the free base. The maximum concentration of 46 ng/ml was reached in 4 h followed by a rapid decrease to a level of 5.75 ng/ml at 12 h. The levels beyond this point were below the detection limit of the method. The half-life of primaquine in the dog was found to be 2.5 h. The major metabolite of primaquine in man has been identified by Baty et al. [5] as 6-methoxy-8-aminoquinoline. The monoHFB derivative which is formed on acylation with HFB anhydride under the derivatization conditions used in the present method does not elute from the GC column at 230°C, the temperature used in the present method.

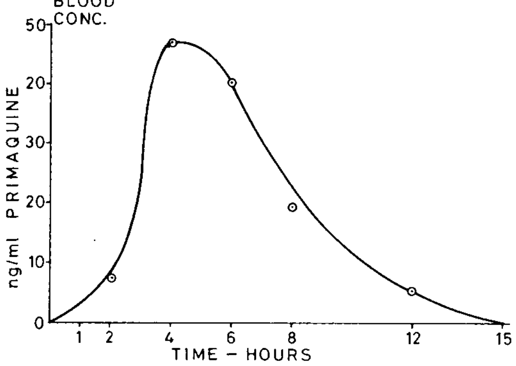

Fig. 4. Concentration of primaquine in dog blood after the administration of an oral dose of 0.5 mg/kg.

The method presented in this paper is simple, fast and extremely sensitive for the quantitative determination of primaquine in biological fluids. Experiments on the application of this technique to study single- and multiple-dose pharmacokinetics of this important antimalarial drug are in progress.

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CHROMBIO. 862

DETERMINATION OF THE ANTIHYPERTENSIVE AGENT 1-(2-AMINOETHYL)-3-(2,6-DICHLOROPHENYL)THIOUREA IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and specific normal-phase (adsorption) high-performance liquid chromatographic (HPLC) assay was developed for the determination of 1-(2-aminoethyl)-3-(2,6-dichlorophenyl)thiourea [I] in plasma and urine. The assay involves the extraction of the compound into methylene chloride from plasma or urine buffered to pH 10, and the HPLC analysis of the residue dissolved in methylene chloride—methanol—heptane (85:10:5). A 10-μm silica gel column was used with methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1) as the eluting solvent. The effluent was monitored at 254 nm and quantitation was based on the peak height vs. concentration technique. The assay has a recovery of 64.5 ± 4.5% (S.D.) from plasma and 96.0 ± 6.3% (S.D.) from urine in the concentration range of 0.1—2 μg per ml and 2—40 μg per 0.1 ml of plasma and urine, respectively, with a limit of detection of 0.05—0.1 μg [I] per ml of plasma using a 1-ml specimen and 0.1 μg per ml urine using a 0.1-ml specimen, respectively. The assay was applied to the determination of plasma levels and urinary excretion of the compound [I] in dog following the oral administration of 28.8 mg of [I] · maleate per kg body weight.

The HPLC assay was also used to determine the stability of [I] and for the measurement of a potential degradation product, clonidine [II] [2-(2,6-dichlorophenylamino)-2-imidazoline] in pooled human plasma stored at —17°C, and pooled human urine stored at —17°C and —90°C, respectively.

INTRODUCTION

The compound 1-(2-aminoethyl)-3-(2,6-dichlorophenyl)thiourea ([I], Fig. 1), is one of a series of compounds, structurally related to clonidine [2-(2,6-dichlorophenylamino)-2-imidazoline] ([II], Fig. 1), which is presently under investigation as an antihypertensive agent [1]. Gas—liquid chromatographic analysis of [I] using either electron-capture or mass spectrometric detec-

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STRUCTURES	DISSOCIATION CONSTANTS
[I]	$PKa_1 = 9.17$ $PKa_2 = 10.35$
[II] CLONIDINE	$PKa = 8.11$

Fig. 1. Chemical structures and dissociation constants of [I] and [II].

tion is not feasible due to thermal rearrangement of [I] in the injection port resulting in the formation of clonidine [II] as a major degradation product [2], necessitating the use of high-performance liquid chromatography (HPLC) analysis, the utility of which was recently described for the determination of [I] in a dietary admix using reversed-phase HPLC [3].

This paper describes a rapid, sensitive, and specific HPLC assay for the determination of [I] in plasma, blood, and urine without chemical derivatization. The assay utilizes normal-phase (adsorption) HPLC with isocratic elution at ambient temperature, monitoring the absorbance of the eluent at 254 nm. The assay was used for preclinical bioavailability studies and for the evaluation of the stability of [I] and its conversion to [II] in pooled human plasma stored at —17°C and urine stored at —17°C and —90°C.

EXPERIMENTAL

Column

The column used was a 30 cm × 4 mm I.D. stainless-steel column containing 10 μm μPorasil silica gel (Waters Assoc., Milford, MA, U.S.A.).

Instumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector, and a Model 440 UV detector with a 254-nm wavelength kit (Waters Assoc.). The mobile phase used for isocratic normal-phase chromatography was a mixture of methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1). The chromatographic system was operated at ambient temperature, with a flow-rate of 2.2 ml/min, and at pressure of 800 p.s.i. Under the above conditions, the capacity factors k' of compounds [I] and [II] were 4.44 and 1.89, respectively. Concentrations of 40 ng of compound [I] and 15 ng of [II] injected yielded peaks of approximately 50%

of full scale at a detector sensitivity of 5×10^{-3} a.u.f.s. The chart speed on a 10-mV recorder was 30 in./h (0.5 in./min) (Speedomax XL, 625 series, Leeds and Northrup, North Wales, PA, U.S.A.).

Upon completion of a day's analysis, the ammonium hydroxide and any accumulated endogenous material are flushed from the column with methanol, which is then equilibrated in methylene chloride. Although it is reported that high alkalinity reduces the useable life span of silica columns, this one has been in use for ten months without any noticeable deterioration.

Standard solution

Weigh out 10.0 mg of the compound [I] ($C_9H_{11}Cl_2N_3S$, mol. wt. = 264.17, m.p. = 135—137°C) into a 10-ml volumetric flask and dissolve in 10 ml methanol. This stock solution [A] contains 1.0 mg [I] per ml and is used to prepare an intermediate solution in 10 ml containing 100 μg [I] per ml in methanol (solution [B]). In addition, stock solution [A] is used to prepare two working solutions [C] and [D] in 10 ml of 1 *M* phosphate buffer (pH 10.0) containing 100 μg and 10 μg [I] per ml, respectively. Solutions [C] and [D] are used to prepare the internal standards for the quantitation of the unknowns and must be prepared fresh, prior to each day's analysis.

A series of working external standards for the plasma assay is prepared by transferring aliquots of 0.050, 0.125, 0.250, 0.375, and 0.500 ml of solution [B] into 10-ml volumetric flasks and diluting to volume with methylene chloride—methanol—heptane (85:10:5) to yield solutions containing 0.5, 1.25, 2.5, 3.75, and 5 μg/ml. Aliquots (20 μl) of these solutions (equivalent to 0.01, 0.025, 0.050, 0.075, and 0.1 μg) are injected to establish an external standard curve for the calculation of percent recovery from plasma.

A series of working external standards for the urine assay is prepared by transferring aliquots of 0.020 ml from solution [B] and 0.02, 0.10, 0.20, and 0.40 ml of solution [A] into separate 10-ml volumetric flasks and diluting to volume with methylene chloride—methanol—heptane (85:10:5) to yield solutions containing 0.20, 2.0, 10.0, 20.0, and 40.0 μg/ml. Aliquots (10 μl) of these solutions (equivalent to 0.002, 0.020, 0.1, 0.2, and 0.4 μg) are injected to establish an external standard curve for calculation of percent recovery from urine.

Reagents

All reagents are of analytical grade purity and are prepared in deionized, distilled water. Phosphate buffer (1.0 *M*, pH 10) is prepared by mixing 847 ml 1 *M* $K_2HPO_4 \cdot 3H_2O$ (228.23 g/l) and 153 ml saturated solution Na_3PO_4. Mix well and adjust to pH 10.0 with $KH_2PO_4 \cdot 3H_2O$ (1 *M*) or saturated solution Na_3PO_4 as needed. Other reagents include ammonium hydroxide, ACS grade (J.T. Baker, Phillipsburg, NJ, U.S.A.), methylene chloride, methanol, and heptane (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). The mobile phase is deaerated in an ultrasonic bath prior to use.

Assay in plasma or whole blood

Into a 15-ml stoppered centrifuge tube (PTFE No. 13 stopper) add 1.0 ml unknown plasma (or 0.5 ml oxalated whole blood) and 1 *M* phosphate buf-

fer (pH 10) to make up to a volume of 2.5 ml. Mix well and extract the sample with 6 ml of methylene chloride by slowly shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.). Centrifuge the sample in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC Corporation, Needham, MA, U.S.A.) at 0—5°C for 10 min at approximately 1200 *g*. Immediately aspirate off as much of the aqueous phase as possible and transfer a 5-ml aliquot of the methylene chloride extract into a tapered 15-ml stoppered centrifuge tube (PTFE No. 13 stopper). Evaporate the methylene chloride extract carefully under a stream of clean, dry nitrogen to dryness at 35—40°C over steam vapor using a N-EVAP evaporator (Organomation, Worcester, MA, U.S.A.). Dissolve the residue in 200 µl of methylene chloride—methanol—heptane (85:10:5) and keep the tube in an ice bath. Inject a 20-µl aliquot for HPLC analysis, Fig. 2A.

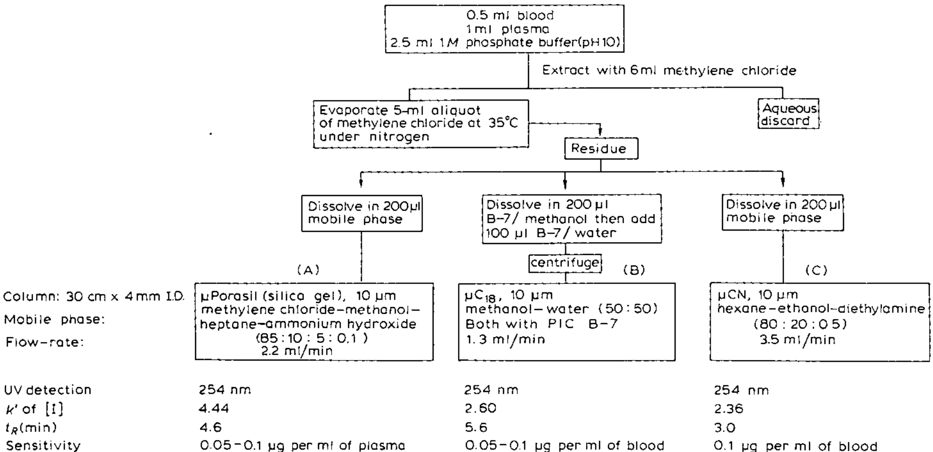

Fig. 2. Flow diagram of HPLC assays for compound [I] in biological fluids. PIC B-7 = 1-heptanesulfonic acid, 0.005 *M*, pH 3.5.

A specimen of 1.0 ml of control plasma and five 1.0-ml aliquots of control plasma containing 0.010, 0.025, 0.050, 0.075, and 0.100 ml of solution [D] (equivalent to 0.1, 0.25, 0.5, 0.75, and 1.0 µg of the compound [I] per ml plasma), respectively, are processed with the samples as recovered standards (Fig. 3). These standards are used to establish a calibration curve for the direct quantitation of the unknowns.

Assay in urine

Into a 15-ml stoppered centrifuge tube, add 0.1 ml urine (0.1 ml of 1:10 dilution of 0—24 h specimen) and sufficient of 1 *M* phosphate buffer (pH 10) to bring the buffer to a volume of 0.5 ml, mix well, and extract the sample with 1 ml of methylene chloride—methanol—heptane (85:10:5) by mixing for 1 min on a Vortex-Genie (Ace Scientific Supply Co., Linden, NJ, U.S.A.). Centrifuge the samples immediately in a refrigerated centrifuge at 0—5°C for 10 min at approximately 1200 *g*. Aspirate off all the aqueous phase, transfer 0.5—0.6 ml of the organic phase in a clean tube and place the tube in an ice bath. Inject a 10-µl aliquot for HPLC analysis.

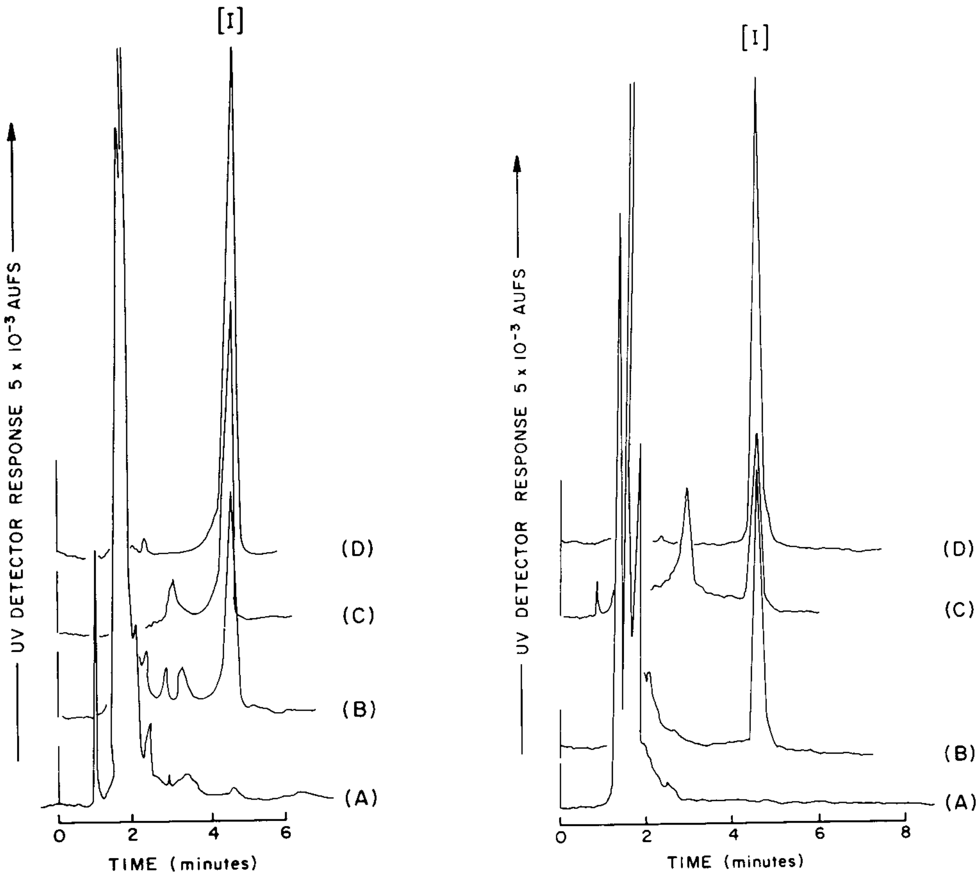

Fig. 3. Chromatograms of HPLC analysis of plasma extracts on a μPorasil silica gel column of (A) control plasma; (B) 6 h dog plasma post dose; (C) control plasma containing added [I] authentic standard, and (D) authentic standard [I].

Fig. 4. Chromatograms of HPLC analysis of urine extracts on a μPorasil silica gel column of (A) control urine; (B) control urine containing added authentic standard [I]; (C) 24—48-h dog urine post dose; and (D) authentic standard [I].

A specimen of 0.1 ml of control urine and five 0.1-ml aliquots of control urine containing 0.200 ml of solution [D] and 0.020, 0.100, 0.200, and 0.400 ml of solution [C] (equivalent to 0.2, 2, 10, 20, and 40 μg of the compound [I] per 0.1 ml urine), respectively, are processed along with the samples as recovered standards (Fig. 4). These standards are used to establish a calibration curve for the direct quantitation of unknowns.

Calculations

The concentration of [I] in μg per ml of plasma or μg per 0.1 ml of urine are determined from their respective standard curves using the peak height vs. concentration technique. For the urine assay, the results are corrected for the appropriate dilutions and calculated as [I] μg per ml of urine.

RESULTS

Statistical validation of the method in plasma and urine

Plasma. The intra-assay linearity and precision of the method was evaluated in plasma over a concentration range of 0.1—2 μg [I] per ml (Table I). Duplicate samples at each concentration of compound [I] were added to 1 ml of plasma, and taken through the analytical procedure. The data shown in Table I are best described by a linear function equation of the form: $y = Bx + A$ ($y = 16.134x + 0.02$) with a correlation coefficient (r) of 0.997, indicating the high degree of linearity of the method. The method showed good precision over the concentration range investigated with an average coefficient of variation of 4.1%. The recovery of compound [I] from plasma was 64.5 ± 4.5% (S.D.) and the sensitivity limit was 0.05—0.1 μg of [I] per ml.

Urine. The intra-assay linearity and precision of the method was evaluated in urine over a concentration range of 2—40 μg per 0.1 ml. Duplicate samples at each of the above concentrations of compound [I] were added to 0.1 ml urine, and then taken through the analytical procedure. The recovery data, shown in Table I, were described by a linear equation at the form: $y = Bx + A$ ($y = 0.443x—0.45$) with a correlation coefficient (r) of 0.998 and with an average coefficient of variation of 3.7%. The recovery of compound [I] from the urine samples was 96.0 ± 6.3% (S.D.) and the sensitivity limit was 1 μg/ml.

Inter-assay linearity and precision data for the method were generated during the stability studies with [I] (Table II). The data showed correlation coefficients (r) of 0.992 and 0.990 with average coefficients of variation

TABLE I

LINEARITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [I] IN PLASMA AND URINE

N = 2 in all cases.

Concentration range	Concn. added	Mean concn. found ± S.D.	Coefficient of variation (%)	Mean recovery (%)
Plasma* 0.1—2 μg/ml	0.10	0.107±0.003	2.8	58.4
	0.20	0.237±0.003	1.3	71.3
	0.50	0.473±0.021	4.4	67.5
	1.00	0.960±0.074	7.7	62.5
	2.00	2.015±0.082	4.1	63.1
Mean ± S.D.			4.1	64.5±4.5
Urine** 2—40 μg/0.1 ml	2.00	2.36±0.15	6.5	83.3
	4.00	4.07±0.35	8.7	100.0
	10.00	10.3 ±0.35	3.5	100.0
	20.00	19.1 ±0.14	0.7	100.0
	30.00	29.4 ±0.52	1.8	92.9
	40.00	40.8 ±0.50	1.2	100.0
Mean ± S.D.			3.7	96.3±6.3

*$y = 16.13x + 0.02$ ($r = 0.997$).

**$y = 0.443x - 0.45$ ($r = 0.998$).

TABLE II

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [I] IN PLASMA AND URINE

Concentration range	N	Concn. added	Mean concn. found ± S.D.	Coefficient of variation (%)	Mean recovery (%)
Plasma* 2—6 μg/ml	15	2.00	1.98±0.09	4.4	60.3±7.2
	3	3.00	3.03±0.07	2.4	58.6±4.2
	17	4.00	4.02±0.21	5.2	70.1±6.8
	15	5.00	5.01±0.15	3.0	74.6±12.5
	9	6.00	5.96±0.32	5.4	69.3±7.5
Mean ± S.D.				4.1	66.6±7.6
Urine** 0.1—0.5 μg/0.1 ml	20	0.100	0.105±0.015	14.2	59.5±14.1
	20	0.200	0.192±0.017	8.6	60.5±10.4
	19	0.300	0.299±0.020	6.6	61.5±9.7
	21	0.400	0.405±0.027	6.6	60.3±18.3
	19	0.500	0.498±0.018	3.6	60.0±8.8
Mean ± S.D.				7.9	60.4±12.3

*$y = 4.91x - 2.75$ ($r = 0.992$). Each determination was combined from eight different dates of assay.
**$y = 24.74x - 0.07$ ($r = 0.990$). Each determination was combined from ten different dates of assay.

of 4.1 and 7.9% for plasma and urine, respectively. The overall recoveries were 66.6 ± 7.6% and 60.4 ± 12.3% in the plasma and urine, respectively. Lower recoveries of [I] were measured in the urine in the inter-assay precision study at concentrations of 0.1—0.5 μg per 0.1 ml as compared to the intra-assay precision study at concentrations of 2—40 μg per 0.1 ml. This lower range of concentrations, near the sensitivity limit of the assay, was selected to measure [I] in the urine at the concentrations anticipated in man.

Stability evaluation of [I] in biological samples

The ionization constants of [I] and [II] (pK_a values, see Fig. 1) are approximately equal which precludes differential solvent extraction of the compounds from each other. The determination of the stability of [I] in biological samples as indicated by its recoverability by HPLC analysis and the rate of formation of [II] measured by radioimmunoassay (RIA) [4] is described below:

Preparation of pooled biological samples. Four 50-μl aliquots of stock solution [A] were added to four separate 10-ml aliquots of control human plasma and combined (total 40 ml) to yield a pooled plasma sample containing 5 μg [I] per ml. The pooled plasma sample was then subdivided into 1-ml aliquots in 15-ml glass-stoppered tubes and stored frozen at −17°C.

Aliquots of 350 μl and 300 μl of standard solution [B] were added to 10 ml of control human urine to yield two pools containing 0.35 μg [I] per 0.1 ml and 0.30 μg [I] per 0.1 ml for stability studies at −17°C and −90°C, respectively. The pooled urine sample was subdivided into 0.1-ml aliquots

in 15-ml polypropylene centrifuge tubes and stored at —17°C and —90°C (Revco Ultra-low temperature freezer, Rheem Refrigeration Products Division, Columbia, SC, U.S.A.).

Assay procedure. Normal-phase HPLC as described in the experimental section was used for analysis which was carried at fixed time interval using five individual plasma or urine test samples which had been stored at —17°C or —90°C.

A stock solution containing 100 μg of the free base of [II] per ml in methanol was prepared by weighing 11.588 mg of [II]·HCl, ($C_9H_9Cl_2N_3 \cdot HCl$, mol. wt. = 266.54) into a 100-ml volumetric flask and dissolving with methanol. Four individual series of freshly spiked plasma and urine standards prepared from solutions of [I] and [II] individually in 1 *M* phosphate buffer (pH 10.0) containing 2—6 μg [I] and 0.2—2 μg [II] per ml of plasma, respectively, and 0.1—0.5 μg [I] and 0.05—0.2 μg [II] per 0.1 ml of urine, respectively, were processed with each set of pooled samples for measurement of [I] and to determine the rate of formation of [II].

