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HIGH-RESOLUTION GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF THE METHYL ESTERS OF ORGANIC ACIDS FROM UREMIC HEMOFILTRATES

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(Received October 7th, 1980)

SUMMARY

The organic acid fraction of hemofiltrates was investigated in the form of methylates by glass capillary gas chromatography—mass spectrometry. The pattern obtained is similar to that of urinary organic acid methylates from healthy individuals. A marked difference was noted for N-phenylacetyl-α-aminoglutarimide, present in hemofiltrate at levels 50—100 times higher than those in urine. Analysis of hemofiltrate samples taken at different times during a hemofiltration with post-dilution technique revealed that the hemofiltrate concentration of most compounds was drastically reduced during the course of the hemofiltration treatment. Compared to the other compounds, the reduction in hemofiltrate concentration of N-phenylacetyl-α-aminoglutarimide was extremely rapid.

INTRODUCTION

Persons whose renal system has failed or is malfunctioning often accumulate high levels of metabolites and toxins in their blood which would normally be excreted in the urine [1]. The symptoms brought on by these high toxin and metabolite concentrations, termed uremia, are relieved by cleansing the blood by an extracorporeal ultrafiltration process [2].

Although the analysis of the hemofiltrate is difficult due to low metabolite and relatively high electrolyte concentrations, it is plentiful and easy to obtain. Its analysis could provide added insight into uremia. Senftleber et al. [3] reported a reversed-phase liquid chromatographic method for the analysis of unfractionated hemodialysis fluid. Identification of 23 compounds was achieved through comparison with standard mixtures. Veening and his group [4] then combined the detection capabilities of a mass spectrometer with a packed-column gas chromatograph to study acidic and neutral compounds separated from hemodialysates. Twelve compounds were resolved; six of these

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were identified through mass spectral data. Niwa et al. [5] used trimethylsilane derivatives for analysing the organic acid fraction of hemofiltrates using glass capillary gas chromatography—mass spectrometry (GC—MS) [6,7] and were able to detect close to 30 different compounds. These mainly consisted of phenol [5], and phenolic, dicarboxylic, and "sugar" acids.

The high-resolution GC—MS investigation of the methyl ester derivatives prepared from this same organic acid fraction is described in this paper. They yield a totally different and complementary profile. "Sugar" acids and many other such compounds of high polarity do not pass through the GC column as methylates. This disadvantage is compensated for by the fact that many other important compounds (aromatic and dicarboxylic acids, for example) in lower concentrations are no longer masked and are rendered detectable.

This allows a comparison of urinary acid patterns obtained from healthy individuals with acid patterns in hemofiltrates. In addition the change in the concentration of acids in the hemofiltrate during the course of a filtration can be studied.

EXPERIMENTAL

Patients

All patients (3 men and 2 women) were hospitalized in the Nephrological Department of the University Clinic in Göttingen, G.F.R. They suffered from chronic renal failure and had little or no kidney function. The patients (aged between 30 and 62 years) had undergone hemofiltration three times a week by means of a Hämoprocessor (Fresenius) for 4 months to 3 years before the sampling date. Medication was typical for chronic uremia.

Dialysate samples

Since the ultrafiltrate often contained blood at the beginning of the hemofiltration sampling could only begin approximately 1 h after the start of the procedure; 0.5—1-l samples were taken.

To study the change of the acid concentration in the hemofiltrate during a treatment, samples were collected at approximately 1 h, 4 h, and 7 h after the beginning of the process.

Urine samples

After a 12-h fast, 100-ml aliquots of morning urine samples were taken for comparison from two healthy males and two healthy females (aged between 22 and 34) working in the laboratories of this department. All samples were stored without preservative at −20°C until needed.

Reagents and materials

Extrelut® columns were obtained from Merck, Darmstadt, G.F.R. Three to four per cent diazomethane solution in diethyl ether was prepared regularly in our laboratories and stored at −20°C. All solvents were at least pro analysis grade and were also obtained from Merck. Deionized water was used.