Inter-assay linearity and precision data for [II] using the assay yielded correlation coefficients (*r*) of 0.998 and 0.998, and coefficients of variation of 4.2% and 2.5%, respectively, for plasma and urine. The recovery of [II] from the plasma and urine was 86.1 ± 9.9% (S.D.) and 76.1 ± 0.8% (S.D.), respectively (Table III). Typical chromatograms of [II] from the stability evaluation in plasma and urine are shown in Figs. 5 and 6, respectively.

TABLE III

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR COMPOUND [II] IN PLASMA AND URINE

Concentration range	*N*	Concn. added	Mean concn. found ± S.D.	Coefficient of variation (%)	Mean recovery (%)
Plasma* 0.2—2 μg/ml	2	0.200	0.193±0.004	2.1	73.5
	2	0.500	0.504±0.043	8.5	74.5
	2	1.00	1.00 ±0.039	3.9	94.4
	2	1.50	1.50 ±0.039	2.6	94.4
	2	2.00	1.99 ±0.074	3.7	93.8
Mean ± S.D.				4.2	86.1±9.9
Urine** 0.05—0.2 μg/0.1 ml	4	0.050	0.050±0.002	4.0	75.8
	4	0.100	0.100±0.002	2.0	75.4
	4	0.150	0.148±0.003	2.0	75.8
	4	0.200	0.201±0.004	2.0	77.5
Mean ± S.D.				2.5	76.1±0.8

*$y = 12.9x - 0.038$ ($r = 0.998$).
**$y = 90.5x - 0.24$ ($r = 0.998$). Each determination was combined from two different dates of assays.

Stability results

The plasma samples stored at —17°C showed no significant degradation for seven days (Table IV). Decomposition of 4.4—19.2%, measured as loss

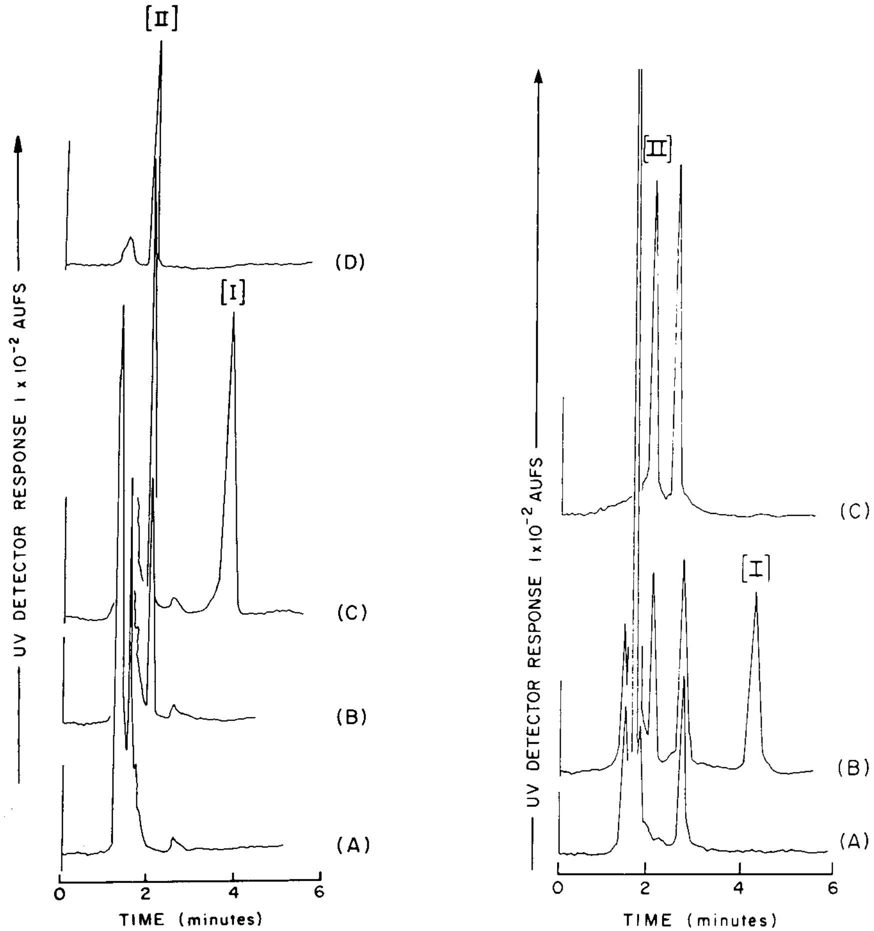

Fig. 5. Chromatograms of HPLC assay of plasma containing added [I], stored at −17°C for stability evaluation: (A) control plasma, (B) control plasma containing added [II] authentic standard, (C) plasma stability sample on day 49, and (D) authentic standard [II].

Fig. 6. Chromatograms of HPLC assay of urine containing added [I], stored at −17°C for stability evaluation: (A) control urine, (B) urine stability sample on day 43, and (C) control urine containing added [II] authentic standard.

of [I], was found from the 24th to 70th day. The degradation product [II] had the same retention volume as authentic clonidine measured by HPLC. Aliquots of this plasma pool assayed by RIA showed an increase in "clonidine like" material of 9 to 16% on the 35th to 45th day [4]. The good agreement between the non-specific RIA and the specific HPLC assay indicated that the product measured by RIA is predominantly clonidine [II].

The urine samples stored at −17°C showed as much as 52% breakdown over a 43-day period (Table V). Only a small portion (11–17%) could be accounted for as [II] by HPLC which suggests other routes of degradation. The urine samples stored at −90°C showed complete stability over a 68-day period (Table V). A third study demonstrated complete stability of [I] in

TABLE IV

STABILITY OF [I] IN PLASMA, STORED AT −17°C DETERMINED BY HPLC ANALYSIS
The pooled plasma concentration was 5.00 μg/ml, for each determination.

Day of study	*N*	Amount [I] found ± S.D.	[I] lost (%)
0	5	4.90±0.19	n.m.*
1	4	5.00±0.12	n.m.
7	5	5.10±0.14	n.m.
24	5	4.78±0.09	4.4
49	5	4.07±0.13	18.6
70	5	4.04±0.06	19.2

*Non-measurable.

TABLE V

STABILITY OF [I] AND ESTIMATION OF [II] IN URINE STORED AT −17°C AND −90°C

Storage temperature	Duration of storage (days)	Pooled urine conc. (μg/0.1 ml)	*N*	Amount [I] found ± S.D.	[I] lost (%)	[II] found (%)
−17°C	0	0.35	5	0.35±0.016	—	—
	1	0.35	4	0.33±0.007	(5.7)	n.m.*
	7	0.35	5	0.25±0.012	28.6	n.m.
	14	0.35	5	0.21±0.030	40.0	10.9
	35	0.35	5	0.17±0.020	51.4	15.1
	43	0.35	5	0.17±0.017	52.0	16.9
−90°C	0	0.30	5	0.30±0.021	—	—
	1	0.30	5	0.32±0.014	—	—
	13	0.30	5	0.30±0.013	—	—
	27	0.30	5	0.34±0.032	—	—
	48	0.30	4	0.32±0.030	—	—
	68	0.30	5	0.29±0.020	—	—

*Non-measurable.

urine for a period of 2 h at 37°C and approximately 20% degradation during the third hour. This would indicate that urine specimens should be frozen immediately on collection.

Application of the method to biological specimens

The assay was applied to the quantitation of [I] in plasma and urine samples (Figs. 3 and 4) in a dog following the oral administration of 28.8 mg of [I] · maleate ($C_9H_{11}Cl_2N_3S \cdot C_4H_6O_4$, mol. wt. = 382.17) per kg of body weight. The samples were frozen (−17°C) upon collection and assayed within one week. Following this dose, a peak plasma concentration of 1.9 μg [I] per ml was measured at 1 h and declined to nonmeasurable amounts

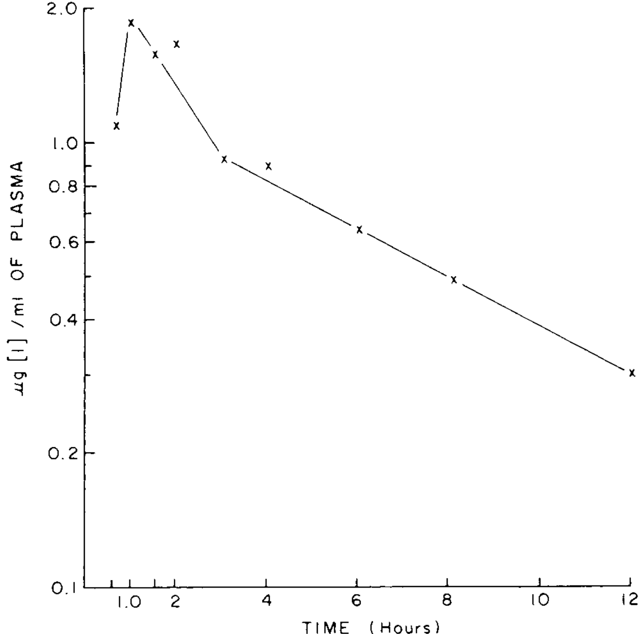

Fig. 7. Plasma level fall-off curve in a dog following oral administration of 28.8 mg [I]· maleate per kg dose, equivalent to 20 mg of free base per kg.

(⩽0.05 µg/ml) after 12 h (Fig. 7). Concentrations of [II] were non-measurable (⩽0.2 µg/ml) in the plasma throughout the experimental period.

The urinary excretion of [I] following this dose was 52.3 and 0.4% of the dose recovered as [I] in the 0—24 and 24—48 h periods, respectively. Non-measurable amounts (⩽2 µg/ml) were observed in the 48—72 h excretion period.

DISCUSSION

Thermal rearrangement of [I] to clonidine [II] in the injection port precludes GLC analysis of the parent drug since it is also metabolized to [II] in vivo, and therefore would result in a non specific assay. Clonidine [II] per se can, however, be analyzed by electron-capture GLC utilizing the pentafluorobenzyl [5] and diheptafluorobutyryl [6] derivatives of the compound. Gas chromatographic—mass spectrometric assays based on selective ion monitoring of clonidine [II] and its deuterated internal standard by electron-impact ionization as either the intact compound [7] or as the dimethyl derivative produced by on-column methylation with trimethylanilinium hydroxide [8] have also been used to determine [II] in plasma. These assays [5, 8] are capable of measuring as little as 25 pg/ml using a 4-ml plasma sample. Plasma concentrations of [II] have been measured in man following oral doses of 50—300 µg of [II] · hydrochloride [6, 9—11]. Due to the aforementioned problems, compound [I] had to be determined by a specific HPLC

assay capable of resolving [I] from [II] and other potential metabolites and/ or breakdown products.

Development of normal-phase HPLC analysis

The choice of 254 nm for measurement of [I] and [II] is based on their UV spectra in methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1) which showed maxima at 247 and 250 nm, respectively. The strongly basic nature of [I] and [II] required extraction at alkaline pH and the use of either a basic mobile phase for normal-phase (adsorption) chromatography or "paired ion" chromatography for reversed-phase HPLC analysis. Both techniques were investigated and normal-phase HPLC was selected eventually as the method of choice since it yielded the best resolution and chromatographic performance in biological fluids.

Studies were initially conducted on the use of normal-phase (adsorption) chromatography utilizing a 30 cm × 4 mm I.D. stainless-steel column containing 10 μm μBondapak CN (chemically bonding a cyano group to μPorasil, 9% by weight, Waters Assoc.) and a mobile phase of hexane—ethanol—diethylamine (80:20:0.5) (Fig. 2C). This procedure provided a solvent system for the assay of [I] in blood, in which the lipid materials in the sample residue are totally soluble in the mobile phase system. The assay is straightforward and obviates the need for clarification of the reconstituted sample extract usually required for reversed-phase HPLC. Normal-phase HPLC analysis at a flow-rate of 3.5 ml/min with the above mobile phase and a head pressure of 950 p.s.i., resulted in a capacity factor (k') for compound [I] of 2.36. The overall recovery of [I] is 77.6 ± 7.4% (S.D.) in the range of 0.05—5 μg per 0.5 ml blood, with a limit of detection of 0.1 μg/ml of blood using 0.5 ml per assay.

Unfortunately, the chromatographic response became nonreproducible following continuous use with this mobile phase, possibly due to the hydrolysis of the cyano groups at the high alkalinity of 0.5% diethylamine in the solvent system and further work using the μBondapak CN column was discontinued.

The successful use of small amounts of concentrated ammonium hydroxide (< 0.5%) as a modifier in the mobile phase for use with silica columns in normal-phase HPLC analysis is well-documented [12—15]. The substitution of concentrated ammonium hydroxide for diethylamine in the mobile phase led to the successful use of methylene chloride—methanol—heptane—ammonium hydroxide (85:10:5:0.1) as the mobile phase for HPLC and resulted in the assay previously described for the preclinical evaluation of [I] in plasma and urine in dog specimens.

Preliminary investigation of reversed-phase HPLC analysis

This mode of analysis is generally used for the determination of polar, ionizable (cationic, anionic or zwitterionic) compounds, which usually chromatograph poorly (as broad tailing peaks) by normal-phase chromatography.

The use of an appropriately buffered mobile phase renders the molecule in a non-ionized "lipophilic" state enabling retention on chemically bonded octadecylsilane (ODS-C_{18}) columns.

Preliminary investigations for the analysis of [I] in body fluids involved reversed-phase chromatography, with water—methanol—acetic acid (80:20:0.5) as the mobile phase, and a 30 cm × 4 mm I.D. stainless-steel column containing 10 μm μBondapak C_{18} (Waters Assoc.). The parent drug [I] was extracted into methylene chloride from blood or plasma buffered to pH 10.0 with 1 *M* phosphate buffer, the residue of which was reconstituted in water—methanol of varying composition. It was noted that coextracted lipids were insoluble in the water—methanol mixtures, resulting in a colloidal suspension which interfered with HPLC analysis.

The reversed-phase chromatographic system was then modified in an attempt to increase the solubility of the residue by increasing the methanol concentration and by the introduction of an ion-pair reagent as the counter-ion. A mobile phase of water—methanol (50:50) containing heptanesulfonic acid (PIC Reagent B-7, Waters Assoc.) with an ionic strength of 0.005 *M* (pH 3.5) was found to be suitable. The assay required reconstitution of the methylene chloride extract in 200 μl of 0.005 *M* heptanesulfonic acid in methanol by vortex mixing, followed by the addition of 100 μl of 0.005 *M* heptanesulfonic acid in water. The lipids which were coextracted from the sample still precipitated out upon addition of the aqueous solution. The sample was clarified by centrifuging at approximately 1200 *g* to obtain a clear supernatant, 30 μl of which were assayed by HPLC (Fig. 2B). The mobile phase and μBondapak C_{18} column previously described were used at a flow-rate of 1.3 ml/min, and a head pressure of 2.1×10^3 p.s.i., and gave a capacity factor (k') for compound [I] of 2.60. The overall recovery of [I] was 83.0 ± 5.7% (S.D.) with a limit of detection of 0.05 μg [I] per ml of plasma or 0.1 μg [I] per ml of blood. However, this procedure was very time-consuming since the mobile phase required filtration through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to use and the reconstituted sample extracts required clarification by centrifuging prior to analysis.

Attempts to increase the sensitivity of measurement by post-column derivatization of [I] with fluorescamine (Fluram, Hoffmann-La Roche, Nutley, NJ, U.S.A.) following the reversed-phase HPLC using mixtures of water, methanol, and acetic acid were unsuccessful. Losses in sensitivity were observed due to the need to dilute the acidic HPLC effluent with an alkaline reagent prior to derivatization. Compound [II] does not react with fluorescamine.

CONCLUSIONS

A normal-phase HPLC assay is described for the measurement of [I] in plasma and urine with sufficient sensitivity and specificity to measure the drug in preclinical bioavailability and toxicology studies. Based on the stability of the drug in biological fluids, it is recommended that the samples be analyzed within one week if stored at −17°C and that storage for extended periods of time should be at −90°C. All samples should be analyzed expeditiously after thawing.

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PERFORMANCE EVALUATION OF A REVERSED-PHASE, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF VALPROIC ACID INVOLVING A "SOLVENT DEMIXING" EXTRACTION PROCEDURE AND PRECOLUMN DERIVATISATION

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SUMMARY

As previously shown by others, the antiepileptic drug valproic acid can be assayed in biological fluids by reversed-phase, high-performance liquid chromatography after derivatisation with a bromomethyl aryl ketone through crown-ether catalysis. It is possible to extract the drug directly into acetonitrile, the solvent used for its derivatisation: when an excess of some salt such as NaCl is added to a mixture of plasma and acetonitrile, the organic solvent separates and valproic acid is extracted into it with a high recovery yield. This "solvent demixing" extraction method has shown excellent reproducibility, as well as promising versatility. Derivatisation, still in acetonitrile, using bromomethyl naphthyl ketone and 15-crown-5 allowed us to get rid of the current heating step without markedly increasing the delay of reaction. Chromatography was performed on a C-18 bonded stationary phase with acetonitrile—water as mobile phase, cyclohexanecarboxylic acid as internal standard and ultraviolet spectrophotometric detection. Statistical analysis of results shows 80% recovery of extraction, good linearity and an inter-extract variation coefficient of 4%, the last mainly ascribable to chromatographic measurements. Recovery is readily improved by increasing the amount of acetonitrile, which was equal to that of plasma in our experiments, since the high sensitivity of detection can tolerate the resulting decrease of valproic acid concentration in the extract.

INTRODUCTION

Valproic acid is a chemically simple drug (2-propyl-pentanoic acid, mol. wt. 144.2) used in the treatment of some types of epilepsy. Therapeutic levels in plasma or serum range from 260 to 840 µmol/l [1, 2] with a suggested threshold of efficiency at 350 µmol/l [3], of which 80—90% is protein-bound and

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which corresponds to daily dosages ranging from 15 to 30 mg/kg. Elimination half-life is estimated at between 13 and 21 h [2].

Opinions differ on the usefulness of blood assays intended for the therapeutic monitoring of this drug. Schobben et al. [1] doubt it, but a very well-planned study of Gram et al. [4] has recently shown that there is a good level—efficiency relationship between 110 and 350 μmol/l and that dosages required for obtaining a given level vary by more than double between individuals. These results, together with the observation of frequent subclinical toxicity to the liver which improves when doses are lowered [5], advocate the usefulness of assay.

Plasma levels of valproic acid are currently measured with gas chromatography [6—12]. Liquid chromatography is difficult owing to the low ultraviolet absorbance of this compound. Colorimetric detection using a pH indicator in the mobile phase has been proposed [13]. We turned to a general method of derivatisation of carboxylic acids by an aryl bromomethyl ketone with crown-ether catalysis [14]. This method using commercial reagents has already been proposed for the liquid chromatography of valproic acid [15] but extraction of the drug from plasma was not dealt with. A closely related method using amine instead of crown-ether catalysis has been recently published [16] which is said to perform as well as the gas chromatographic method [12] from which it stemmed.

We present a quantitative evaluation of a whole procedure applied to biological samples. Some original features are a rapid and reliable extraction method involving salting out of a water-miscible solvent (acetonitrile) and the suppression of heating the derivatisation mixture. We tried a smaller crown ether (15-crown-5) instead of 18-crown-6 currently used [14, 15, 17] merely because it was easier to obtain in this country.

MATERIALS AND METHODS

Reagents and solvents

Potassium chloride and carbonate Normapur, analytical grade, were from Rhone-Poulenc, (Paris, France). Acetonitrile for far UV was from Fisons (Loughborough, Great Britain) through Touzart et Matignon (Paris, France). Cyclohexanecarboxylic acid for synthesis was from Merck-Schuchardt (Darmstadt, G.F.R.). Valproic acid was kindly given by Labaz. Bromomethyl naphthyl ketone (α-bromo-2′-acetonaphthone, 99%) was from Aldrich (Milwaukee, WI, U.S.A.). 15-Crown-5 purum (1,4,7,10,13-pentaoxacyclopentadecane) was from Fluka (Buchs, Switzerland).

Working solutions

All solutions were made up in acetonitrile.

A solution of bromomethyl naphthyl ketone and a solution of 15-crown-5 both at twice the desired concentration were mixed in equal parts to make the so-called ketone—crown solution. In the same way a solution of valproic acid (VA) and a solution of cyclohexanecarboxylic acid (internal standard, IS), both at twice the desired concentration, were mixed to make the so-called VA—IS solution.

Chromatographic apparatus

An SP 8000 chromatograph from Spectra-Physics was used, equipped with a Valco loop injector (10 μl volume). The column was a Hibar RT 250-4 pre-packed column from E. Merck (Darmstadt, G.F.R.) filled with LiChrosorb RP-18 bonded reversed phase, particle size 5 μm.

The detector was a Model 770 variable-wavelength, spectrophotometric detector from Schoeffel.

Solvent demixing extraction

In a 5-ml glass tube add successively 1 vol. (0.5 ml) of VA—IS solution (IS 0.40 mmol/l, VA either zero or at the indicated concentration), 0.1 vol. of 1 *N* HCl, and 1 vol. of water or plasma. Mix briefly by vortex (when plasma is used an homogeneous precipitate of proteins should be obtained). Add excess KCl (about 0.3 g) and mix vigorously (say ten brief vortex strokes at maximum speed) to saturate. Centrifuge (20°C, for at least 5 min at 1500 *g*); the extraction mixture separates into (from bottom upwards) excess KCl, a saturated aqueous layer, a compact disc of plasma proteins, and an acetonitrile layer of about two-thirds of the added volume.

Derivatisation

In a second tube add K_2CO_3 (about 20 mg) and 200 μl of acetonitrile supernatant. Mix by vortex to prepare potassium salts of the extracted acids (1 h). Add 200 μl of the ketone—crown solution, mix and leave to react at room temperature either for the time chosen (determination of the time course) or for 1 h to completion.

Chromatography

The reaction mixture was injected (10 μl) into an isocratic mobile phase of acetonitrile—water (83:17) at a flow-rate 1 ml/min at room temperature. Detection was made at 280 nm.

Experimental design and calculations

To determine the time course of derivatisation, salting out extractions from water were performed as described above with one VA—IS solution (VA 1.9 mmol/l, IS 0.40 mmol/l), derivatisation was carried out with three ketone—crown solutions at different concentrations indicated in the legend of Fig. 1, and the three derivatisation mixtures were chromatographed through time.

For the overall evaluation of the whole procedure, extractions were made both from water and from human serum with three VA—IS solutions (IS constant, VA as in Table I), each combination being replicated four times. Derivatisation was made with VA—IS solutions (subscript S), water extracts (subscript W) and plasma extracts (subscript P) and the $3 \times 3 \times 4 = 36$ samples were chromatographed following the order of numbers indicated in Table I to minimise any systematic factor of variation linked to time.

Peak heights (h) were measured on the chromatograms. The expected relationship

$$h = a\,C \qquad (1)$$

with concentrations C of VA may be written (decimal logarithms)

TABLE I

EXPERIMENTAL DESIGN FOR EVALUATION OF THE PROCEDURE

The 36 samples were chromatographed successively following the sequences given.

	IS; 0.40 mmol/l VA: 0.24 mmol/l	IS: 0.40 mmol/l VA: 0.48 mmol/l	IS: 0.40 mmol/l VA: 0.96 mmol/l
S*	1.18.19.36	2.17.20.35	3.16.21.34
W**	4.15.22.33	5.14.23.32	6.13.24.31
P***	7.12.25.30	8.11.26.29	9.10.27.28

*VA—IS solutions.
**Water extracts.
***Plasma extracts.

$$\log h = \log C + \log a \tag{2}$$

Thus the linear regression log h vs. log C was expected to be a line of slope 1 if eqn. 1 was valid.

Under the assumption of a constant relative indetermination of measurements (a constant coefficient of variation of h), the logarithmic transformation warrants homogeneity of variance (homoscedasticity) needed for the regression to be valid.

Calculations were made as follows:

(1) Homoscedasticity of the 9 sets of 4 replications was first tested through Bartlett's test [18]. If not significant at the probability level $P = 0.1$,

(2) Three separate regressions (log h_S, log h_W, log h_p vs. log C) were then performed and their linearity was tested through one-sided F tests. If not significant at $P = 0.1$, analysis of the common regression log h vs. log C was started. Then

(3) Parallelism of the three lines was tested through a one-sided F test. If not significant at $P = 0.1$,

(4) Departure from 1 of the common regression coefficient was then tested through two-tailed t-test. If not significant at $P = 0.1$, the validity of eqn. 1 was admitted.

Further calculations included:

(5) Comparison of elevations [19, 20] of the lines corresponding to water or plasma extracts and to solutions in order to estimate the recovery of the extraction method, and

(6) An estimation of the overall coefficient of variation (C.V.) from the common residual variance s_C^2 about regression lines. This was made through the approximations

$$\text{C.V.} \approx \frac{\mathrm{d}m}{m} = \mathrm{d}\log_e m = 2.3026\ \mathrm{d}\log_{10} m \approx 2.3026\ s_C$$

which assimilate ($\approx$) standard deviations with differentials and arithmetic with geometric means m, and which work well as long as C.V. is not too large, say lower than 10%.

RESULTS AND DISCUSSION

Time course of derivatisation

Fig. 1 shows the time course of derivatisation at room temperature. Of the three reagent concentrations tried, the highest one reached equilibrium after 1 h in a parallel manner for IS and VA. In fact, due to the parallelism of evolution, VA/IS peak ratios as used in an assay were stable after half an hour, which is not disproportionate to the 15-min reaction time proposed in the method of Durst et al. [14] and avoids heating at 90°C.

Values obtained with the two highest reagent concentrations did converge, not with the lowest one; this probably confirms that the reaction goes to completion if the bromoketone concentration is large enough [17]. Consequently, the proposed solvent demixing extraction, and particularly the probable hydration of the salted-out acetonitrile phase did not appear to affect the reaction seriously.

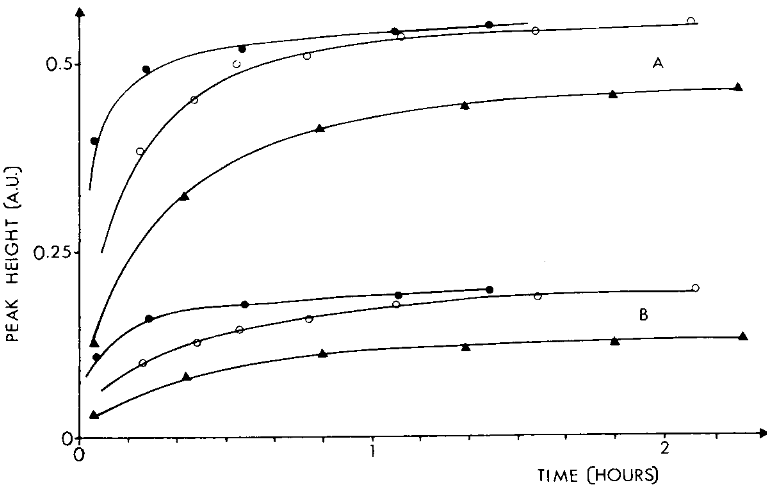

Fig. 1. Kinetics of derivatisation of valproic acid (VA) and of cyclohexanecarboxylic acid (internal standard, IS) with the following final concentrations of reagents in the reaction mixture: (A) valproic acid 1 mmol/l (corresponding to a plasma level of about 3 mmol/l or 300 mg/l); (B) internal standard 0.20 mmol/l; (▲) bromoketone 2.5 mmol/l, crown ether 0.188 mmol/l; (○) bromoketone 5 mmol/l, crown ether 0.375 mmol/l; (●) bromoketone 10 mmol/l, crown ether 0.75 mmol/l.

Evaluation of performance

Fig. 2 shows three typical chromatograms obtained with the VA—IS solution (a), a water extract (b) and a normal serum extract (c); no troublesome extraneous peak appears in the case of serum.

Fig. 3 illustrates logarithmic relationships obtained between VA concentrations and either peak heights h (A) or the ratio k of VA/IS peak heights (B), the variable used in assays with IS, which will be referred to as "peak ratio". Bartlett's test was significant in neither case ($0.10 < P < 0.25$ and $0.25 < P <$

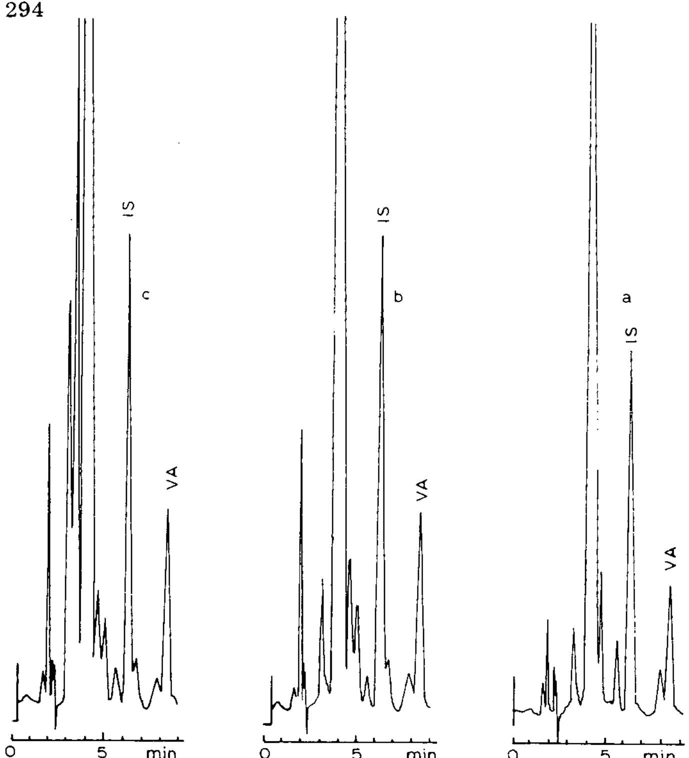

Fig. 2. Chromatograms of (from left to right): (c) an extract from normal serum; (b) an extract from water; (a) the VA—IS solution used for preceding extractions. Retention times of internal standard and valproic acid are 6.2 and 8.4 min, respectively.

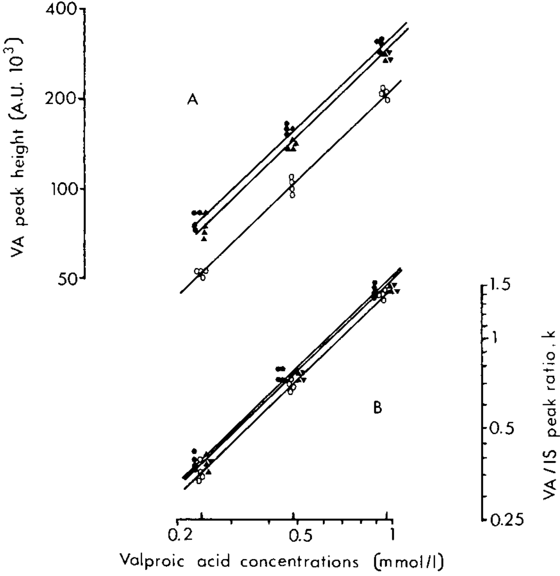

Fig. 3. Relationship between valproic acid concentrations (abscissa) and, in ordinate (both scales logarithmic): valproic acid peak height (A), and ratio of valproic acid over internal standard peak heights (B). (○), VA—IS solutions; (●), water extracts; (▲), plasma extracts. The sets of points corresponding to extracts have been slightly shifted from their true abscissa for the sake of clarity.

0.50 for h and k, respectively) which allows unweighted regressions to be performed.

Analysis of regressions log h vs. log C

Separate analysis of the three regressions (Fig. 3A) showed that none departed significantly from linearity ($F_{1/9} < 1$ in the three cases). Overall analysis showed that neither did the three lines depart significantly from parallelism ($F_{2/30} < 1$) nor did the common slope from one ($0.70 < P < 0.80$). The common residual variance was 0.00 04 98 12 (log unit)^2, corresponding to a 5.14% coefficient of variation, calculated as described in Methods.

Values obtained from water and plasma extracts were significantly ($P \ll 0.001$) higher than those from VA—IS solutions. This is due to the fact that the salted-out volume of acetonitrile was smaller than the added volume. The ratio of salted-out to added volume was separately measured on larger volumes and was found to be (mean ± standard deviation) $V_W/V_S = 0.613 \pm 0.02$ ($n = 9$), and $V_P/V_S = 0.593 \pm 0.012$ ($n = 9$). Comparison of elevations of the three parallel lines resulted in

$\log a_W - \log a_S = 0.1709$	$a_W/a_S = 1.482$ (1.414—1.550)*
$\log a_P - \log a_S = 0.1415$	$a_P/a_S = 1.385$ (1.322—1.448)*

*confidence interval 95%.

Thus the average yield of the described extraction method can be estimated to be: from water, $(a_W/a_S)(V_W/V_S) = 91\%$; and from plasma, $(a_P/a_S)(V_P/V_S) = 82\%$.

Analysis of regressions log k vs. log C

As above, for these regressions (Fig. 3B) statistical tests did not deny linearity ($F_{1/9} < 1$ in the three groups), parallelism ($F_{2/30} < 1$) and unit value of the common slope ($0.60 < P < 0.70$), so that the peak ratio k may be deemed proportional to VA concentrations in the range studied. The common residual variance about the lines was 0.00 02 82 94 (log unit)^2 corresponding to a 3.87% variation coefficient. Thus precision was not reduced, and was even better in fact, when using IS. This could mean that the elimination of variations of the injected volume (even with a loop injector) overcompensates for the double measurement of peak heights.

Unexpectedly, elevations were still significantly different ($P < 0.001$):

$\log a_W - \log a_S = 0.0376$	$a_W/a_S = 1.0905$ (1.050—1.132)*
$\log a_P - \log a_S = 0.0312$	$a_P/a_S = 1.0740$ (1.040—1.110)*

*confidence interval 95%.

This results shows that the extraction yield of IS was smaller than that of VA by nearly 10% and requires that the calibration of assays be made with standards extracted from a blank plasma.

Comments on the "solvent demixing" extraction procedure

The principle of this procedure was first applied by Bastos et al. [21] to the extraction of basic drugs from urine into ethanol through salting out with potassium carbonate, then extended to plasma extractions into isopropanol by Horning's team [22].

As already mentioned, variance was not significantly different among the nine sets of replications corresponding to solutions and to extracts. This means that the proposed extraction procedure did not impair precision significantly, i.e. that its reproducibility was good. This was to be expected, since the solvent is expelled from an homogeneous solvent—water mixture and the extraction yield is not exposed to such critical factors as duration of agitation, degree of dispersion, and interfacial "wetting", which can affect extractions into immiscible solvents. An incidentally useful consequence of initial homogeneity is that it does not matter that valproic acid be incorporated to the extraction solvent instead of the plasma; this feature greatly simplifies calibrations. The recovery was good for valproic acid. It has proved good too for other anionic anticonvulsants (phenobarbital, phenytoin, ethosuximide, carbamazepine) which we currently extract in the same way. A symmetrical basic extraction from plasma, using NaOH instead of HCl, was not so efficient for cationic drugs. It is probably a matter of adsorption on proteins, a difficulty currently met with with this kind of drug. Attempts to apply this method to other drugs and metabolites are in progress.

A further development of the "solvent demixing" principle is also under study: water demixing. The acetonitrile supernatant is transferred and a water-immiscible solvent, such as dichloromethane, is added to it in convenient amount (for example, 0.1 vol.). Then water, either acid or alkaline depending on the ionic species of the drug to be extracted, is added. Under mild motion, most of the acetonitrile dissolves in the water which results in a new two-phase system: water (and diluted acetonitrile) and dichloromethane. This method has shown promising efficiency and usefulness in preliminary experiments. Of course, solvent demixing extraction can be made more efficient if needed by increasing the volume of the water-miscible solvent, and more versatility by changing to other solvents.

Interferences

It is to be expected that all drugs with a carboxylic moiety are likely to react in the same way as valproic acid, and may interfere either with it or with the internal standard. Among the currently used antiepileptic drugs — ethosuximide, phenobarbital, phenytoin and carbamazepine — only phenobarbital appears to react with the derivatising reagent. Fig. 4 shows that the phenobarbital peak stands far enough apart from that of valproic acid not to impair measurement of the latter. This finding demonstrates, however, that reaction with a brominated ketone is not restricted to carboxylic acids.

Liquid vs. gas chromatography of valproic acid

We devised this method as an attempt at utmost technical uniformity in the routine determination of drug levels by reversed-phase liquid chromatography. However, gas chromatography (GC) still remains the current analytical stan-

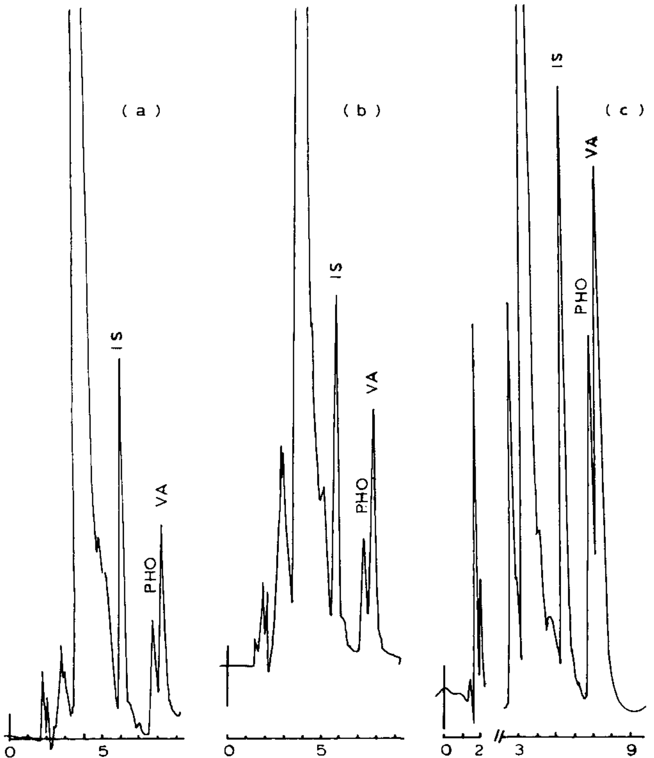

Fig. 4. Chromatograms of extracts from three plasma samples of patients receiving both valproic acid (VA) and phenobarbital (PHO). Levels were as follows (μmol/l), PHO being measured by another method: (a) VA = 330, PHO = 75; (b) VA = 460, PHO = 77.5; (c) VA = 590, PHO = 190.

dard. Gupta et al. [12] reviewed and solved the main difficulties met with by many authors in devising GC assays of valproic acid, the most troublesome being probably the instability of separation parameters due to the stationary phase being contaminated by extraneous organic solutes. This, together with the risk of evaporation losses of VA, led Gupta et al. to devise a method including both a purification step and derivatisation before concentrating the extract. The high absorbance of the aromatic derivative prompted them to apply their derivatisation procedure to liquid chromatography with "similar results" [16].

On technical grounds, the 1-h long sample preparation of Gupta et al. compares with ours, as does their 250-μl sample volume with our 500 μl. Perhaps our method is more liable to sample volume reduction than theirs, since we injected only 10 μl of our 400-μl derivatisation mixture, which allows us to use ten-times smaller volumes if necessary. Gupta et al. [12] did not state how much of their 50-μl final methanolic solution they injected. Finally, neither retention times nor peak shape showed any pejorative trend throughout about a thousand chromatograms. The column needs no washing after use, owing to the absence of any buffer in the mobile phase; nor does any clogging appear

from injecting acetonitrile extracts, owing to its high acetonitrile content. In fact, our columns usually die from channelization, which we are tempted to ascribe to mechanical strains exerted by our harshly regulated pumping device. As for the quality of routine determinations, accuracy can hardly be stated otherwise than through external quality control [23]. Our method shows an acceptable precision, though our 4% within-batch coefficient of variation is larger than the mere 1—2% of Gupta et al. [12], the latter estimation coming down, in fact, to the usual precision of the required initial double volumetric measurement of sample and internal standard solution.

CONCLUSION

Direct extraction into the solvent used for the derivatisation, suppression of heating and the possibility to perform calibrations with solutions made in the extracting solvent instead of using spiked plasmas, make the liquid chromatography of valproic acid quick and easy. Analysis of our results has shown that this simplification was not obtained at the expense of precision, and even that the proposed solvent demixing extraction combines simplicity and a high reproducibility. One point remains to be checked: how large is the variability of peak ratio k between different plasma samples? Owing to the incomplete (70—80%) extraction of valproic acid and of internal standard, it is not excluded, though improbable, that inter-plasma peak ratio variability may be larger than the mere intra-plasma component which was evaluated in this work. Should it so happen, extraction yield could be increased by increasing the amount of acetonitrile beyond the volume-for-volume ratio arbitrarily adopted here. This would dilute the extract and decrease the sensitivity, but the high ultraviolet absorbance of the naphthyl ketone allows an appreciable margin.

We are currently using this method for the therapeutic monitoring of valproic acid. Besides the usual reasons for such a monitoring, the suspicion that valproic acid could have a concentration-related metabolic toxicity seems to justify extensive studies.

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DETERMINATION OF 2,4-DIAMINO-5-BENZYLPYRIMIDINES IN COMBINATION WITH SULPHADIAZINE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatographic methods for the simultaneous analysis of tetroxoprim (TXP)/sulphadiazine (SDZ) and metioprim (MTP)/SDZ in serum and prostatic secretion (PS) are described. The detection limits in serum and PS were 50 ng TXP per ml and 100 ng SDZ per ml, and 40 ng MTP per ml and 100 ng SDZ per ml, respectively. The intra-assay coefficients of variation were in the range of 2.7—2.19%. Some preliminary data from a pharmacokinetic study in geriatric patients and a distribution study in dogs are presented. These methods enable the investigator to process a large number of TXP/SDZ and MTP/SDZ samples in one working day.

INTRODUCTION

As a consequence of co-trimoxazole (trimethoprim and sulphamethoxazole) favourable therapeutic properties in the selective inhibition of folic acid metabolism [1, 2], numerous diaminobenzyl analogues of trimethoprim were investigated for their antibacterial activity.

Tetroxoprim (TXP; 2,4-diamino-5-[3,5-dimethoxy-4-(2-methoxyethoxy)]-benzylpyrimidine), in combination with sulphadiazine [SDZ; 4-amino-N-(pyrimidyl)-benzenesulfonamide], is the first product of this research to reach the market [manufactured as Sterinor® (Heumann-Pharma) and Tibirox® (Roche)]. Metioprim (MTP; 2,4-diamino-5-[3,5-dimethoxy-4-(methylthio)]-benzylpyrimidine) is at present undergoing thorough clinical investigation. Methods currently available for determining TXP/SDZ concentrations use either microbiological assays [3] or high-performance liquid chromatography (HPLC) [4]. However, methods reported up to date, require extensive sample work-up. We are now in the position to present a new procedure involving only a simple serum-protein precipitation step, and yet allowing one to

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determine the respective benzylpyrimidine and SDZ simultaneously. In order to make distribution studies possible on TXP/SDZ and MTP/SDZ in experimental animals we have expanded the methods to body fluids, i.e. prostatic secretion. The latter can be injected directly onto the column.

EXPERIMENTAL

Materials

All chemicals and solvents were of at least p.a. quality, water glass-distilled; all were prefiltered using a GV 100/1 glass filtration apparatus (Ref. No. 392700) and filter-discs, RC 58, 0.2 μm (Ref. No. 371628) both from Schleicher and Schüll (Dassel, G.F.R.). Acetic acid, ethyl acetate and KH_2PO_4 were obtained from E. Merck (Darmstadt, G.F.R.) and acetonitrile HPLC Grade S from Rathburn Chemicals (Walkerburn, Great Britain). For preparing standard curves, TXP charge No. 79-04923, MTP charge no. Gu 2409790 and SDZ charge No. 3268800 were used (Heumann-Pharma, analytical department).

Instrumentation

All investigations were carried out on a Waters ALC/GPC 204 high-performance liquid chromatograph (Waters, Königstein, G.F.R.), which was equipped with an UV absorbance detector (254 nm), Model 440 and a Waters Data Module 730 integrator. Injections were made with a 10-μl Hamilton syringe through a Waters U6K injector. A 120 × 4.6 mm reversed-phase column packed with 5 μm ODS-Hypersil (Shandon Southern Products, Ashmoor, Great Britain) was used. The packing of the column was performed principally according to Bristow et al. [5].

Sample preparation

Serum was collected by venipuncture from healthy volunteers (MTP/SDZ) and geriatric patients (TXP/SDZ) who had received a single oral dose of 200 mg MTP and 500 mg SDZ and of 200 mg TXP and 500 mg SDZ, respectively. Acetonitrile (1 ml) is added to 1 ml serum. After the sample was shaken for 30—60 sec (Reax 1 DR, Heidolph) it was centrifuged for 15 min at 2700 *g* using a Hettich EBA 3 S centrifuge.

Prostatic secretion (PS) was collected from anaesthetized mongrel dogs, which had been dosed with 5 mg/kg of the respective benzylpyrimidine and 12.5 mg/kg SDZ by an intravenous bolus and followed by constant infusion of 0.5 mg/kg/h TXP (MTP) and 1.25 mg/kg/h SDZ [6]. Aliquots (10 μl) of PS were then injected onto the column in the untreated state.