Sample preparation

A 200-ml volume of hemofiltrate was measured into a 1-l flask and freeze-dried overnight or until nearly dry (in the case of urine, a 20-ml sample was diluted to 200 ml with deionized water). The residue was taken up in 19 ml of deionized water. Then 200 μg of 4-phenylbutyric acid standard in 1 ml of water were added and the pH was adjusted to 1 with 6 N H_2SO_4. The aqueous solution was then poured into an Extrelut® column. After absorption on to the column (ca. 15 min), the sample was eluted with 70 ml of acetic acid ethyl ester in three portions. Each portion was used to rinse out the 1-l flask. After evaporation to near dryness (Rotovap), the sample was taken up in ca. 1 ml of methanol, cooled on ice, and reacted with fresh CH_2N_2 solution until the characteristic yellow diazomethane color persisted as described by Spiteller and Spiteller [8]. A stream of dry nitrogen was used to remove excess diazomethane and to concentrate the sample to a volume below 0.2 ml (actual volume depended on the acid concentration in the sample). Care was taken not to allow the sample to go dry under the nitrogen stream as it was observed that many of the more volatile esters were then lost. Benzene or tetrahydrofuran containing a trace of methanol was used as the final solvent; 0.8—1.2 μl were injected into the gas chromatograph.

Gas chromatography

Gas chromatograms were taken on a Carlo Erba Model 2900 equipped with a flame-ionization detector. The column was a 30-m open tubular glass capillary (0.3 mm I.D.) wall-coated with OV-101. Hydrogen carrier gas pressure was 0.6 kg/cm^2. Temperature program was 80°C isothermal for 7 min then 2°C/min to 275°C. Detector temperature was 280°C while the injection port was kept at 260°C. Split ratio was 1:20. Peak area integration was performed by an Autolab System 1 computing integrator from Spectra Physics.

Kovats' retention indices were determined using a standard mixture of even-carbon-number hydrocarbons from C8 to C26.

Gas chromatography—mass spectrometry

GC—MS work was performed on an LKB 2091 with separate oil diffusion pumps for inlet and source. The ion source temperature was 250°C, the electron energy 70 eV, acceleration voltage 3.5 kV, and the TIC signal registered at 20 eV. The gas chromatograph—mass spectrometer separator was a two-step molecular jet separator (Becker-Ryhage), temperature 250°C. The chromatograph was a Pye-Unicam one-column instrument. The column and temperature program were identical to those listed above. Data collection was accomplished by an LKB 2030, PDP-11 data system.

Normalization of data

The total quantities of organic acids in the various samples varied widely. In an attempt to compare profiles of hemofiltrate with those of urine from normal subjects, the integrated gas chromatogram areas were subjected to a normalization process suggested by Gates et al. [9], similar to that used by Dirren et al. [10]. As reported by Gates et al., the normalized area A_{ij}* of the ith component in the jth sample is calculated from the uncorrected area A_{ij} by

$$A_{ij}^* = \frac{A_{ij} \times 10^2}{n' A_{ij}}$$

where the summation is for all GC peaks except for major, less-reliable, or poorly resolved components. The factor of 10^2 in the numerator is a slight modification of the procedure of Gates et al. in which a factor of 10^4 was used.

RESULTS AND DISCUSSION

Reproducibility

Reproducibility was seen to be better and analysis time faster using Extrelut® column extraction than using conventional techniques, confirming a previous report to this effect [11]. The ratio of the peak area of each identified peak to the standard peak area in a triplicate analysis of the same sample gave values whose standard deviation was usually less than 10% of their mean. Exceptions were noted for very poorly resolved peaks where integration error was significant.

Recoveries

Recoveries of acids were checked with an "artificial urine" standard mixture. Since each sample had to be subjected to freeze-drying, appreciable amounts of compounds of higher volatility were lost. This resulted in poor recoveries for compounds of volatility equal to or higher than that of benzoic acid. Due to absorption on the Extrelut® column, the recoveries of extremely polar compounds such as citric acid were also very poor, ranging much lower than 50% after correction for detector response. An XAD-4 extraction procedure followed by ion exchange (described in ref. 8) yielded much higher recoveries of citric acid and other polar compounds. Yet this procedure was not suitable for analysis of hemofiltrates due to the co-extraction of large amounts of "sugar" acids which are not chromatographed as methylates, resulting in rapid column deterioration.