Method 1: Measurement of TXP and SDZ in serum and PS

Serum standard curves were prepared as follows. A 62 μg/ml TXP and 155 μg/ml SDZ solution in water was prepared from a tenfold concentrated methanolic stock solution. A 0.5-ml aliquot of this standard solution was added to 2.5 ml of drug-free human serum to make up a 12.4 μg/ml TXP and a 31.0 μg/ml SDZ standard. It was then serially diluted with drug-free serum to yield concentrations of 6.20/15.5, 3.10/7.75, 1.55/3.88, 0.78/1.94, 0.39/0.97 and 0.195/0.48 μg/ml TXP/SDZ. After protein precipitation with

acetonitrile and centrifuging as described above, 10-μl aliquots of the clear supernatant were injected. The mobile phase was composed of 800 ml 0.1 *M* KH_2PO_4 (containing 1% acetic acid and 1% ethyl acetate) and of 200 ml acetonitrile. The flow-rate was 1.3 ml/min. The UV detector setting for both drugs was 254 nm. For determining TXP and SDZ concentrations in PS without extraction the same system conditions as described for serum were used. Standard curves with drug-free PS were prepared with the same stock solution and the same serial dilution steps as serum.

Method 2: Measurement of MTP and SDZ in serum and PS

Serum standard curves were prepared from a 62 μg/ml MTP and 155 μg/ml SDZ aqueous solution by serial dilution with drug-free human serum exactly in the same way as described in Method 1. After protein precipitation with acetonitrile and centrifuging at 2700 *g*, 10-μl aliquots of the clear supernatant were injected into the chromatograph. The mobile phase was as used in Method 1. In addition, a flow programme consisting of 1.3 ml/min for the first 3 min and thereafter directly increasing to 2.3 ml/min was employed. Both drugs were detected at 254 nm.

The simultaneous determination of MTP and SDZ from PS was achieved under the conditions given for serum. For details on preparing standard curves with drug-free PS see Method 1.

RESULTS AND DISCUSSION

Fig. 1 includes chromatograms of blank serum and representative serum from a patient, who had received 200 mg TXP and 500 mg SDZ by oral administration. The total elution time per assay is 8 min. Unknown serum constituent peaks from the blank (Fig. 1A) can be in no way whatever seen to interfere with those of either SDZ and TXP. The limit of detection using 1-ml serum samples is 50 ng TXP per ml and 100 ng SDZ per ml respectively. The standard curves for TXP and SDZ were linear from the limits of detection up to 12.4 μg/ml TXP and 31.0 μg/ml SDZ with correlation coefficients (r) greater than 0.99.

The precision and accuracy of the assay are shown in Table I. Mean recoveries of 92% with mean intra-assay coefficients of variation (C.V.) of 2.7% were obtained for TXP as well as for SDZ over the concentration range of 0.195—12.4 μg/ml (TXP) and of 0.48—31.0 μg/ml (SDZ) respectively. The chromatographic procedure is reproducible with retention times of 2.22—2.24 min for SDZ and 4.02—4.04 min for TXP. This, and the fact that the compounds are not destroyed in the column, make it possible to measure TXP and SDZ without the use of internal standards.

A pharmacokinetic study of TXP and SDZ in geriatric patients who had received a single dose of 200 mg TXP and 500 mg SDZ has recently been accomplished with this method [7]. Some results are shown in Fig. 2. The TXP and SDZ serum levels found in this controlled group of patients and the main pharmacokinetic parameters, half-life of elimination and volume of distribution, were in correlation with the literature values [8, 9], i.e. they compared well with the results obtained from the double extraction technique pre-

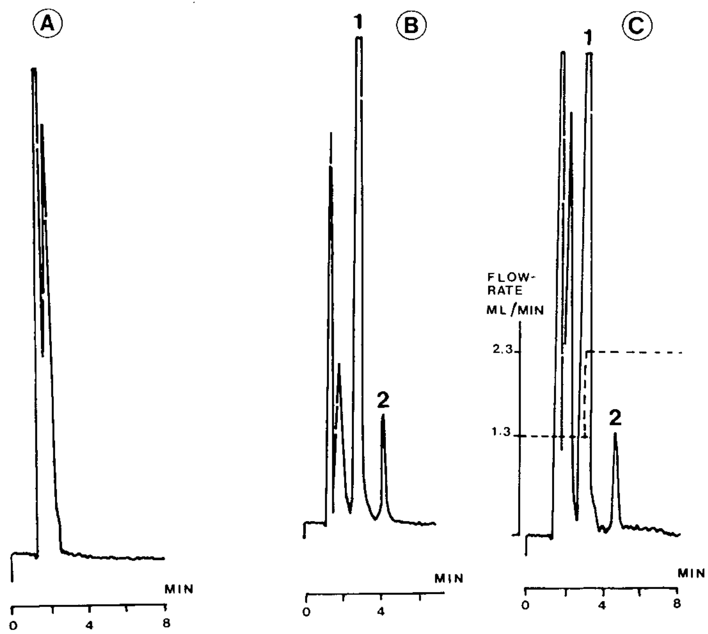


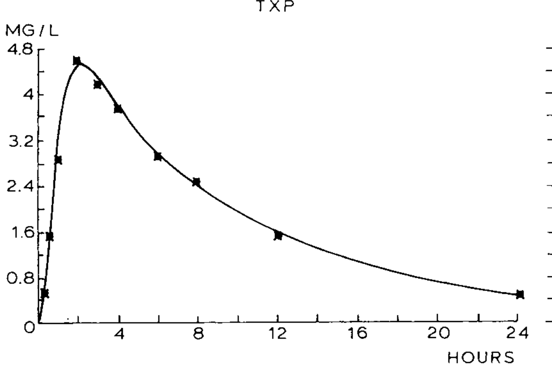
Fig. 1. Assay of tetroxoprim (TXP) and sulphadiazine (SDZ) and metioprim (MTP) and SDZ in serum (see Methods 1 and 2). (A) Drug-free human serum; (B) 3-h serum sample obtained from a geriatric patient taking a single dose of 200 mg TXP and 500 mg SDZ; (C) 3-h serum sample obtained from a healthy volunteer taking a single dose of 200 mg MTP and 500 mg SDZ. Peaks (B): 1 = SDZ, 2 = TXP; (C) 1 = SDZ, 2 = MTP, UV detector setting: 254 nm.

TABLE I

INTRA-ASSAY VARIATION OF TXP AND SDZ IN SERUM

Tetroxoprim (TXP)		Sulphadiazine (SDZ)	
Spiked concentration (μg/ml)	Intra assay C.V. (%) (n = 5)	Spiked concentration (μg/ml)	Intra assay C.V. (%) (n = 5)
0.195	3.1	0.48	3.4
0.39	n.d.*	0.97	n.d.*
0.78	1.9	1.94	1.7
1.55	2.8	3.88	3.2
3.10	2.6	7.75	2.7
6.20	2.7	15.50	2.1
12.40	3.0	31.0	2.9
Mean $\bar{x}$	2.7	Mean $\bar{x}$	2.7

*Not determined.



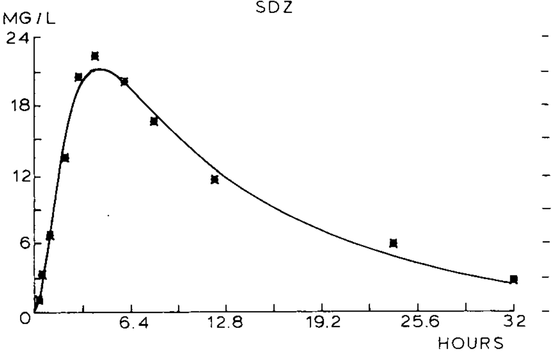


Fig. 2. TXP and SDZ concentrations in serum from 6 geriatric patients after oral administration of 200 mg TXP and 500 mg SDZ. Computer fits carried out on a Wang MVP-system with the aid of the Heinzel Topfit program [12].

viously developed in our laboratories [4]. This consisted of alkalinizing a serum sample, TXP extraction with chloroform (C.V. = 2.8%), lyophilisation of the remaining aqueous phase and hence SDZ extraction (C.V. = 2.2%) with methanol. Comparison of the values obtained using the two techniques to assay five samples from urinary tract infections patients differed from one another by 4.9—1.8%.

The detection limits for TXP and SDZ in PS of dogs were the same as in human serum. In the PS samples no interfering peaks were observed, as shown in Fig. 3. The recoveries of TXP and SDZ added to drug-free PS of dogs were quantitative (Table II). For both compounds standardisation can be carried out externally and PS can be measured by direct injection.

Peak areas shown in Fig. 3 demonstrate a reciprocal concentration relation of SDZ and TXP in PS compared to serum. Thus, under steady-state conditions in the dog (experimental set-up as previously described) approximately 20 μg/ml TXP and 4 μg/ml SDZ can be found in PS. These results are as would be expected [10, 11] and can be attributed to the different pK_a values of TXP and SDZ, and to the weakly acidic nature of PS (pH = 6.5).

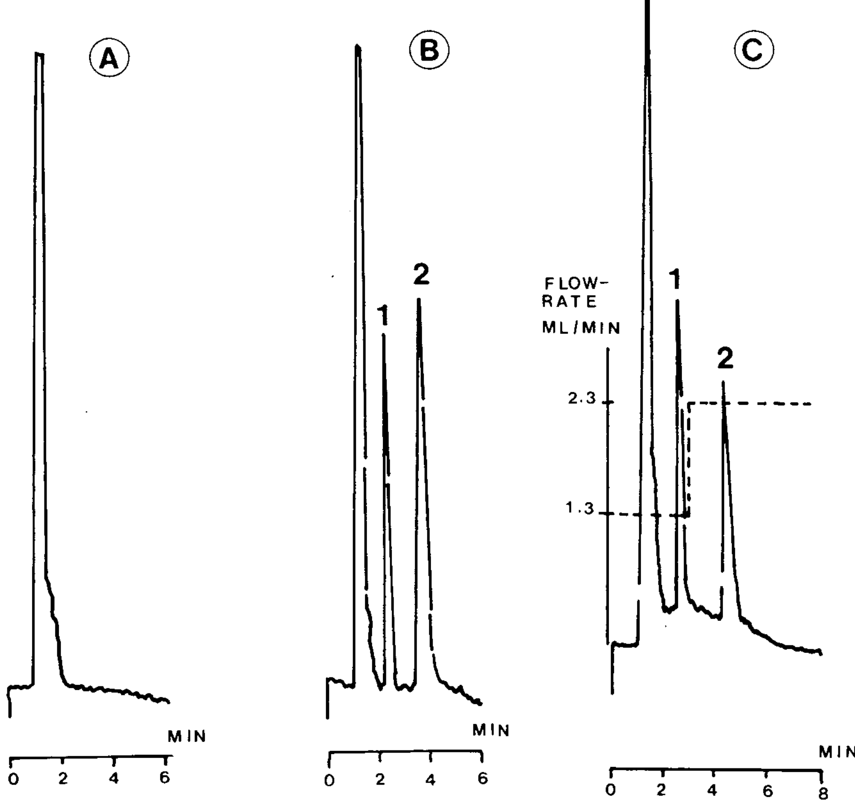


Fig. 3. Assay of tetroxoprim (TXP) and sulphadiazine (SDZ) and metioprim (MTP) and SDZ in prostatic secretion (see Methods 1 and 2). (A) Drug-free canine prostatic secretion (PS); (B) 1-h PS sample obtained from an anaesthetized mongrel dog, dosed with 5 mg TXP per kg + 12.5 mg SDZ per kg by intravenous bolus injection followed by constant infusion of 0.5 mg/kg/h TXP and 1.25 mg/kg/h SDZ; (C) = 1-h PS sample obtained from an anaesthetized mongrel dog, dosed with 5 mg MTP per kg + 12.5 mg SDZ per kg by intravenous bolus injection followed by constant infusion of 0.5 mg/kg/h MTP and 1.25 mg/kg/h SDZ. Peaks (B): 1 = SDZ, 2 = TXP; (C) 1 = SDZ, 2 = MTP. UV detector setting: 254 nm.

TABLE II

RECOVERIES OF TXP AND SDZ FROM PROSTATIC SECRETION (PS) WITHOUT EXTRACTION

Amount of TXP added to 1 ml PS (μg)	Recovery (%)	Amount of SDZ added to 1 ml PS (μg)	Recovery (%)
0.78	102	1.94	103
3.10	96	7.75	96
12.40	101	31.00	102
Mean $\bar{x}$	99.7	Mean $\bar{x}$	100.3

Fig. 1 shows chromatograms of blank serum and a typical serum from a volunteer who had received a single oral dose of 200 mg MTP and 500 mg SDZ. The limit of detection using 1-ml serum samples is 40 ng MTP per ml and 100 ng SDZ per ml respectively. Standard curves prepared for both drugs were linear from the limits of detection up to 12.4 μg/ml MTP and 31.0 μg/ml SDZ ($r > 0.99$). The mean recovery of MTP added in a wide concentration range to drug-free human serum was almost 96% (intra-assay C.V. = 2.19%), whereas recoveries of simultaneously added SDZ of 92% (intra-assay C.V. = 2.7%) were of the same magnitude as obtained for SDZ in the TXP/SDZ assay. Reproducible retention times were found to be 2.22—2.24 min for SDZ and 4.39—4.41 min for MTP. Table III shows precision and accuracy of the MTP/SDZ assay. Due to the high degree of reproducibility for both drugs, standardisation for routine analysis can be carried out externally. Preliminary pharmacokinetic results in human volunteers show maximum serum levels of 2.5—3 μg/ml MTP and 20—24 μg/ml SDZ after administration of a single oral dose consisting of 200 mg MTP and 500 mg SDZ.

TABLE III

INTRA-ASSAY VARIATION OF MTP AND SDZ IN SERUM

Metioprim (MTP)		Sulphadiazine (SDZ)	
Spiked concentration (μg/ml)	Intra-assay C.V. (%) (n = 5)	Spiked concentration (μg/ml)	Intra-assay C.V. (%) (n = 5)
0.195	2.2	0.48	3.0
0.39	2.5	0.97	3.2
0.78	1.4	1.94	1.9
1.55	2.9	3.88	3.0
3.10	1.8	7.75	2.4
6.20	n.d.*	15.50	n.d.*
12.40	2.3	31.00	2.7
Mean $\bar{x}$	2.19	Mean $\bar{x}$	2.7

*Not determined.

The simultaneous determination of MTP and SDZ from canine PS can be achieved by direct injection of the latter. Analogous to the TXP/SDZ system, recovery values were found to be quantitative for both drugs (Table IV). There was also no interference by endogenous substances (Fig. 3).

A distribution study of MTP and SDZ in dogs, which had received an intravenous bolus followed by constant infusion of both drugs has recently been initiated with this method [6]. Some results are shown in Fig. 4. MTP shows a specific affinity to the prostatic secretion, whereas SDZ, as expected, demonstrates lower values in PS than in serum.

The present HPLC methods allow simultaneous determination of the synergisticly acting chemotherapeutic agents TXP/SDZ and MTP/SDZ. By using these assays an extremely rapid sample clean-up and quantification of the two partner drugs can be achieved, without forgoing the high reproducibility. The methods will be used in further pharmacokinetic and clinical studies.

TABLE IV

RECOVERIES OF MTP AND SDZ FROM PROSTATIC SECRETION (PS) WITHOUT EXTRACTION

Amount of MTP added to 1 ml PS (μg)	Recovery (%)	Amount of SDZ added to 1 ml PS (μg)	Recovery (%)
0.78	101	1.84	101
3.10	96	7.75	96
12.40	103	31.00	103
Mean $\bar{x}$	100	Mean $\bar{x}$	100

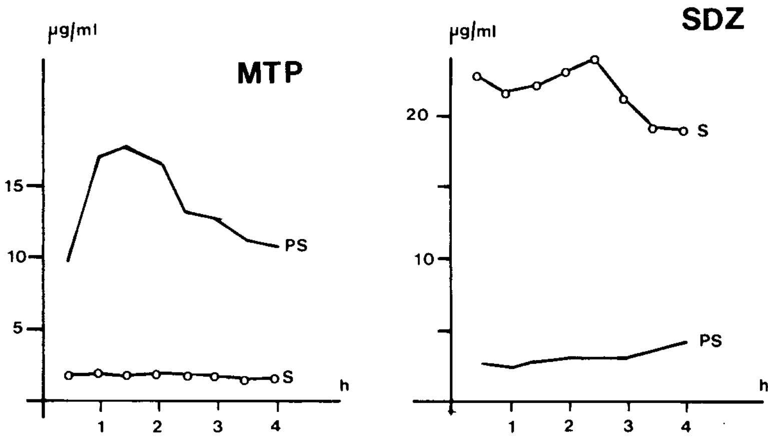


Fig. 4. Metioprim (MTP) and sulphadiazine (SDZ) concentrations in prostatic secretion (PS) and serum (S) in dogs (n = 4), dosed with 5 mg MTP per kg + 12.5 mg SDZ per kg by intravenous bolus injection followed by constant infusion of 0.5 mg/kg/h MTP and 1.25 mg/kg/h SDZ.

The correlation between serum and body fluid and tissue levels of the drugs and the relationships to microbiological and clinical variables will also be investigated.

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Note

Determination of N-acetyldopamine by liquid chromatography with electrochemical detection

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High-performance liquid chromatography with electrochemical detection has proved very useful for the determination of catecholamines in animal tissues [1]. Suitable columns and mobile phases can be found that allow catecholamines to be assayed with detection limits at the picogram level. We report here an assay for N-acetyldopamine using liquid chromatography with electrochemical detection. N-Acetyldopamine is a substance long known to be present in insects and to be involved in biosynthesis of the cuticle. In recent years evidence has accumulated that indicates that N-acetyldopamine might also occur in insect nervous tissues [2—5], where it presumably has some function related to neural activity. Very recently an enzymatic—radiochemical assay for N-acetyldopamine has become available [6], but it has not been applied to insect or other arthropod tissues. Using the liquid chromatographic method with electrochemical detection described here we have obtained evidence that N-acetyldopamine occurs widely in insect nervous tissues and that smaller amounts of it occur in other non-cuticular insect tissues, as reported elsewhere [7]. Quantitative methods for the determination of N-acetyldopamine may be of clinical interest in view of the demonstrations of N-acetyldopamine in patients with phaeochromocytoma and neuroblastoma [8,9].

EXPERIMENTAL

Materials

The catecholamines and related compounds were purchased from Sigma (St. Louis, MO, U.S.A.); all other chemicals were of analytical-reagent grade. Alumina was purchased from Bioanalytical Systems (West Lafayette, IN,

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U.S.A.) and used without further purification. N-Acetylnorepinephrine was synthesized by the method of Wolfe and Thorn [10]. Except for the mobile phase and physiological saline, all solutions were prepared in water redistilled in glass from alkaline permanganate (0.05 *M* NaOH and 0.05 *M* $KMnO_4$).

Cockroaches (*Periplaneta americana* L.) were reared in the laboratory and fed a diet of dog pellets ad libitum. Only adult males were used. Mice were adult males of the Cox (Swiss) strain obtained from Laboratory Supply (Indianapolis, IN, U.S.A.).

Apparatus

The chromatographic system consisted of a Milton-Roy mini-pump (Milton-Roy, Riviera Beach, FL, U.S.A.), pulse damper (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Rheodyne Model 70-10 injection valve with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a Partisil 10 ODS II analytical reversed-phase column (25 × 0.46 cm I.D.) (Whatman, Clifton, NJ, U.S.A.) fitted with a 6 × 0.5 cm I.D. pre-column packed with Bondapak C_{18} Corasil (Waters Assoc., Milford, MA, U.S.A.). The electrochemical detector consisted of a carbon paste, wax-impregnated graphite—oil electrode (CP-W) and LC-2A potentiostat from Bioanalytical Systems. The working electrode was set at +0.72 V relative to an Ag/AgCl reference electrode. Peaks were recorded with an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). All chromatograms were obtained at ambient temperature (20—22°C).

Procedures

The insect tissues were dissected out under ice-cold saline (160 m*M* NaCl, 3 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.2 m*M* Na_2HPO_4, 1.8 m*M* NaH_2PO_4, pH 7.2 [11]) saturated with phenylthiourea to inhibit phenoloxidase activity. Once the tissue had been freed of extraneous tissues, including tracheae as far as possible, excess of saline was removed by absorption with a piece of filter-paper and the tissue was frozen on a porcelain plate kept on dry-ice. The collected tissues were subsequently weighed to the nearest 0.1 mg on a Cahn 7500 electrobalance, transferred to an all-glass homogenizer (200-μl capacity; Radnoti Glass Technology, Arcadia, CA, U.S.A.) and homogenized in 100 μl of 0.1 *M* $HClO_4$ containing 0.0004 *M* sodium bisulfite. Homogenization was continued until fragments or strings of intact tissue were no longer visible. The homogenates were centrifuged for 10 min at 500 *g*. Duplicate 40-μl aliquots of each supernatant were pipetted into 1.5-ml Eppendorf polypropylene micro test-tubes, 10 pmol of N-acetyldopamine in 0.1 *M* $HClO_4$ were added to one of these aliquots and the volume of this and the duplicate sample were adjusted to 100 μl with 0.1 *M* $HClO_4$. By comparing peak heights with and without added N-acetyldopamine it was possible to calculate the recovery of endogenous N-acetyldopamine.

N-Acetyldopamine and the other catechol compounds were adsorbed on to alumina by adding to each tube 22.5 mg of a mixture of alumina and Na_2EDTA [12] (2:1, w/w) and 1.0 ml of 0.5 *M* Tris—HCl buffer (pH 8.6). The tubes were capped and shaken vigorously on a wrist-action shaker for 15 min. After brief centrifugation (2 min at 12,800 *g* in an Eppendorf 5412 centrifuge) to sediment the alumina, the supernatant was aspirated off and discarded. The alumina

was then washed once as follows: 1.0 ml of 0.0004 *M* sodium bisulfite was added, the tubes were shaken vigorously on the wrist-action shaker for 2 min and then centrifuged to sediment the alumina. The supernatant was next aspirated off and discarded. Care was taken to remove as much of the wash solution as possible during aspiration. This operation was facilitated by aspirating through a Pasteur pipette that had been drawn to a fine point.

N-Acetyldopamine was desorbed from the alumina by adding 100 μl of 1.0 *M* acetic acid containing 0.0004 *M* sodium bisulfite. The mixture was shaken for 15 min on the wrist-action shaker. After brief centrifugation to pellet the alumina, the supernatant containing N-acetyldopamine and other catechol compounds was collected and injected into the chromatograph. The mobile phase was 0.06 *M* sodium citrate, 0.04 *M* Na_2HPO_4 [13] and 0.0001 *M* Na_2-EDTA containing 20% (v/v) of methanol (final pH 3.6) and was pumped at a rate of 1 ml/min.

RESULTS AND DISCUSSION

Table I gives the retention times of catecholamines and other compounds that might interfere in the determination of N-acetyldopamine. The chromatogram shown in Fig. 1A shows that N-acetyldopamine is well separated from numerous catechol compounds or derivatives that might occur in animal tissues together with N-acetyldopamine. Fig. 1B shows the presence of an N-acetyldopamine peak in an extract of cerebral ganglion from the cockroach *P. americana*, the peak representing 1.3 pmol of N-acetyldopamine. After correction for recovery, the concentration of N-acetyldopamine in cerebral ganglion in the sample analysis shown was calculated to be 1.9 μg/g wet weight. The mean value for N-acetyldopamine in adult male *P. americana* cerebral ganglion was 2.09 ± 0.25 μg/g wet weight (n=25). The peaks near the solvent front in Fig. 1B are due to dopamine and several additional polar catechol compounds that are adsorbed on to alumina but not retained on the reversed-phase column. The only naturally occurring catechol compound tested that elutes near N-acetyldopamine under the chromatographic conditions described is 3,4-dihydroxyphenylacetic acid, but it elutes early enough not to interfere seriously with N-acetyldopamine determination. We have never been able to detect 3,4-dihydroxyphenylacetic acid in insect tissues but it is easily demon-

TABLE I

RETENTION TIMES OF CATECHOLAMINES AND RELATED COMPOUNDS

Conditions as described under *Procedures*

Compound	Retention time (min)	Compound	Retention time (min)
Norepinephrine	3.3	Metanephrine	4.2
Epinephrine	3.5	Epinine	4.3
3,4-Dihydroxynorephedrine	3.5	N-Acetylnorepinephrine	4.9
3,4-Dihydroxybenzylamine	3.6	3,4-Dihydroxyphenylalanine methyl ester	5.8
3,4-Dihydroxyphenylalanine	3.7	3,4-Dihydroxyphenylacetic acid	8.1
Normetanephrine	3.8	N-Acetyldopamine	10.0
Dopamine	4.0	N-Acetyl-5-hydroxytryptamine	18.4
Isoproterenol	4.2		

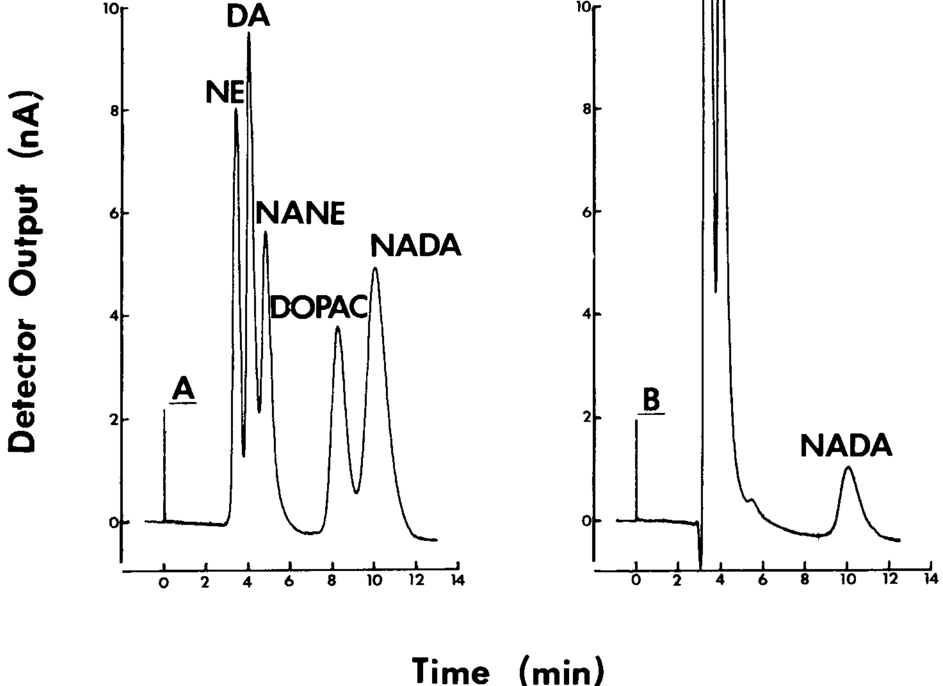

Fig. 1. High-performance liquid chromatograms showing electrochemical detector output after injecting (A) 5 pmol of N-acetyldopamine (NADA), norepinephrine (NE), dopamine (DA), N-acetylnorepinephrine (NANE) and 3,4-dihydroxyphenylalanine (DOPAC), and (B) extract of cerebral ganglion from an adult male *P. americana*.

strable and quantifiable in normal mouse brain using the present procedure. On the other hand, we have been unable to demonstrate the presence of N-acetyldopamine (< 0.02 μg/g) in whole brain extracts from normal adult Cox (Swiss) laboratory mice.

The detector responses to increasing amounts of N-acetyldopamine were linear over the range up to 20 pmol. Over the same range, the recovery from the alumina adsorption step was also linear, with a mean value of 51.0 ± 2.0%. Although alumina adsorption procedures for the isolation and assay of catecholamines frequently involve multiple washing steps after adsorption of the amines, we found that a single washing step eliminated any interfering substances. Further washes served only to reduce the recovery. Correlation coefficients determined by linear regression of the amount of N-acetyldopamine injected versus peak height were $r^2 = 0.99$ for authentic N-acetyldopamine injected directly into the chromatograph and $r^2 = 0.99$ for standard N-acetyldopamine carried through the alumina adsorption step.

When the concentration of methanol in the mobile phase was increased above 20%, N-acetyldopamine eluted faster but the life of the carbon paste electrode was markedly reduced. Decreasing the concentration of methanol below 20% increased the retention time of N-acetyldopamine and resulted in a marked broadening of the peak. The methanol concentration of 20% (v/v)

represents the best compromise between speed of elution, peak height, and convenience for analysis.

The selectivity and specificity of the method depends on the selectivity of the extraction of catechol compounds on to alumina, the retention of N-acetyldopamine as a moderately polar compound on the reversed-phase column and the ease of its oxidation in the electrochemical detector. The minimum amount of N-acetyldopamine detectable with our method was ca. 0.12 pmol or 25 pg. This amount of N-acetyldopamine produced a peak that was approximately twice as high as the baseline noise on the strip-chart recorder.

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CHROMBIO. 874

Note

Resolution of 52 ninhydrin-positive compounds with a high-speed amino acid analyser

Determination of carnosine and homocarnosine in biological materials

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(First received January 13th, 1981; revised manuscript received February 20th, 1981)

Many cases of inborn errors of amino acid metabolism have been described [1]. Perry et al. [2] described carnosinaemia, a metabolic disorder associated with neurological disease and mental defects involving impaired cleavage of imidazole dipeptides. Sjaastad et al. [3] found elevated concentrations of homocarnosine in cerebrospinal fluid of family members having spastic paraplegia.

In such cases, identification of carnosine (β-alanylhistidine) and homocarnosine (γ-aminobutyrylhistidine) in physiological fluids was complicated. Perry et al. [4] described two-dimensional paper chromatographic and high-voltage electrophoretic methods, but they were time-consuming and only semi-quantitative. They also used an amino acid analyser to identify these peptides [4]. Carnosine and homocarnosine were eluted after 1,111 and 1,114 min, respectively. As each peak width was about 10 min, it was difficult to resolve the peaks completely as the difference in retention times between carnosine and homocarnosine was only 3 min.

Adriaens et al. [5] reported the identification of these peptides with an amino acid analyser using lithium buffers. The peak resolution of the peptides was about 50% and the retention times were over 21 h.

In this paper, we report the determination of carnosine and homocarnosine in physiological fluids with a high-speed amino acid analyser.

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EXPERIMENTAL

Materials

L-Carnosine was purchased from Tokyo Chemical Industry (Tokyo, Japan) and L-homocarnosine sulphate, δ-amino-*n*-valeric acid and D-glucosaminic acid from Nakarai Chemical (Kyoto, Japan). Titanium(III) chloride was purchased from Pierce (Rockford, IL, U.S.A.) and other reagents from Wako (Osaka, Japan) and Nakarai Chemical.

A standard solution of ninhydrin-positive compounds was prepared as described in a previous paper [6].

Amino acid analysis

A Hitachi 835 high-speed amino acid analyser equipped with an autosampler, data processer, graphic printer and recorder was used. The experimental conditions are summarized in Table I. The programs for changing the buffer and temperature were modified following the Hitachi instruction manual.

TABLE I

EXPERIMENTAL CONDITIONS FOR AMINO ACID ANALYSIS

Column: 250 × 2.6 mm I.D. stainless steel, packed with Hitachi Custom Ion-exchange resin 2619.

Flow-rates: pump 1 (buffer), 0.275 ml/min (190 kg/cm^2)
pump 2 (ninhydrin), 0.300 ml/min (30 kg/cm^2)

Programme:

Buffer change		Temperature change	
1 → 2	66 min	34 → 43°C	32 min
2 → 3	82 min	43 → 47°C	69 min
3 → 4	128 min	47 → 68°C	101 min
4 → 5	164 min	68 → 45°C	153 min
5 → 6	191 min	45 → 34°C	218 min
6 → 1	203 min		

Buffer compositions:

Buffer	Lithium concentration (*N*)	pH	LiOH (g/l)	Li citrate (g/l)	LiCl (g/l)	Citric acid (g/l)	Ethanol (ml/l)	Thio-diglycol (ml/l)	25% Brij-35 (ml/l)
1st	0.155	3.00	—	9.80	2.12	35	40	5	4
2nd	0.255	3.70	—	9.80	6.36	13	30	5	4
3rd	0.805	3.46	—	9.80	29.67	12	—	—	4
4th	1.000	4.40	—	9.80	38.14	3.3	—	—	4
5th	1.200	7.07	—	47.00	29.68	—	—	—	4
6th	0.200	—	8.40	—	—	—	—	—	4

Ninhydrin reagent:

Ninhydrin	80 g
Methylcellosolve	3 l
5 *M* sodium acetate buffer (pH 5.5)	1 l
Titanium(III) chloride (15%)	6.8 ml

All of the buffers were passed through a stainless-steel pre-column (120 × 4 mm I.D.) packed with Hitachi Custom Ion-exchange Resin 2650 to remove ammonia.

Sample preparation

Urine. Samples from a band-shaped keratopathy patient were used. A 10-ml volume of 24-h urine that had been preserved by adding toluene was deammoniated by the method of Benson and Patterson [7]. The residue was resuspended in and diluted to 10 ml with 0.01 *N* hydrochloric acid.

Cerebrospinal fluid (CSF). CSF was obtained by lumbar puncture from a patient with Reye's syndrome, collected in a centrifuge tube and after 10 mg/ml of sulphosalicylic acid had been added, was centrifuged at 10,000 *g* for 10 min. The deproteinized supernatant was analysed.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms of 52 ninhydrin-positive compounds obtained with the Hitachi 835 high-speed amino acid analyser. The total analysis time was 195 min.

Excellent resolution of these compounds was obtained, except for cystathionine—*allo*-isoleucine and 5-hydroxylysine—*allo*-hydroxylysine. δ-Amino-*n*-valeric acid (AVA) (144.68 min) and D-glucosaminic acid (13.94 min) were used as internal standards instead of norleucine, the peak of which overlapped the tyrosine peak. These internal standards were used because the sample size in the high-speed amino acid analyser was only 50 μl.

The colour yields of carnosine, asparagine and anserine were lower than in a standard Hitachi KLA-5 amino acid analyser. As the ninhydrin reaction time with the high-speed amino acid analyser was only 1.706 min, it was unavoidable that the peak areas at 570 nm were smaller than those at 440 nm, and we quantified these compounds at 440 nm, as for proline.

Carnosine and homocarnosine were eluted after 175.18 and 177.18 min, respectively, and the resolution was about 30% at 570 nm and 80% at 440 nm. The colour yield of homocarnosine was greater than that of carnosine at 570 nm, the peak-area ratios (440/570 nm) being 1.26 for carnosine and 0.18 for homocarnosine. These ratios were used for the identification of these peptides.

Fig. 2 shows a typical chromatogram of the amino acids in CSF. A large amount of glutamine was present, but the other amino acids were found in only very low concentrations. Homocarnosine was clearly identified by the retention time and the peak-area ratio, which agreed with those of the authentic compound.

Carnosine in the urine was also identified by the same method, as shown in Fig. 3. Some homocarnosine was present in this sample. This urine sample contained many ninhydrin-positive compounds that could not be identified clearly.

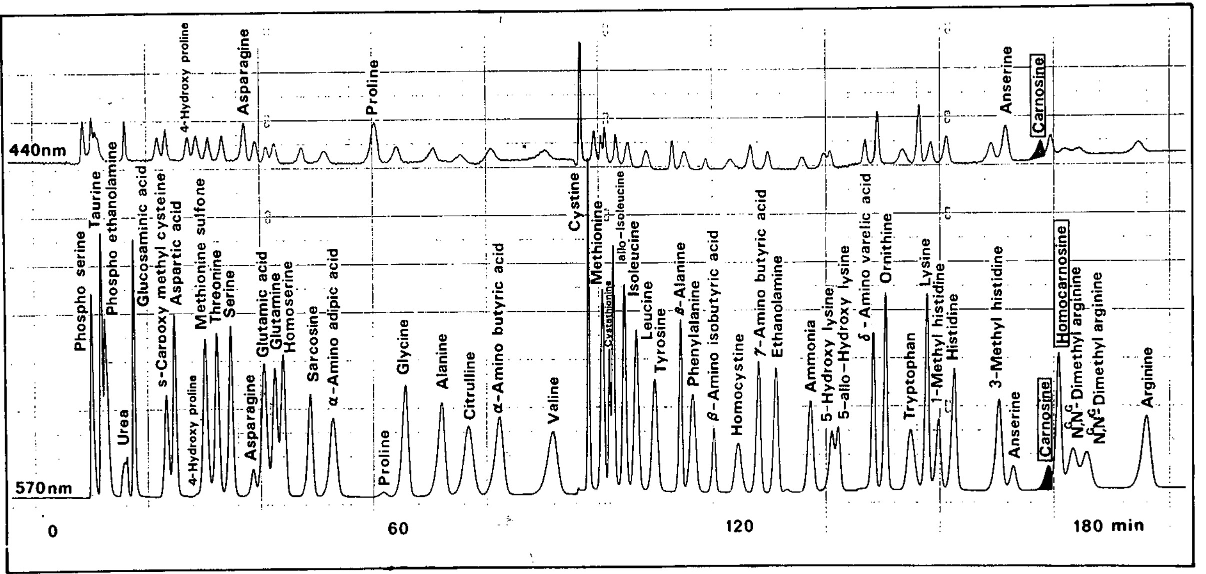

Fig. 1. Typical chromatogram of 52 ninhydrin-positive compounds obtained with a high-speed amino acid analyser. The amount of each amino acid to be analysed was 2.5 nmole. The following amino acids were prepared: homoserine (0.625 nmole); cystathionine (1.0 nmole); S-carboxymethylcysteine, N^G,N^G-dimethylarginine and N^G,N'^G-dimethylarginine (1.25 nmole); proline, sarcosine, β-alanine and anserine (5 nmole); and urea (250 nmole).

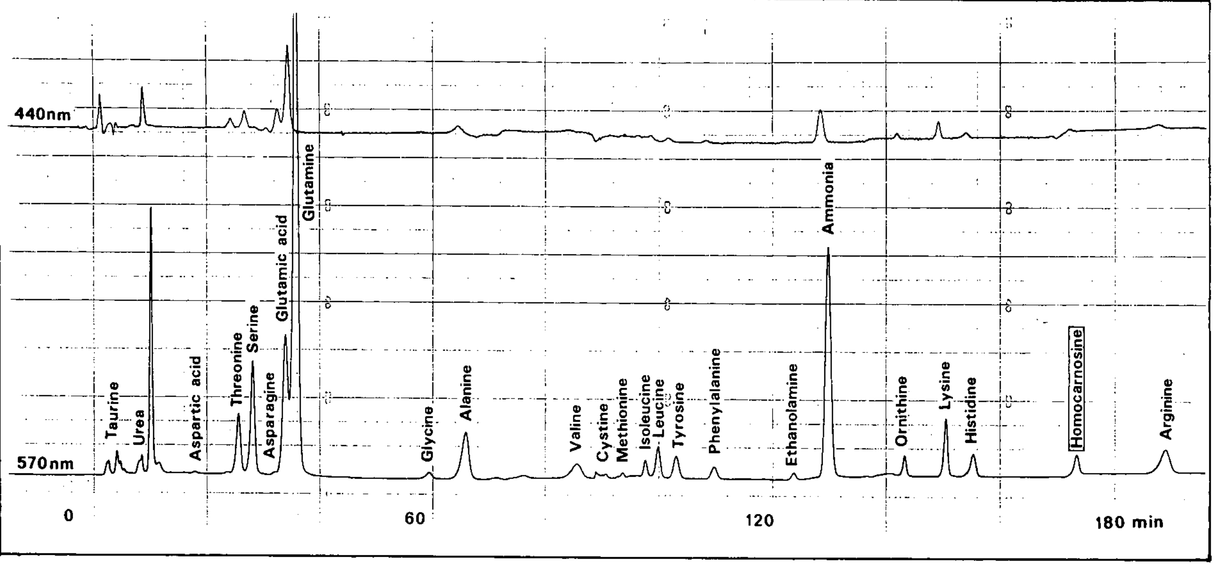

Fig. 2. Chromatogram of amino acids in 33.3 μl of CSF collected from a Reye's syndrome patient.

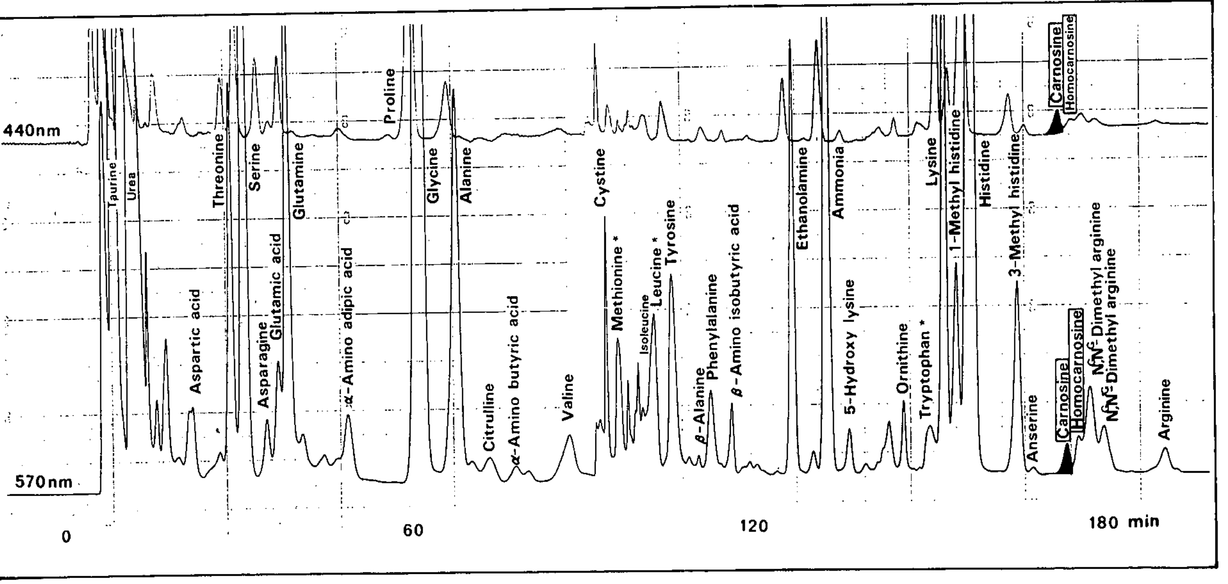

Fig. 3. Chromatogram of amino acids in 50 μl of urine collected from a band-shaped keratopathy patient. The asterisks indicate peaks that seemed to contain other unknown compounds.

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CHROMBIO. 872

Note

Analysis of middle molecular peptides in normal and uremic body fluids by high-performance gel chromatography

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(First received December 19th, 1980; revised manuscript received February 10th, 1981)

In recent years, middle molecular weight uremic solutes in the molecular weight range of 300–5000 as postulated in the middle molecule hypothesis [1] have been considered to play a major role in uremic toxicity. However, the middle molecule hypothesis was derived originally from clinical observations. The methods termed high-speed gel filtration [2] and high-performance gel chromatography [3] were used to separate middle molecular weight uremic solutes. Strictly speaking, however, these methods reported are not high-performance liquid chromatographic (HPLC) methods.

We report here the first high-performance gel chromatographic analysis of middle molecular peptides in normal and uremic body fluids with the use of a newly developed small-particle hydrophilic gel column, TSK-GEL 2000SW [4–6]. The detection system used was specific for substances containing primary amino groups, such as proteins, peptides and amino acids.

EXPERIMENTAL

Subjects

Fifteen patients undergoing maintenance hemodialysis or hemofiltration were selected randomly from the Hemodialysis Division of Nishijin Hospital.

Eight healthy subjects were used as normal controls.

Chemicals

Cytochrome *c* and aprotinin were obtained from Mochida Pharmaceutical Co. (Tokyo, Japan). Glucagon was from Novo Industries (Bagsvaerd, Denmark). Tetracosactide was from Organon (Oss, The Netherlands). All other peptides were from the Peptide Institute (Osaka, Japan). Spermine, spermidine

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and α-chymotrypsinogen A were from Sigma (St. Louis, MO, U.S.A.). All other chemicals were from Nakarai (Kyoto, Japan). All chemicals used were of analytical or HPLC grade.

Sample preparation

Serum samples were obtained by venipuncture and allowed to clot in siliconized glass tubes at room temperature. Freshly voided urines were used as urine samples. The total amounts of spent hemodialysate and hemofiltrate were about 75 and 18 l, respectively. All samples were stored frozen at −30°C until analyzed. Prior to analysis, all samples were ultrafiltrated through a Centriflo CF-25 (Amicon, Lexington, MA, U.S.A.) which has a nominal molecular weight cut-off of about 25,000. All sample sizes were 250 μl except standard samples.