Quantification

The reproduced gas chromatograms do not give a picture of the absolute quantities of the indicated acids due to different flame-ionization detector response factors for each individual compound. Yet absolute quantitative measurements do not seem necessary to us since the absolute amounts of metabolites are dependent on individual factors such as size, etc., while large relative changes in metabolite patterns are of diagnostic significance [12]. These changes can be recognized from GC data, independent of detector response.

Comparison of urinary and hemofiltrate acid patterns

In Fig. 1 the gas chromatogram of the methylated organic acid fraction from hemodialysate obtained during the dialysis procedure of a 52-year-old uremic woman is represented. Peak numbers correspond to compounds whose GC and

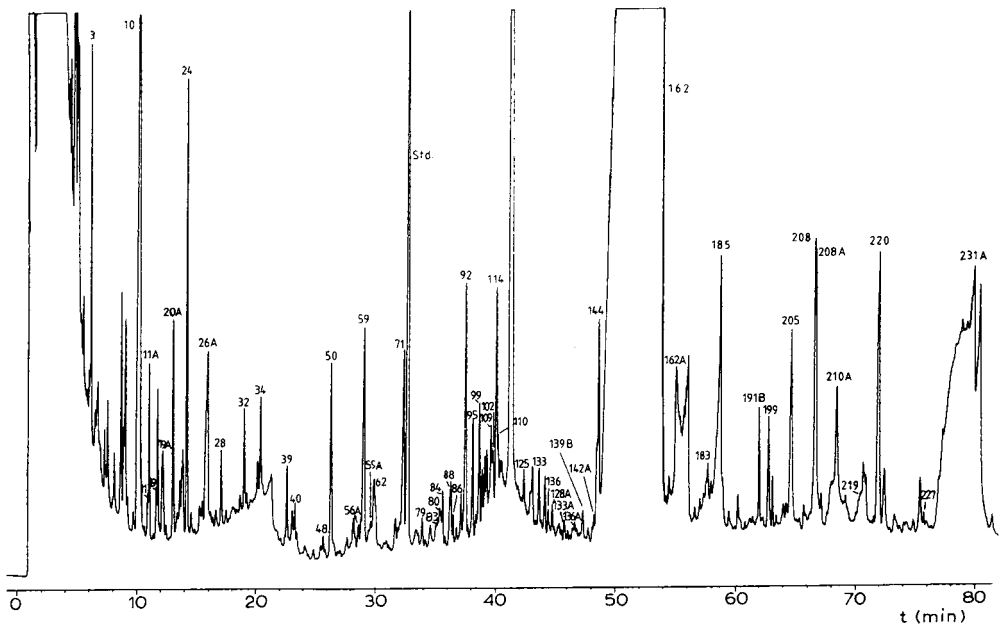


Fig. 1. Glass capillary GC profile of the organic acid methylates from hemofiltrate obtained during the hemofiltration procedure of a 52-year-old uremic woman.