High-performance gel chromatography

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used which included a Model SIL-1A injector, a Model CRD-5A chemical reaction detector and a Model RF-500LC spectrofluorimeter equipped with a 12-μl flow cell. The detection system was post-column fluorescence derivatization with fluorescamine [7, 8]. A prepacked TSK-GEL 2000SW column (particle size 10 ± 2 μm, 60×0.75 cm) was obtained from Toyo Soda (Tokyo, Japan). This column is reported by the manufacturer to have a fractionation range of 500—60,000 molecular weight. No information is available on either the chemical or the physical composition of the column. Analyses were performed using a mobile phase of 0.05 *M* sodium phosphate buffer (pH 7.2) containing 0.3% sodium dodecyl sulfate (SDS) at a flow-rate of 0.3 ml/min. All chromatograms were run at room temperature.

Preliminary experiments revealed that the addition of SDS to the mobile phase was effective in alleviating the adsorption of peptides on the column. Resolution was dependent chiefly on the concentration of SDS. The most suitable concentration of SDS was in the range 0.3—0.5%. In this method, also, no pretreatment of samples was required.

RESULTS AND DISCUSSION

The elution volume and molecular weight of standard samples are shown in Table I. Most peptides tested were well separated from amino acids. Spermine and spermidine, however, eluted in the range of peptides, but their concentration in both serum and urine [9—11] had no significant effect on the analysis of serum and urine. Kato et al. [6] have reported that the separation range of columns of TSK-GEL type could not be extended below a molecular weight of 10,000 even by use of a 2000SW column which has a smaller pore size, and the elution behaviour of proteins was greatly affected by the sodium phosphate concentration in the eluent. Our preliminary experiments, however, revealed that the resolution of peptides was dependent chiefly on the concentration of SDS and the addition of 0.3—0.5% SDS was effective.

Typical elution profiles of serum and standard samples are shown in Fig. 1. Fluorescamine is a selective reagent for substances containing primary amino groups, such as proteins, peptides and amino acids [12]. By comparing the

TABLE I

ELUTION VOLUME AND MOLECULAR WEIGHT OF STANDARD SAMPLES

Sample	Elution volume (ml)	Molecular weight
α-Chymotrypsinogen A	13.2	25,700
Cytochrome *c*	14.5	12,400
Aprotinin	14.5	6,520
Glucagon	14.8	3,485
Tetracosactide	14.8	2,934
Angiotensin I	15.0	1,297
Angiotensin II	15.8	1,046
Oxytocin	15.0	1,007
Met-enkephalin	20.2	574
Leu-enkephalin	19.0	556
Tuftsin	16.4	501
Glutathione	20.7	307
Spermine	15.7	202
Spermidine	17.7	145
Amino acids and putrescine	>20.7	

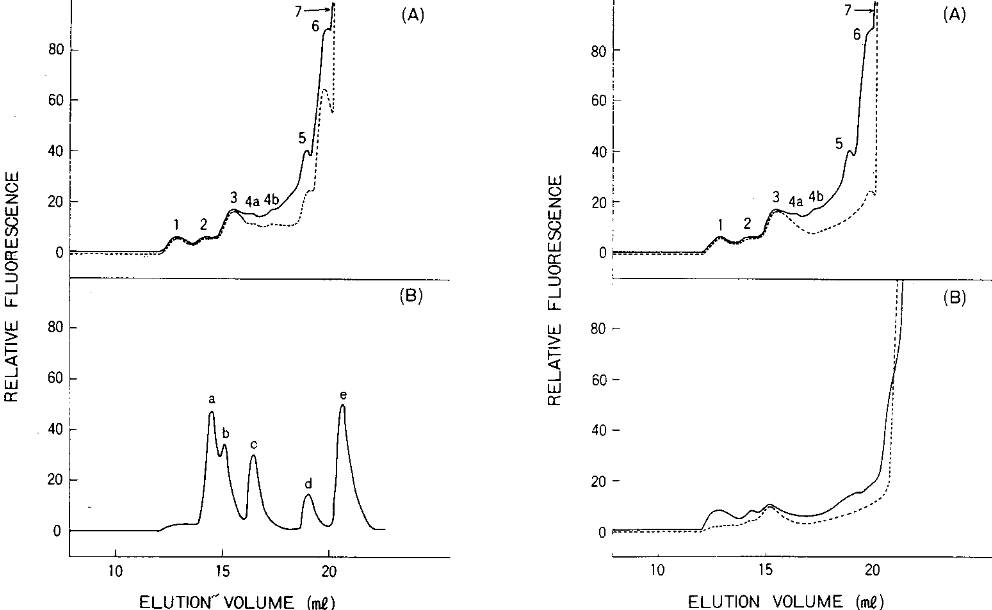

Fig. 1. Typical elution profiles of (A) normal (— — —) and uremic (——) serum, and (B) standard samples. Standard samples: (a) cytochrome *c*, 180 pmol; (b) oxytocin, 1.5 nmol; (c) tuftsin, 580 pmol; (d) Leu-enkephalin, 640 pmol; (e) glutathione, 2.6 nmol.

Fig. 2. Typical elution profiles of (A) pre-dialysis (——) and post-dialysis (— — —) serum, and (B) spent hemodialysate (— — —) and hemofiltrate (——).

elution profiles of standard samples, therefore, peaks 1 and 2 are proteins, and peak 7 is amino acids and low molecular weight amines. Peaks 3--6 can be considered to be a mixture of peptides. The molecular weight range is about 1000—3000 for peak 3, about 500—1000 for peak 4, and below 500 for the others. Subpeaks 4a and 4b were not always detected as specific peaks. In general, peptides having molecular weights below 1000 were increased in uremic serum compared with normal serum, but there was no significant difference in peak 3. No peak unique to uremia was detected. Serum concentrations of peptides decreased after hemodialysis except peak 3 (Fig. 2A). There were some differences in the elution profiles between spent hemodialysate and hemofiltrate due to the characteristics of the membranes used for therapy (Fig. 2B). Spent hemodialysate and hemofiltrate, however, contained moderate amounts of peptides. There were significant differences between normal and uremic urine. The elution profiles of normal urine was similar to serum, and normal urine contained a large amount of peptides (Fig. 3).

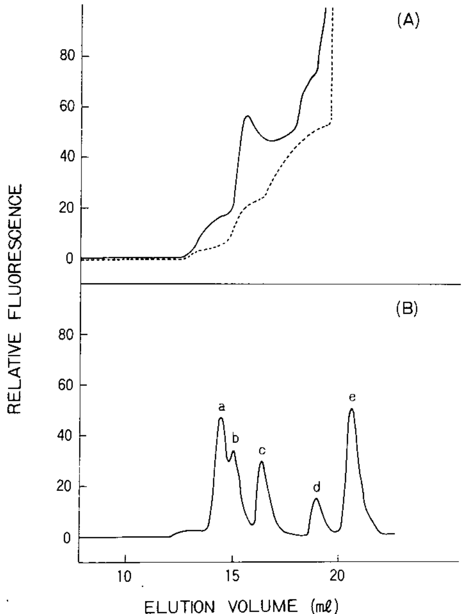

Fig. 3. Typical elution profiles of (A) normal (——) and uremic (— — —) urine, and (B) standard samples. Key to the standard samples is as given in Fig. 1.

Most gel-chromatographic methods reported for the separation of middle molecular weight uremic solutes have used ultraviolet absorption for flow-monitoring at different wavelengths [2, 3, 13]. Peters et al. [14] used the fluorescamine reaction to detect peptides in fractions obtained by Sephadex gel filtration. This was not flow-monitoring, and the separation of peptides from low molecular weight amines was not satisfactory. Fürst et al. [2] detected ten peaks in uremic plasma using the method termed high-speed gel filtration, and all peaks were abnormal when compared with normal plasma. In particular, peak 7 (molecular weight range 1000—2000) was unique to uremic plasma and

correlated more closely with symptoms of uremia. Funk-Brentano et al. [3] reported a middle molecular weight peak named b (molecular weight range of 600—1300) using a method termed high-performance gel chromatography. Our results contrast with these reports described above. The ultraviolet absorbance at 200--220 nm in highly sensitive to peptides; however, the main disadvantage is the lack of specificity compared with the fluorescamine method. Therefore, it remains to be elucidated in future studies whether the reported peaks are peptide(s) or not. Moreover, the other methods reported have used Sephadex G-15 which is relatively soft, and column performance is relatively poor compared with polystyrene gels or porous silica packings, which restricts its application in HPLC [15]. In a more precise sense, therefore, high-performance gel-chromatographic analysis of middle molecular substances in uremic body fluids has not been carried out until now.

In conclusion, this is the first report of the analysis of peptides in normal and uremic body fluids using high-performance gel chromatography combined with post-column fluorescence derivatization with fluorescamine.

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Note

Determination of benzbromarone, bromobenzarone and benzarone in plasma by gas chromatography—mass spectrometry

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Benzbromarone, a benzofuran derivative, lowers serum urate and increases urinary urate excretion [1—4]. The drug undergoes extensive dehalogenation in the liver to form bromobenzarone and benzarone which also display hypouricaemic activity. Twelve hours after ingestion approximately 75% of the absorbed drug has been converted to benzarone [5].

A high-performance liquid chromatographic method for the determination of benzarone from human urine and serum was described by Lücker and Wetzelsberger [6]. The analytical procedure was based on a gradient elution on a reversed-phase column after chloroform extraction. Besides a study using tritium labelled compounds by Broekhuysen et al. [5], Yu [7] reported a gas chromatographic (GC) analysis of acetylated derivatives. This paper describes a gas chromatographic—mass spectrometric (GC—MS) method for the measurement of benzbromarone and its dehalogenated analogues after extraction from plasma and derivatization with trifluoroacetic anhydride. Accurate estimates of benzbromarone, bromobenzarone and benzarone plasma concentrations can be made following the ingestion of pharmacological amounts of the uricosuric agent.

MATERIALS AND METHODS

Chemicals

Benzarone, bromobenzarone and benzbromarone were obtained from Labaz (Düsseldorf, G.F.R.); trifluoroacetic anhydride was purchased from EGA (Steinheim, G.F.R.).

Apparatus

A Varian 3700 gas chromatograph in combination with a MAT 44 mass

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spectrometer was used. The silanized glass column (2 m × 2 mm I.D.) was packed with 1% OV-17 on 80—100 mesh Chromosorb W (Perkin Elmer, Überlingen, G.F.R.). Prior to its initial use, it was conditioned overnight at 300°C with the carrier gas helium at a flow-rate of 30 ml/min. Operating temperatures were: column, 260°C; injection port, 290°C; separator, 280°C; transfer line, 280°C; ion source, 250°C. The electron energy was kept at 80 eV, the emission current at 0.8 μA.

Analytical procedure

Concentrations of benzarone, bromobenzarone and benzbromarone in the range between 10 and 2000 ng were added to 1 ml pooled male plasma samples. The addition of 1 ml 0.1 *N* hydrochloric acid and 5.0 ml chloroform was followed by an extraction for 1 h. After centrifugation (ca. 1950 *g*, 15 min) the water layer was removed by aspiration and 4.0 ml of the remaining chloroform phase were transferred with a Pasteur pipette to clean test tubes. Known concentrations of diazepam in chloroform solution were added for internal standardization. After evaporation to dryness trifluoroacetate (TFA) derivatives were formed by reacting the residue with 50 μl of a 20% solution of trifluoroacetic anhydride in acetonitrile. The samples were shaken ultrasonically for 30 sec. A 2—5-μl volume was analyzed by GC—MS. Benzarone/diazepam, bromobenzarone/diazepam and benzbromarone/diazepam peak height ratios were determined at least in triplicate for each benzarone, bromobenzarone and benzbromarone concentration to establish a standard curve.

RESULTS AND DISCUSSION

The molecular ions after derivatization with TFA, *m/e* 520, *m/e* 440 and *m/e* 360, represented the base peaks and were used for sinlge ion monitoring (Figs. 1, 2 and 3). Therefore a rapid GC analysis without interference from endogenous substances was possible; retention times for benzarone, bromobenzarone and benzbromarone derivatives were 0.9, 1.2 and 1.5 min, respec-

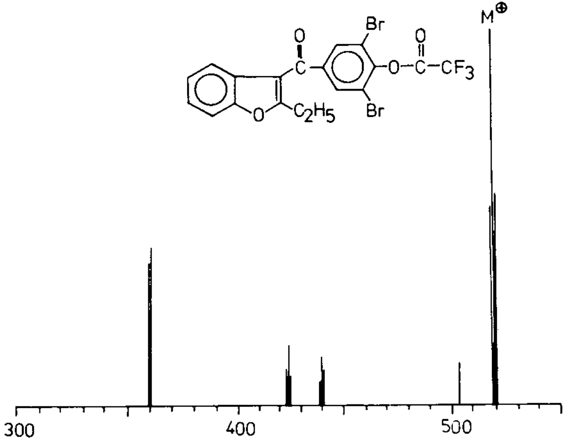

Fig. 1. Mass spectrum of derivatized benzbromarone.

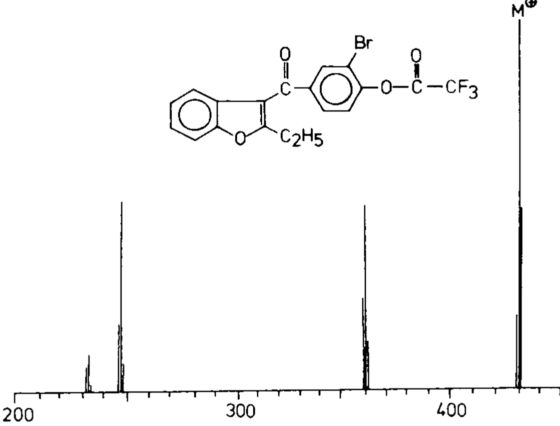

Fig. 2. Mass spectrum of derivatized bromobenzarone.

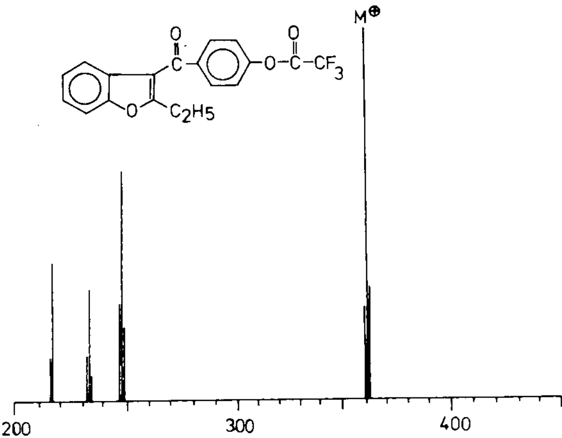

Fig. 3. Mass spectrum of derivatized benzarone.

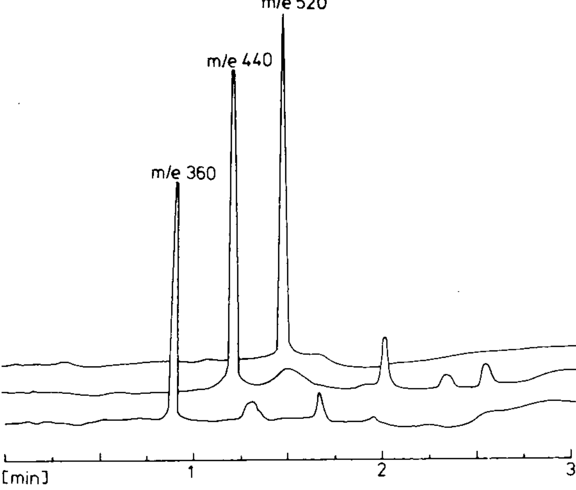

Fig. 4. Single ion monitoring after GC separation.

tively. Specificity was promoted by monitoring molecular ions. Fig. 4 shows a typical gas chromatogram of a spiked plasma sample. Derivatization with TFA was uncomplicated and rapid. A 20% solution of the reagent in acetonitrile was required to obtain complete derivatization (Fig. 5). Other acetylating and methylating techniques can be applied, but longer reaction times, fragmentation because of less stability of the molecular ions and less response have to be taken into account. Derivatization with silylating agents was unsuccessful because of the bulky bromide ions in the case of benzbromarone. Recovery from plasma by chloroform extraction was nearly quantitative: 91.7 ± 5.5% was found for benzarone, 92.0 ± 3.5% for bromobenzarone and 95.4 ± 3.2% for benzbromarone. Similar results were reported by Lücker and Wetzelsberger [4]. Linear and reproducible relationships between benzbromarone/diazepam, bromobenzarone/diazepam and benzarone/diazepam concentrations were obtained over the tested concentration range (Fig. 6).

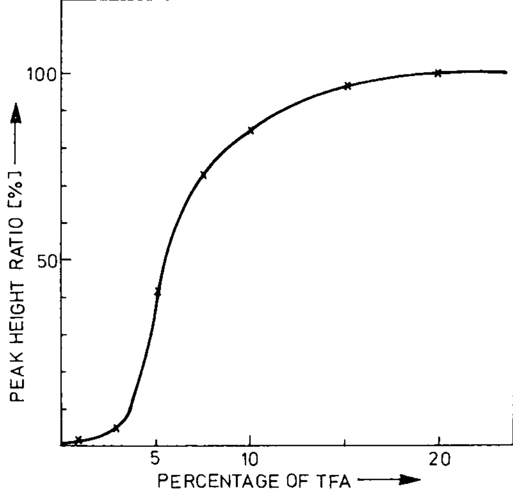

Fig. 5. Peak height ratio of benzbromarone/diazepam as a function of the percentage of TFA.

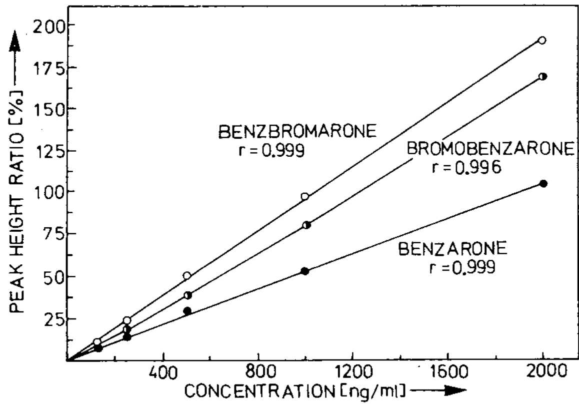

Fig. 6. Calibration curves of benzbromarone, bromobenzarone and benzarone.

The standard deviations at 100 ng benzarone, bromobenzarone and benzbromarone per ml plasma were 10.0, 5.3 and 3.7%, respectively, and at 2 μg per ml plasma were 2.5, 2.4 and 2.9%, respectively. The lowest concentrations that could be measured were 10 ng/ml plasma.

According to the literature data [7] plasma concentrations reach the maximum of 2—3 μg benzbromarone per ml plasma after the ingestion of a 80-mg dose, whilst even higher plasma levels were measured for benzarone. Therefore, the present method allows pharmacokinetic and metabolism studies of benzbromarone after therapeutic oral doses.

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CHROMBIO. 866

Note

Contribution au dosage de quatre antipaludéens, chloroquine, quinine, pyriméthamine et sulfadoxine, seuls et en mélange dans les milieux biologiques

II. Chromatographie en phase gazeuse

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(Reçu le 14 novembre 1980; manuscrit modifié reçu le 16 février 1981)

Devant l'extension de la résistance de diverses souches de *Plasmodium falsiparum*, la thérapeutique antipalustre s'oriente de plus en plus vers l'utilisation de subtances en association: pyriméthamine—sulfadoxine [1—6], pyriméthamine—sulfadoxine—quinine [7], et chloroquine—quinine [1].

Dans un travail déjà publié [8], nous avons étudié les possibilités offertes par la spectrophotométrie UV pour la détermination dans les mileux biologiques de ces quatre molécules. Une méthode utilisant un détecteur thermoionique spécifique azote—phosphore en chromatographie en phase gazeuse est ici proposée, pour l'analyse de ces même substances, pyriméthamine, sulfadoxine, quinine et chloroquine, dans le sang et l'urine.

TECHNIQUES EXPÉRIMENTALES

Appareillage

L'appareil utilisé est un chromatographe Hewlett-Packard type 5710A équipé d'un détecteur thermoionique spécifique azote—phosphore modèle 18789A et d'un enregistreur type W + W recorder 1100.

Conditions chromatographiques

La colonne est constituée d'un tube de verre de 183 cm de long sur 2 mm de diamètre intérieur; elle est remplie avec les phases 2% d'OV-17 sur Chromosorb W AW DMCS 100—120 mesh. Les gaz utilisés sont: gaz vecteur, azote à raison de 30 ml/min; gaz détecteurs: air médical à raison de 60 ml/min et hydrogène à raison de 6 ml/min.

Les températures de l'injecteur et du détecteur sont maintenues respective-

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ment à 250°C et 350°C. La température de four est programmée de 200 à 290°C, à raison de 8°C/min avec un temps initial de 2 min à 200°C et un temps final de 4 min à 290°C.

Réactifs

Nous avons utilisé les solutions tampons Merck (Darmstadt, R.F.A.) à pH 13 et 4.1, du méthanol Prolabo (Paris, France), de l'éther diéthylique Prolabo et du dichloro-1,2-éthylène Prolabo.

Solutions étalons

Les solutions mères sont préparées à raison de 1 mg de chaque substance par ml de méthanol soit 50 mg de chloroquine base (à partir du sulfate de chloroquine fourni par les Laboratoires Specia, Paris, France) à dissoudre dans 50 ml de méthanol, 50 mg de quinine base (à partir du formiate de quinine fourni par les Laboratoires Delacroix, Paris, France) à dissoudre dans 50 ml de méthanol, 50 mg de pyriméthamine (des Laboratoires Roche, Paris, France) à dissoudre dans 50 ml de méthanol, 50 mg de sulfadoxine (des Laboratoires Roche) à dissoudre dans 50 ml de méthanol.

Les solutions d'essai sont préparées par dilution extemporanée des solutions précédentes dans le méthanol.

En ce qui concerne les solutions d'étalon interne, préparer au moment de l'emploi des dilutions à 2 μg/ml de chloroquine ou de pyriméthamine à partir des solutions mères.

Extraction

Urines. (1). Dans un tube à centrifuger en verre de 15 à 20 ml bouchant Téflon, introduire 1 ml d'urine à analyser. Ajuster à pH 12.5 à l'aide de la solution de soude à 60%. Extraire par 8 ml d'éther diéthylique par agitation au Vortex. Centrifuger et séparer phase organique (Fraction a) et phase aqueuse (Fraction b).

(2). Introduire 4 ml de la phase organique (a) dans un tube conique. Ajouter 1 ml de la solution d'étalon interne choisie. Homogénéiser par agitation au Vortex. Evaporer à sec à 45°C sous courant d'azote. Reprendre le résidu d'évaporation par 100 μl de méthanol. Agiter au Vortex. Injecter 4 μl de la solution précédente dans le chromatographe. La quinine. la pyriméthamine et la chloroquine sont dosées dans cette première fraction.

(3). Reprendre la totalité de la phase aqueuse (b). Acidifier à pH 4.1 par addition d'une solution de 1 *N* HCl. Extraire comme précédemment par 8 ml de dichloro-1,2-éthylène. Centrifuger. Recueillir 4 ml de la phase organique dans un tube conique. Ajouter 1 ml de la solution d'étalon interne. Homogénéiser au Vortex. Evaporer à sec à 45°C sous courant d'azote. Reprendre le résidu d'évaporation par 100 μl de méthanol. Homogénéiser et injecter 4 μl dans le chromatographe. La sulfadoxine est alors dosée dans cette fraction.

Sang. Pour libérer les substances de leur combinaison protéique, le sang est soumis à une hydrolyse préalable au protocole d'extraction déjà mentionné. Dans un tube à centrifuger, introduire 1 ml de sang total. Ajouter 1 ml de tampon phosphaté à pH 13.0. Porter au bain-marie bouillant pendant 3 min en agitant de temps en temps. Laisser refroidir et pratiquer l'extraction.

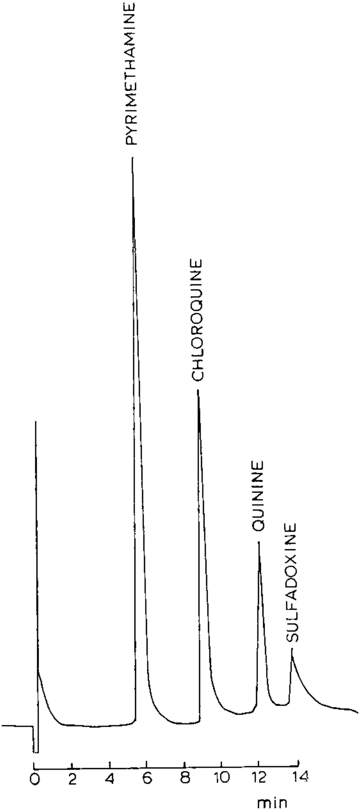

Fig. 1. Chromatogramme obtenu à partir d'un mélange témoin de pyriméthamine, chloroquine, quinine et sulfadoxine.

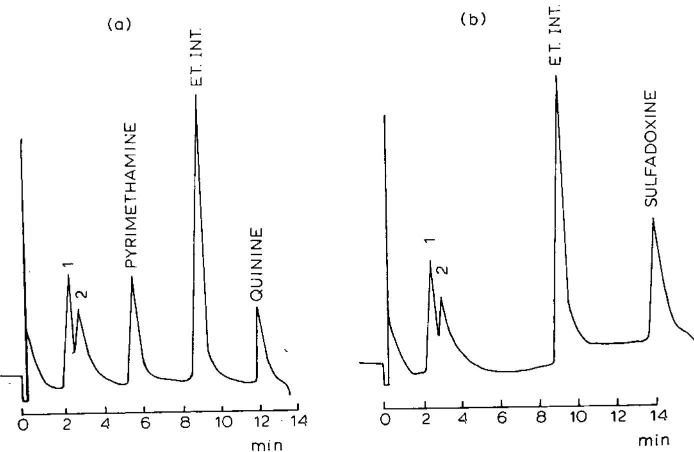

Fig. 2. Chromatogramme d'un mélange pyriméthamine, quinine et sulfadoxine après extraction simulée du sang. Étalon interne: chloroquine. Fraction a: pyriméthamine et quinine. Fraction b: sulfadoxine. 1 et 2: pics parasites provenant du sang.

RESULTATS

Identification

La Fig. 1 montre le chromatogramme obtenu à partir d'un mélange témoin des quatre molécules. Les temps de rétention sont respectivement de 5 min 40 sec, 9 min 00 sec, 12 min 6 sec et 13 min 47 sec pour la pyriméthamine, la chloroquine, la quinine et la sulfadoxine. La Fig. 2 montre les chromatogrammes du mélange pyriméthamine—quinine—sulfadoxine, après extraction du sang.

Courbes d'étalonnage

Les courbes d'étalonnage ont été établies soit à partir de solutions de produit pur dans le méthanol, soit après extraction à partir de l'urine et du sang. La Fig. 3 montre un exemple de graphique d'étalonnage pour la sulfadoxine.

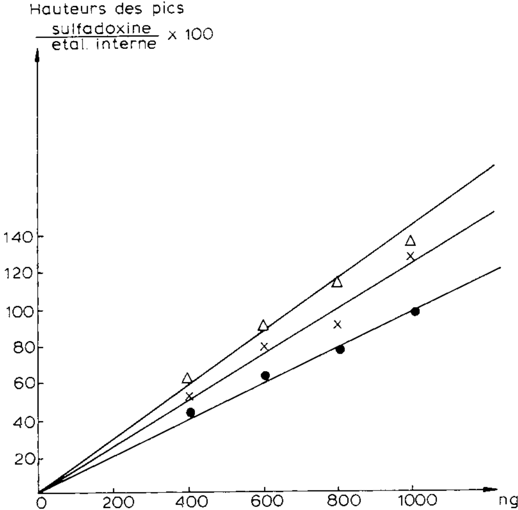

Fig. 3. Courbes d'étalonnage de la sulfadoxine. (△) Solutions pures, (×) après extraction simulée à partir de l'urine, (●) après extraction simulée à partir du sang.

Récupération

L'application de la méthode après extraction d'un mélange contenant 0.75 μg de sulfate de chloroquine, 0.75 μg de pyriméthamine, 1 μg de quinine et 10 μg de sulfadoxine par ml de sang ou d'urine, a permis de déterminer les pourcentages de récupération suivants: chloroquine: urine = 94.5 ± 7.1%, sang = 87.3 ± 6.7%; quinine: urine = 92.8 ± 13%, sang = 84.3 ± 11%; pyriméthamine: urine = 89.5 ± 4.3%, sang = 82.3 ± 8.8%; sulfadoxine: urine = 82.4 ± 4%, sang = 70.2 ± 8.1%.

Reproductibilité

Dans les milieux biologiques, que la substance considérée soit seule ou en mélange, le pourcentage de récupération est toujours le même. Les Figs. 4 et 5 établies à partir des moyennes de dix essais illustrent bien cette propriété.

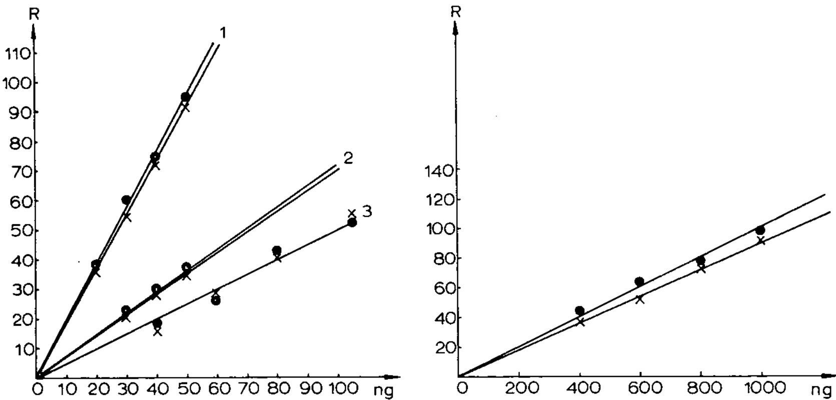

Fig. 4. Courbes d'étalonnage de la pyriméthamine, de la chloroquine et de la quinine en solution pure et en mélange après extraction à partir du sang. 1, Pyriméthamine (• = pure, × = en mélange); 2, chloroquine (• = pure, × = en mélange); 3, quinine (• = pure, × = en mélange). R = rapport des hauteurs des pics produit/étalon interne × 100.

Fig. 5. Courbes d'étalonnage de la sulfadoxine en solution pure et en mélange après extraction à partir du sang. • = pure, × = en mélange. R = rapport des hauteurs des pics produit/étalon interne × 100.

Sensibilité

Les limites de détection de la technique sont respectivement de 0.23 ng, 0.52 ng, 0.191 ng et 2.31 ng pour la chloroquine base, la quinine base, la pyriméthamine et la sulfadoxine.

DISCUSSION

VandenHeuvel et al. [9] et Robinson et al. [10] utilisèrent une phase SE-30 pour la séparation et le dosage des dérivés quinoléiques. Kazyak et Permisohn [11] et Holtzman [12] effectuèrent des études comparatives des phases OV-1, OV-17, QF-1, SE-30 et NGS en ce qui concerne les temps de rétention et la sensibilité de la chloroquine. Evans et al. [13] utilisèrent une phase à 3% d'OV-17, Viala et al. [14] employèrent la même phase sur Gas-Chrom Q (100—120 mesh) pour la détermination de la chloroquine dans les milieux biologiques. L'utilisation d'une colonne à 2% d'OV-17 sur HP Chromosorb W AW DMCS nous a donné les meilleurs résultats.

Considérant les associations médicamenteuses employées en thérapeutique antipaludéenne, on constate que trois types de mélanges sont susceptibles d'être rencontrés dans les milieux biologiques, à savoir pyriméthamine—sulfadoxine [1—6], pyriméthamine—sulfadoxine—quinine [7] et quinine—chloroquine [1]. Partant de ces observations, le choix de l'étalon interne a porté sur l'une des quatre molécules, absente des mélanges considérés, soit la chloroquine pour le premier et le deuxième mélange, soit la pyriméthamine pour le troisième.

Viala et al. [14] préconisèrent pour le dosage de la chloroquine, l'extraction simultanée de la substance à doser et de l'étalon interne. Cette technique donne de bons résultats, toutefois elle ne peut être appliquée au cas des mélanges où la sulfadoxine est présente. Il faudrait alors utilisé deux étalons in-

ternes, l'un extractible en milieu alcalin, l'autre en milieu acide. C'est pourquoi l'étalon interne est ajouté dans la phase terminale avant d'effectuer l'évaporation du solvant. Ce procédé a de plus l'avantage de donner un pic d'étalon interne stable, assurant une bonne reproductibilité à la technique.

Lors de l'étude en spectrophotométrie UV, nous avons mis au point un procédé général d'extraction des quatre substances [8]. Le même principe a été repris ici, les seules modifications apportées concernent la prise d'essai, beaucoup plus faible et les quantités de solvant, plus minimes. Cette prise d'essai peut être encore réduite de moitié lorsque le résidu d'évaporation est repris par 50 μl de méthanol.

La sensibilité est satisfaisante puisque la limite de détection se situe au-dessous du nanogramme pour la chloroquine, la quinine et la pyriméthamine, et de l'ordre de 2 ng pour la sulfadoxine. Cette sensibilité est donc compatible avec les quantités de produits présents dans le sang ou les urines lors d'un contrôle thérapeutique. En effet, les concentrations plasmatiques au cours d'un traitement par les antipaludéens sont de l'ordre de 0.215 mg/l pour la chloroquine [15], 2.5—9.5 mg/l pour la quinine [16], 0.4 ± 0.13 mg/l pour la pyriméthamine [17] et 10.5 ± 2.5 mg/100 ml pour la sulfadoxine [17].

Cala et al. [18] après extraction de la pyriméthamine dans les tissus trouvèrent un taux de récupération de l'ordre de 86 ± 20%. Viala et al. [14] après extraction simulée de la chloroquine dans l'urine, obtinrent un graphique d'étalonnage superposable à celui tracé à partir des solutions pures. Les pourcentages de récupération trouvés par notre procédé d'extraction sont en accord avec ceux obtenus par les autres auteurs à partir de l'un des produits mais présent à l'état unique dans les milieux biologiques. Enfin, cette technique est applicable au dosage de routine puisque le temps moyen mis pour effectuer l'extraction et le dosage est de l'ordre de 30 min.

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Note

Method for the measurement of melphalan in biological samples by high-performance liquid chromatography with fluorescence detection

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The alkylating agent melphalan (Alkeran; L-phenylalanine mustard) (I) was synthesized in the 1950s by Bergel and co-workers [1,2] and has been used clinically as an antitumour agent [3—5] and in the treatment of various neoplastic diseases [6,7].

Very few pharmacokinetic data on melphalan are available, despite the drug having been used for 20 years [8], because, in common with other alkylation agents, it is rapidly hydrolysed [9—11]. The main product of hydrolysis is the dihydroxy compound (II), but the intermediate monohydroxy compound (III) may also be detected as both are also formed metabolically.

NH_2 — CH — CH_2 — C_6H_4 — N(CH_2CH_2Cl)(CH_2CH_2Cl); COOH

(I)

NH_2 — CH — CH_2 — C_6H_4 — N(CH_2CH_2OH)(CH_2CH_2OH); COOH

(II)

NH_2 — CH — CH_2 — C_6H_4 — N(CH_2CH_2OH)(CH_2CH_2Cl); COOH

(III)

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Gas–liquid chromatographic [12] and high-performance liquid chromatographic (HPLC) [13,14] methods for the determination of melphalan have been described, in addition to a spectrophotofluorometric method [15]. This paper describes a sensitive method for measurement by reversed-phase HPLC using fluorescence detection, after separation from the hydrolysis products in rat plasma, liver and kidney.

EXPERIMENTAL

Materials

Methanol was of Distol grade (Fisons Scientific Apparatus, Loughborough, Great Britain). Water was glass-distilled. Melphalan (Wellcome Foundation, Dartford, Great Britain) was obtained in pure form. Standard solutions of melphalan were prepared in methanol and stored at −20°C.

Analytical methods

Extraction. Drug plus metabolites were extracted by a slight modification of published procedures [14]. To plasma or tissue homogenates (up to 3 ml) at 4°C were added two volumes of chilled methanol and the mixture vortex-mixed for 20 sec prior to centrifugation at 1500 *g* for 15 min. The clear methanolic supernatant was removed after centrifugation and the protein was re-suspended using a further volume of methanol. The two solvent extracts were pooled and stored at −20°C until processed further.

Separation of melphalan. The methanolic extract was added to four volumes of ice-cold water to produce a 15% methanol solution and passed through a reversed-phase C_{18} Sep-Pak cartridge (Waters Assoc., Stockport, Great Britain), where the melphalan was adsorbed. The eluent, which contained any dihydroxymelphalan present in the sample, was retained so that this metabolite could be measured later if required. The cartridge was washed with 10 ml of ice-cold 15% methanol and then the melphalan was eluted with 2 ml of methanol (the first 0.4 ml being discarded as the void volume) and stored at −20°C until assayed by HPLC. The fate of any monohydroxymelphalan present was not followed, but it was shown that this compound was eluted with the unretained peaks during the subsequent HPLC assay of melphalan and therefore did not interfere in the measurements.

HPLC assay method. A Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Winnersh, Great Britain) was attached to a Hitachi MPF2A fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, Great Britain). The fluorescence of melphalan was measured in a 20-μl flow cell (Hellma, Westcliff-on-Sea, Great Britain), employing excitation and emission wavelengths of 260 and 350 nm, respectively.

Chromatography was carried out isocratically at 60°C using 40% methanol in water at 3 ml/min as the mobile phase through a 250 × 4.9 mm I.D. stainless-steel column packed with 5-μm Spherisorb S5 ODS (Phase Separations, Queensferry, Great Britain). A run time of 15 min was satisfactory for all extracts.

Calibration method. Because of the instability of melphalan the chromatographic performance was checked by running a standard solution of melphalan before, during and after any run of samples.

RESULTS

Despite its lability at room temperature, melphalan is known to be stable in plasma at −20°C for at least 3 weeks [14]. Further, solutions in methanol were found to retain full integrity for more than 12 h at room temperature and for at least 4 weeks at −20°C.

Peak heights were linearly related to concentration for standard solutions of melphalan over the range 10 ng to 2.5 μg injected on to the column, as were tissue samples spiked at 250 ng/g to 12.5 μg/g (Fig. 1). The lower limit of detection (signal-to-noise ratio = 2) was about 500 pg injected.

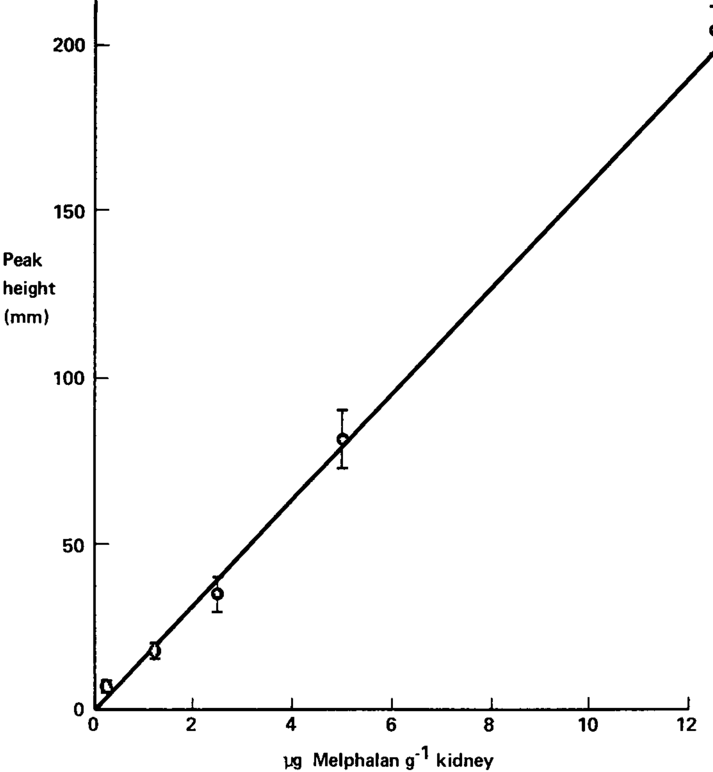

Fig. 1. Melphalan calibration graph for spiked kidney samples. Melphalan added to 3-ml samples of kidney homogenate then treated as described in the text. Final extract assayed by HPLC. For all concentrations n = 4.

Extracts from plasma, kidney and liver were sufficiently free from interfering substances to allow the melphalan to be measured directly. Representative chromatograms are shown in Fig. 2.

Following single oral doses to rats over a range from 0.5 to 5 mg/kg the concentrations of melphalan determined 1 h later were 0.14—1.9 μg/ml in plasma, 0.02—0.2 μg/g in liver and 0.17—4.8 μg/g in kidney.

The coefficient of variation of the extraction and assay of melphalan from

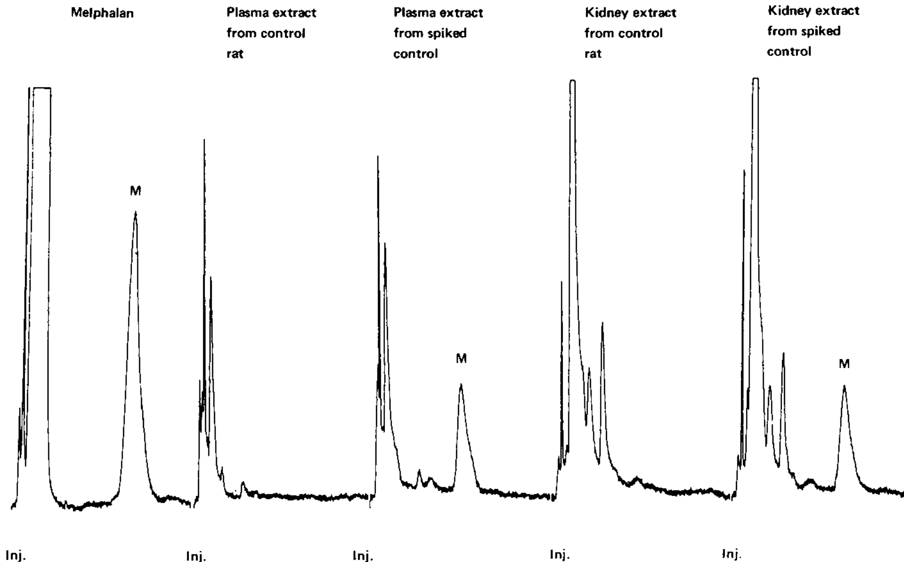

Fig. 2. Chromatograms of melphalan (M) in different samples.

spiked control plasma and tissues was consistently below ± 10%, usually below ± 5%. Standard melphalan solutions in methanol, when measured by HPLC, had coefficients of variation of less than ± 1.5% in all instances. The recovery of melphalan from plasma and tissues was 82% before and 62% after Sep-Pak separation, with a coefficient of variation of less than ± 7%.

DISCUSSION

The separation and measurement of melphalan is hampered by its instability and by the very high solubility of the compound in water. In addition, the amphoteric nature of the molecule makes solvent extraction unsatisfactory, and consequently previously described liquid chromatographic methods [13, 14,16] have depended on precipitation of the protein followed by direct measurement in the supernatant, which inevitably involves dilution of the original biological sample.

Measurements of substances eluted from the HPLC column have been carried out mainly with a UV detector [13,14,16], although Brox et al. [16] additionally used a fluorescence detector, but the advantages of fluorescence detection were not discussed.

In the present method, which depends on adsorption of melphalan on a Sep-Pak C_{18} cartridge and its subsequent elution, the volume of eluate is 1.6 ml regardless of the volume of the original sample, and consequently this step can lead to concentration rather than dilution of the drug. By separating melphalan from the major hydrolysis product before chromatography the drug was easily measured with a simple isocratic system. Dihydroxymelphalan separated by this method was available for subsequent assay without the use

of chromatographic gradients and in addition the background interference due to the biological samples was reduced.

By this means and by exploiting the native fluorescence of the drug, the sensitivity was improved over that of previously reported methods such that the lower limit of detection was well below 5 ng/ml in plasma.

The method, which was applied to rat plasma and tissues in this study, can also be used for human samples.

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CHROMBIO. 863

Note

High-performance liquid chromatographic analysis of a new antiarrhythmic drug, pirmenol, in biological fluids

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Pirmenol hydrochloride (Fig. 1) is a new antiarrhythmic drug [1, 2] that is currently undergoing initial human studies in our institution. In general,

Fig. 1. Structure of pirmenol hydrochloride.

such compounds have a narrow therapeutic index so that early definition of the therapeutic range of plasma concentrations and pharmacokinetics of the drug are of great importance. Initial animal studies [1, 2] reported effective plasma concentrations of the order of 1—3 μg/ml. The method used was a fluorometric dye technique developed by the Drug Metabolism Group at Warner-Lambert/Parke Davis, but no details of the procedure were given.