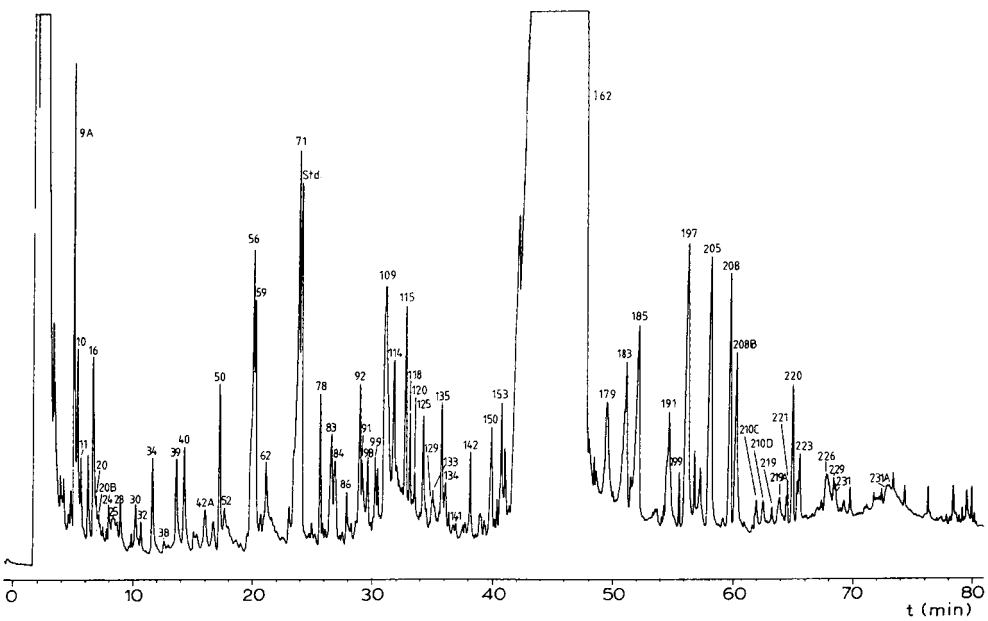


Fig. 2. Glass capillary GC profile of the organic acid methylates from urine obtained from a 33-year-old healthy woman.

TABLE I

MASS SPECTROMETRIC DATA FOR UNKNOWN COMPOUNDS

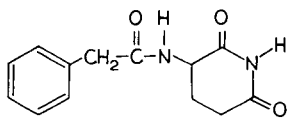
Compound number*	R.I.**	Mass spectrometric data***
UK 1	1014	139(72%), 137(80), 123(6), 121(7), 103(41), 93(6), 87(11), 75(5), 69(7), 59(23), 57(86), 55(38), 43(100), 41(15)
UK 2	1047	129(2%), 128(2), 103(4), 101(27), 85(3), 69(6), 59(12), 58(5), 45(4), 43(100), 41(7)
UK 3	1050	143(4%), 125(9), 115(89), 108(6), 100(7), 99(9), 85(24), 83(17), 81(10), 73(13), 69(10), 67(8), 59(24), 57(32), 55(25), 45(84), 43(100), 41(53), 39(25)
UK 4	1109	89(18%), 88(24), 61(3), 59(10), 57(7), 45(100), 43(13)
UK 5	1580	211(23%), 193(11), 182(10), 169(16), 155(18), 150(27), 137(30), 123(16), 115(17), 109(45), 108(28), 95(45), 91(84), 81(100), 79(30), 67(50), 59(32), 55(32), 45(28), 41(24), 39(21)
UK 6	1596	223(55%), 208(8), 192(100), 191(33), 182(8), 165(15), 164(38), 155(16), 151(8), 132(9), 123(23), 120(25), 105(9), 104(10), 94(10), 81(23), 77(24), 67(15), 63(14), 59(30), 53(12), 43(28), 41(32)
UK 7	1722	216(63%), 201(10), 175(10), 174(100), 173(25), 156(4), 145(5), 129(10), 118(6), 104(9), 91(16), 77(48), 64(9), 55(11), 51(8), 43(27), 39(17)
UK 8	2049	223(7%), 196(6), 179(6), 165(6), 145(29), 143(73), 113(27), 111(68), 101(100), 87(23), 85(25), 81(21), 74(15), 71(17), 69(16), 67(15), 59(56), 55(50), 43(32), 41(23), 29(24), 18(29), 15(13)
UK 9	2058	215(5%), 183(5), 165(6), 145(25), 144(17), 143(100), 116(11), 113(29), 111(83), 101(62), 83(30), 73(10), 69(19), 67(23), 59(28), 57(12), 55(40), 43(11), 41(18), 29(15), 15(23)

*Compound numbers refer to compounds listed in Table II.
**Kovats' retention indices.
***See text for MS parameters.