The present paper describes a rapid, sensitive, selective and accurate high-performance liquid chromatographic (HPLC) method for the measurement of pirmenol in blood plasma and urine.

EXPERIMENTAL

Reagents and materials

Pirmenol (*cis*-(±)-α-[3-(2,6-dimethyl-1-piperidinyl)propyl]-α-phenyl-2-pyridinemethanol monohydrochloride) was kindly supplied by Warner-Lambert/Parke Davis (Ann Arbor, MI, U.S.A.) and the internal standard, disopyramide

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[4-diisopropylamino-2-phenyl-2-(2-pyridyl)butyramide] by Searle Labs. (Chicago, IL, U.S.A.). Stock solutions of pirmenol in methanol and disopyramide in 0.01 *M* phosphoric acid were stored at 4°C for 4 months without detectable decomposition. Ammonium dihydrogen phosphate and triethylamine were purchased from Aldrich Chemical (Milwaukee, WI, U.S.A.). Acetonitrile was of HPLC grade obtained from MC/B-EM (LiChrosolv; Cincinnati, OH, U.S.A.). All other solvents and reagents were of reagent grade.

Sample preparation

Whole blood or plasma (0.5 ml) is placed in a 15-ml capacity culture tube, fitted with a PTFE-lined screw cap and the internal standard (50 μl containing 1 μg of disopyramide), 100 μl of 1 *N* sodium hydroxide and 4 ml of diethyl ether were added. The samples are then immediately extracted using a Labquake automatic shaker for 10 min, followed by centrifugation at 1000 *g* for 5 min, to separate organic and aqueous phases. The lower, aqueous phase is frozen by immersion of the tube in a dry ice—acetone bath and the organic phase transferred to another tube with an elongated cone of about 100 μl capacity. Then 80 μl of 0.1 *N* phosphoric acid are added and the mixture agitated in a Vortex mixer for 40 sec. After a brief centrifugation, 50 μl are sampled from the cone and injected into the chromatograph.

In the case of urine, 500 μl are mixed with 2 ml of 0.1 *N* sodium carbonate (pH 11.23) and 500-μl aliquots extracted in the same way as blood and plasma, except that sodium hydroxide is not added.

Chromatography

The HPLC system consists of a solvent delivery system (Constametric III pump, LDC System, Riviera Beach, FL, U.S.A.) and a 50-μl fixed volume loop injector (Rheodyne 7125, Berkeley, CA, U.S.A.). The analytic column is a reversed-phase Dupont's Zorbax™ TMS (250 × 4.6 mm I.D., particle size 6 μm). The mobile phase is a mixture of acetonitrile—0.05 *M* ammonium dihydrogen phosphate—triethylamine (15:85:0.5, v/v), the pH being adjusted to 2.6 with 1.0 *M* phosphoric acid. The solvent flow-rate is 1.1 ml/min with a column inlet pressure of 1800 p.s.i. The eluate is monitored continuously for absorbance changes at 262 nm using a Spectro-Monitor III (LDC Liquid Chromatogram) and the chromatograms displayed on a Linear Instruments Model 858 dual pen recorder (Irvine, CA, U.S.A.).

Calibration and accuracy

Calibration curves were constructed for each series of unknowns by adding standards of 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 and 3.0 μg of pirmenol and the internal standard (1 μg disopyramide) to control samples of blood and plasma. In the case of urine the standards were 5, 10, 15, 20, 25, 30 and 35 μg of pirmenol. The peak height ratio (PHR) of pirmenol to the internal standards was divided by the amount of pirmenol to derive the normalised PHR. The normalised PHR was used to calculate the amount of pirmenol in the samples and the standard deviation of the normalised PHR used to estimate the accuracy of the method.

The reproducibility of the method was examined by analysing eight replicate plasma samples containing 0.1—2.0 μg/ml pirmenol.

Application of the method

A male patient volunteer with premature ventricular ectopic beats received a 30-min infusion of 150 mg of pirmenol. Samples of venous blood were collected at various intervals for 12 h into heparinised (10 units per ml blood) glass tubes. Aliquots (500 μl) of whole blood were transferred to extraction tubes and stored at −70°C until analysis. The remaining blood was centrifuged to obtain plasma which was also stored at −70°C until analysis. Urine was collected for 24 h and frozen until assay.

RESULTS AND DISCUSSION

The present HPLC method for pirmenol analysis involves an ether extraction after sample alkalinization, followed by back extraction into phosphoric acid and reversed-phase chromatography using a Zorbax TMS column and UV detection. Diethyl ether was chosen as the extraction solvent after an initial comparison with ethyl acetate which resulted in lower and more variable recoveries and methylene chloride which, though efficient, extracted interfering peaks. Back extraction was chosen over evaporation of the organic phase over nitrogen because more reproducible results were obtained. The extraction procedure gave recoveries of 70 ± 1.5, 47 ± 2.9, and 91 ± 3.8% from four samples of plasma, blood and urine, respectively. The Zorbax TMS column was chosen after comparison with a Waters Assoc μBondapak C_{18} column which proved less satisfactory because of tailing.

Chromatograms from control plasma and plasma from the patient after pirmenol administration are shown in Fig. 2. Although our experience is, as yet, limited we have analysed control plasma from patients who were receiving quinidine, digoxin, diazepam, ibuprofen, propranolol, hydrochlorothiazide and indomethacin and found no interfering peaks. However, the chosen internal standard, disopyramide, is also an antiarrhythmic drug, so that its use would not be appropriate were patients to receive this particular drug combination. During the initial investigations of pirmenol, other antiarrhythmic drugs will be avoided, but we are currently exploring pirmenol analogues as potential internal standards. Estimates of the accuracy of the method are shown in Table I. Coefficients of variation averaged 6.7%. The reproducibility of the estimate of pirmenol in plasma was similar at all concentrations studied (Table II) and the coefficient of variation averaged 7.6%.

Application of the method for measuring blood and plasma pirmenol in a patient who received an infusion of the drug is illustrated in Fig. 3. Both concentrations reached a maximum at the end of the infusion and then declined biexponentially, with a terminal half-life of 9 h. Throughout, plasma concentrations were greater than those in blood such that the blood/plasma drug concentration ratio was 0.60 ± 0.06 S.D. in the samples analysed. In this patient, pirmenol concentration in a 24-h urine collection (total volume, 1645 ml) was 25.5 μg/ml. Thus of the 150 mg administered only 42 mg were recovered in the urine. This is consistent with metabolic transformation also being a route of elimination. In this particular patient ventricular ectopic beats disappeared during the infusion and began to return 4 h after drug administration when plasma pirmenol concentrations were 1.06 μg/ml. This

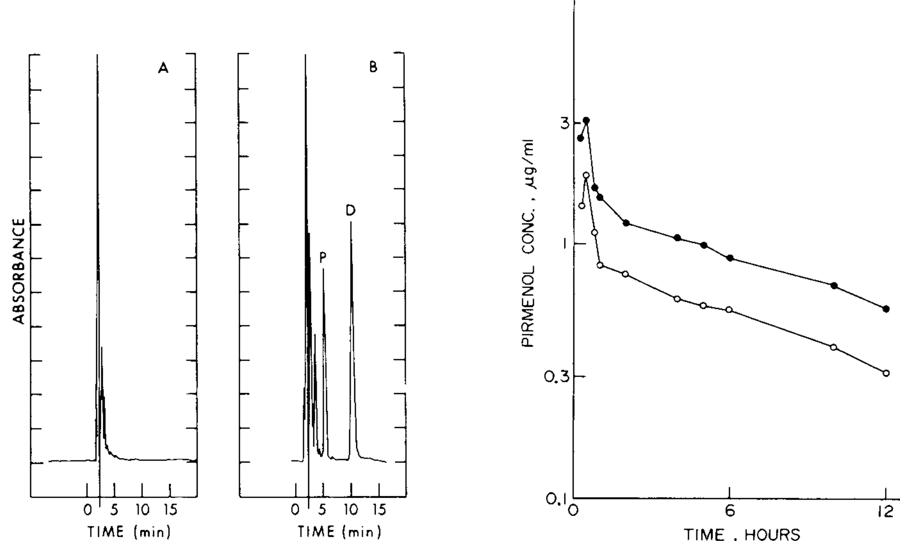

Fig. 2. Chromatograms of (A) control plasma and (B) plasma from a patient who had received pirmenol (P) to which the internal standard disopyramide (D) had been added. Each vertical division represents 0.01 a.u.f.s.

Fig. 3. Semi-logarithmic plot of plasma (•) and blood (○) concentrations of pirmenol during and after a 150-mg infusion of the drug over 30 min.

TABLE I

ESTIMATES OF THE ACCURACY OF THE METHOD

Biological fluid	Concentration range (μg/ml)	No. of studies	Coefficient of variation (%)	
			mean	range
Plasma	0.1—3.0	8	7.4	5.1—9.2
Blood	0.1—3.0	3	6.5	5.8—7.9
Urine	5.0—35.0	2	3.9	2 —5.8
All	0.1—35.0	13	6.7	2 —9.2

TABLE II

REPRODUCIBILITY OF THE METHOD FOR PLASMA

n = 8 in all cases.

Concentration (μg/ml)	NPHR*	Coefficient of variation (%)
0.10	0.91	5.8
0.25	0.93	7.5
0.50	0.99	6.5
1.00	1.01	9.6
1.50	1.03	9.5
2.00	1.08	6.4

*Mean peak height ratio.

is consistent with the effective plasma concentrations in arrhythmias following coronary artery ligation in the dog [1] and in blood superfused Purkinje fibers [2] determined using a fluorometric dye assay.

The presently HPLC method described for the analysis of pirmenol in biological samples is accurate within the putative range of effective concentrations and rapid enough that one person can manually analyse 15—20 samples during an average working day.

ACKNOWLEDGEMENTS

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Note

Simultaneous quantitation of glafenine and its major metabolites, glafenic and hydroxyglafenic acid, by high-performance liquid chromatography

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The metabolism of glafenine, a widely used analgesic drug, has been studied in animals using a radioisotopic technique [1]; known metabolites are glafenic acid, hydroxyglafenine, hydroxyglafenic acid and the N-oxide of glafenic acid, which can be analysed by spectrophotometry [2].

In this paper we describe a high-performance liquid chromatographic (HPLC) method for the separation and determination of glafenine and glafenic and hydroxyglafenic acid.

EXPERIMENTAL

The high-performance liquid chromatography system consisted of a Waters M 6000A solvent delivery pump, a Spherisorb R-Sil C_{18} column (250 × 4.6 mm I.D.) with a particle size of 5 μm and a variable-wavelength spectrophotometer (Schoeffel 6 M 770). A 10-μl sample was injected with a Micromeritics M 725 automatic injector. A Hewlett-Packard 3385A automation system was used for data analysis. Peak areas were measured with an integrating recorder.

Method

The mobile phase was methanol—water—acetic acid (67:23:10) to which 0.3% of 0.91 ammonia solution was added to give a pH of 4.3. Before use, the eluent was degased by sonication for 15 min. The chromatographic system was operated at room temperature with an eluent flow-rate of 0.5 ml/min. As the UV maxima of glafenic acid (364 nm), hydroxyglafenic acid (369 nm) and glafenine (362 nm) are very close, the wavelength of the detector was set at 360 nm.

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Standard solutions

As glafenic acid and especially hydroxyglafenic acid are soluble only at highly alkaline or acidic pH, all solutions were prepared by dissolving 5 mg of either glafenine, glafenic acid, hydroxyglafenic acid or floctafenic acid (internal standard) in 100 ml of methanol—ammonia solution (99.5 : 0.5) (pH 10.5) and were stored at 4°C.

Extraction procedure

To 1 ml of plasma sample were added 10 μg of floctafenic acid as an internal standard and 5 ml of the methanol—ammonia solution (99.5 : 0.5). After vigorous shaking with a reciprocal shaker and centrifugation for 15 min at 3000 *g*, the supernatant was transferred into a cone-shaped tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 1 ml of mobile phase and, after mixing on a Cyclomixer, the sample was filtered on a 0.2-μm Fluoropore filter. Standard samples were prepared from blank plasma spiked with 10 μg of glafenine, glafenic acid and hydroxyglafenic acid and were analysed together with unknown samples.

Fig. 1 shows typical HPLC elution patterns for blank plasma samples spiked either with 10 μg of floctafenic acid (A) or 10 μg of floctafenic acid and 10 μg of each of the three compounds (B), and for a plasma sample obtained from a subject 1 h after oral administration of 400 mg of Glifanan (C) spiked with 10 μg of floctafenic acid.

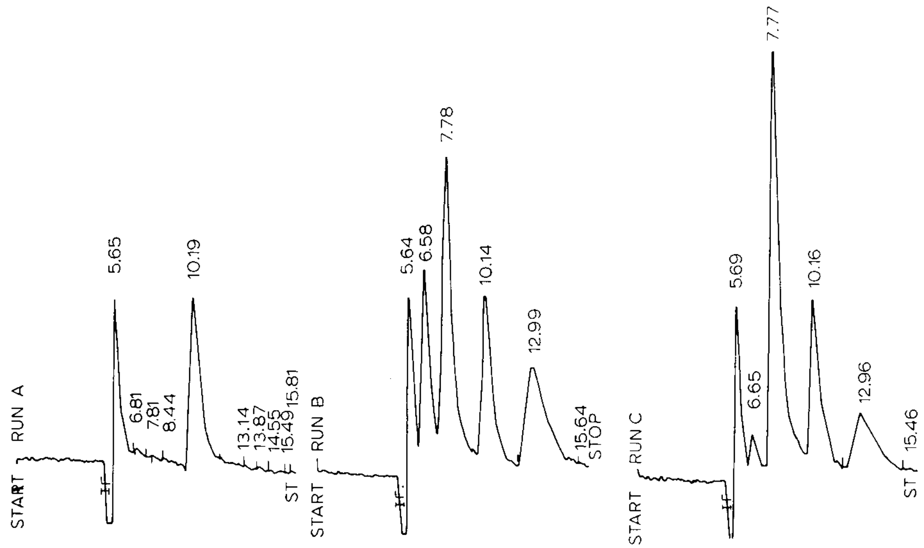

Fig. 1. HPLC elution patterns. (A) Blank plasma spiked with 10 μg of floctafenic acid (retention time = 10.19 min). (B) Blank plasma spiked with 10 μg each of floctafenic acid (retention time = 10.14 min), hydroxyglafenic acid (6.58 min), glafenic acid (7.78 min) and glafenine (12.99 min). (C) Plasma sample obtained from one volunteer subject 1 h after oral administration of 400 mg of Glifanan tablets spiked with 10 μg of floctafenic acid.

RESULTS AND DISCUSSION

The structures of glafenine and its metabolites are shown in the metabolic pathway established in the rat (Fig. 2) [1]. The excretion patterns in rat and human urine are very similar, indicating that the metabolic pathways should be similar in the two species. With HPLC the separation of the different compounds was accomplished in less than 16 min.

The correlation coefficients (r = 0.999, 0.998 and 0.996 for glafenine, glafenic acid and hydroxyglafenic acid, respectively) show a linear relationship between the peak area and the amounts of the compounds present in each plasma injection. The standard deviations for each compound are given in Table I.

The detection limits were 0.2 μg/ml for glafenic acid and 0.5 μg/ml for glafenine and hydroxyglafenic acid, with a signal-to-noise ratio of 2:1. The overall accuracy of the assay (Table II) was calculated from two series of experiments on the determination of six samples corresponding to 1 ml of plasma spiked with (a) 10 μg each of glafenine, glafenic acid and hydroxyglafenic acid, and 5 μg of the internal standard, and (b) 5 μg of each of the three compounds and 5 μg of the internal standard.

To test the validity of the method, 400 mg of glafenine were given orally

Fig. 2. Metabolic pathway of glafenine (I) in the rat (excluding conjugation), according to Pottier et al. [1].

TABLE I

DETERMINATION OF GLAFENINE, GLAFENIC ACID AND HYDROXYGLAFENIC ACID (n = 5)

Results are means ± standard deviations, with coefficients of variation (C.V.).

Concentration (μg/ml)	Peak area (mV)		
	Glafenine	Glafenic acid	Hydroxyglafenic acid
20	320,039 ± 24,985 C.V. = 7.8%	340,745 ± 20,290 C.V. = 5.5%	179,316 ± 8,053 C.V. = 4.7%
10	147,258 ± 6,416 C.V. = 4.3%	158,506 ± 4,966 C.V. = 3.2%	105,409 ± 2,416 C.V. = 2.2%
5	72,247 ± 3,008 C.V. = 4.1%	63,375 ± 4,078 C.V. = 5.8%	50,565 ± 1,643 C.V. = 3.2%
2.5	30,338 ± 2,596 C.V. = 8.5%	30,491 ± 3,595 C.V. = 11.7%	35,637 ± 2,016 C.V. = 5.6%

to one healthy volunteer in the form of two tablets of Glifanan. Blood samples were collected at various times and the plasma was stored at 4°C or frozen until taken for analysis. Plasma levels versus time elapsed after administration are illustrated in Fig. 3. When samples were kept at room temperature, glafenine could not be detected because of hydrolysis to glafenic acid by a plasma esterase.

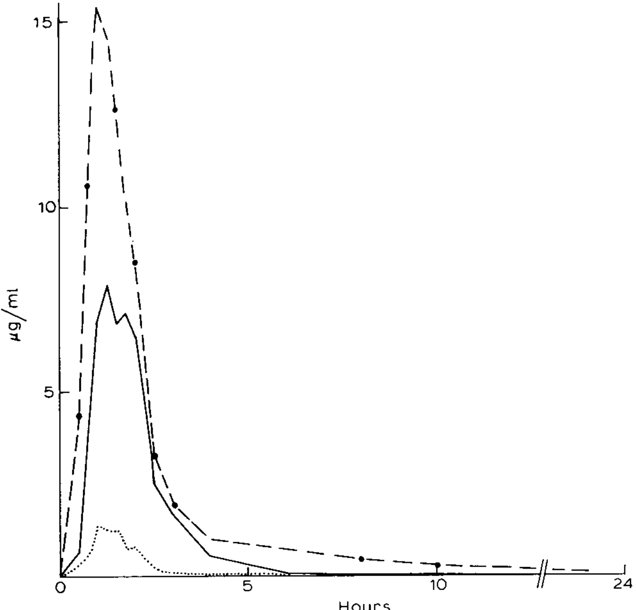

Fig. 3. Plasma levels of glafenine (——), glafenic acid (— — —) and hydroxyglafenic acid (. . . .) after oral administration of 400 mg of glafenine as two tablets of Glifanan.

TABLE II

OVERALL EXTRACTION RECOVERIES OF GLAFENINE, GLAFENIC ACID AND HYDROXYGLAFENIC ACID ($n = 6$)

Amount added to plasma (μg)	Recovery (mean ± S.D.) (%)			Coefficient of variation		
	Glafenine	Glafenic acid	Hydroxyglafenic acid	Glafenine	Glafenic acid	Hydroxyglafenic acid
10	106 ± 8.1	90.8 ± 2.4	89.7 ± 3.8	7.6	2.6	4.3
5	115 ± 7.9	100.7 ± 4.0	84.2 ± 6.3	6.8	4	7.4

The HPLC method described is rapid and reliable and has several advantages over spectrophotometry. It is more specific and it allows the simultaneous quantitative determination of glafenine and its two major metabolites, glafenic acid and hydroxyglafenic acid, in less than 16 min. Moreover, derivatization is not necessary.

ACKNOWLEDGEMENTS

The authors thank Roussel-Uclaf for the gift of pure glafenine, glafenic acid, hydroxyglafenic acid and floctafenic acid.

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CHROMBIO. 876

Book Review

Recent developments in mass spectrometry in biochemistry and medicine, Vol. 2, edited by A. Frigerio, Plenum, New York, London, 1979, x + 492 pp., price US$ 45.00, ISBN 0-306-40294-7.

Volume 2 of "Recent Developments in Mass Spectrometry in Biochemistry and Medicine", edited by Alberto Frigerio (Milan), contains, in about half of its space, a comprehensive series of articles devoted to pharmaceutical drug analysis, most being concerned with drug metabolism. A Foreword by A. Benakis (Geneva) stresses the growing importance of mass spectrometry alone or coupled with gas—liquid chromatography (GC—MS) and emphasizes the quantitative analysis that mass fragmentography provides. Better knowledge of the pharmacokinetics and the pharmaco-availability of drugs is linked to the development of GC—MS methods and the synthesis of radioactive and stable isotope-labelled drugs with a high isotopic abundance. Nevertheless, GC—MS cannot ensure good results without adequate acquaintance with the methods and analyst competence. Twelve drugs are therefore used as examples for the description of excellent methods which demonstrate clearly that structure identification and the determination of metabolic pathways are closely linked. Owing to the large variety of drugs these papers constitute almost a handbook of drug analysis. In addition, two carcinogenic substances and their metabolites have been analysed by GC—MS: 2-acetylaminofluorene and benzo[*a*]pyrene.

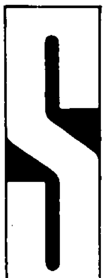
The second part of the book contains twelve articles dealing with the biochemistry and the clinical chemistry of hormones, peptides and substrates such as polyamines, bile acids, organic acids, biogenic amines, prostaglandins, steroids, amino acids and tripeptides. Again the reader will find the most advanced methodologies. They demonstrate clearly that efficient biological surveys and evaluations rely on the excellence of the separation and the quantitative assay of each compound.

Then follows an important aspect of the use of mass spectrometry in physiology, the continuous analysis of expired gases from the pulmonary tract or of blood gas, oxygen and carbon dioxide in the vascular circulation through a catheter interfaced directly to the mass spectrometer.

The two halves of this book are separated by an important article on the use of negative ion mass spectrometry with the combination of electron impact and chemical ionization in forensic science. Going beyond its initial purpose, this article opens up a large field of application in pharmacology and clinical chemistry.

This contribution to the well known annual series edited by Alberto Frigerio is an excellent reference book with comprehensive information for chemists, biochemists, pharmacologists and toxicologists who need the latest methodological developments, and it widens the scope of the mass spectrometer, the last resort for unsolved bioanalytical problems. For this reason, it is regrettable that the use of this book as a reference source is partially lost as no articles have been cited by their titles. On this occasion, we strongly deplore that biochemists and chemists are the last writers in the biosciences who continue to quote references by authors and journals only, omitting the most interesting part, the title.

Dijon (France) P. PADIEU



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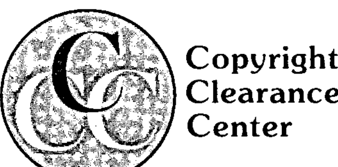

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