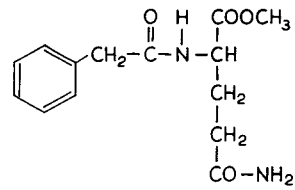
MS data have been reported in a previous publication [8]. MS data for compounds whose number is followed by a letter are given in ref. 13. Unknown compound peaks are marked UK followed by a number. The MS data for these compounds are listed in Table I. To allow a visual comparison of this hemofiltrate acid pattern with that of a typical urine the gas chromatogram of the methylated urinary acid fraction obtained from a healthy 33-year-old woman is reproduced in Fig. 2.

Table II lists the compounds found in the samples along with the minimum, median, and maximum values of their normalized areas. Mean values and standard deviations were not calculated for each compound due to the low number of samples (n = 4 for urine and n = 5 for hemofiltrate). The normalization process used allows comparison of the acid patterns between different groups of samples. Due to the wide range of values found for the normal urines

(further confirming previous reports to this effect [14]) and to the semi-quantitative methods used, only large differences (a factor of five or more is suggested in ref. 15) can be considered as significant. These points taken into consideration, the hemofiltrate acid pattern was quite similar to that of the urinary acids. Values for hippuric acid (number 162, Table II), for example, were quite close to one another. Perhaps the only significant difference was noted for N-phenylacetyl-α-aminoglutarimide (231A, Table II) which is formed



N-phenylacetyl-α-aminoglutarimide



phenylacetylglutamine

by heat-induced ring closure of the corresponding glutamine conjugate in the injection port of the gas chromatograph [13,16]. Normalized areas for this compound reached values two orders of magnitude higher in ultrafiltrate than in urine and were consistently at least 50 times greater. The glutamine conjugate of phenylacetic acid has been discussed in connection with a number of diseases [16].

Slightly elevated values were repeatedly noted in hemofiltrate for many unsaturated aliphatic acids (see compounds 34, 39, 83A, 153 and 191B in Table II), while correspondingly lower values were found in hemofiltrate for certain saturated aliphatic acids (see compounds 40, 86 and 125). These differences were so slight in the light of the statements made above that definite conclusions can not be drawn.

Consistently greater values for the normalized area of the glutamic acid conjugate of phenylacetic acid (compound 220, Table II) in hemofiltrate were observed.

Slightly lower values for certain methoxy ring substituted aromatic acids in hemofiltrate (see compound 102) and correspondingly lower values for a few phenolic acids in urine (see compound 83) cannot be deemed significant due to the derivatization procedure used where phenols run the risk of being non-quantitatively methylated.

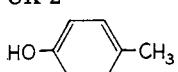
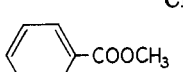
Progressive sampling during hemofiltration treatment

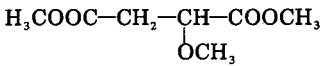
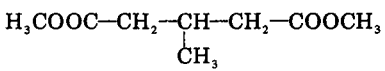
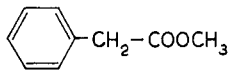
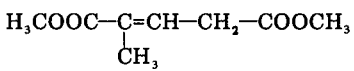
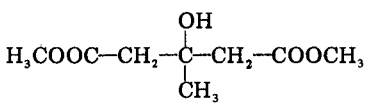
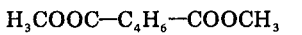
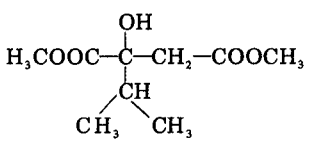
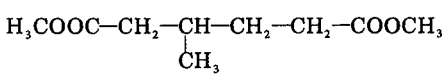
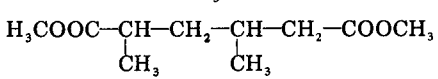
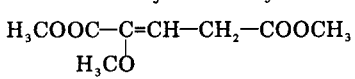
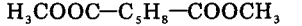
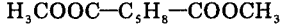
The much higher values of the glutamic acid conjugate and the glutamine conjugate of phenylacetic acid in hemofiltrate compared to the urine raised the question of the behaviour of these and other compounds during hemofiltration treatment.

Fig. 3a—c show the gas chromatograms of the methylated organic acids in hemofiltrate samples taken at different times (1, 4, and 7 h after the beginning) during a routine 8-h hemofiltration of a 62-year-old male uremic patient. Peak numbers again refer to the compounds listed in Table II and in the literature cited above. The ratios of individual peak areas to that of the internal standard

TABLE II

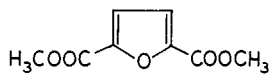
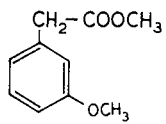
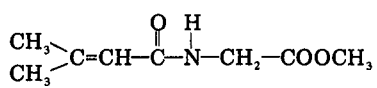
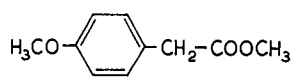
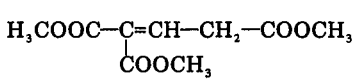
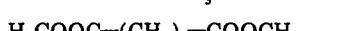
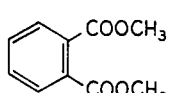
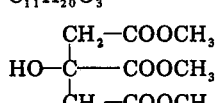
COMPOUNDS FOUND IN THE SAMPLES AND THEIR NORMALIZED PEAK AREAS

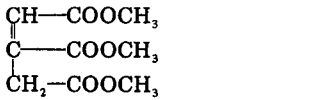
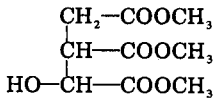
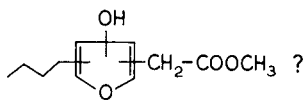
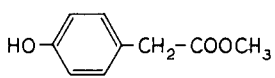
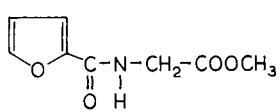
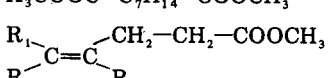
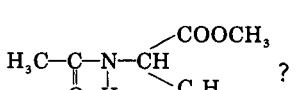
Compound number*	Structural formula** (or partial)	R.I.***	Normalized peak areas§					
			Hemofiltrate ($n=5$)			Urine ($n=4$)		
			Minimum	Median	Maximum	Minimum	Median	Maximum
10	$H_3COOC-CH_2-CH_2-COOCH_3$	996	0.97	2.61	20.6	2.00	4.29	10.0
11	$H_3COOC-\underset{\displaystyle CH_2-CH_3}{\underset{\vert}{CH}}-COOCH_3$	1011	N.D.	0.44	4.83	1.11	3.34	8.04
11A	UK 1	1014	N.D.	N.D.	2.10	N.D.	N.D.	N.D.
16	$H_3COOC-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-COOCH_3$	1035	N.D.	N.D.	2.85	N.D.	0.93	4.31
19	$C_4H_9-\underset{\displaystyle OH}{\underset{\vert}{CH}}-CH_2-COOCH_3$	1047	N.D.	N.D.	1.04	N.D.	N.D.	N.D.
19A	UK 2	1047	N.D.	1.20	2.22	N.D.	N.D.	N.D.
20		1048	N.D.	N.D.	2.09	N.D.	N.D.	0.66
20A	UK 3	1050	N.D.	0.68	2.99	N.D.	N.D.	N.D.
20B	$H_3COOC-CH=\underset{\displaystyle CH_3}{\underset{\vert}{C}}-COOCH_3$	1055	N.D.	N.D.	1.53	N.D.	N.D.	N.D.
24		1072	N.D.	6.76	41.9	N.D.	0.65	1.65
25	$H_3COOC-\underset{\displaystyle CH_3}{\underset{\vert}{\underset{\displaystyle OH}{\underset{\vert}{C}}}}-CH_2-COOCH_3$	1087	N.D.	N.D.	2.97	N.D.	N.D.	N.D.
26A	UK 4	1109	0.52	2.22	5.88	N.D.	N.D.	N.D.

28		1113	N.D.	N.D.	1.15	N.D.	1.06	3.29
30		1137	N.D.	N.D.	1.05	N.D.	0.80	1.28
32		1149	0.76	1.36	3.84	N.D.	0.87	1.03
34		1163	0.98	5.75	10.5	N.D.	1.95	2.36
38		1191	N.D.	N.D.	2.98	N.D.	N.D.	1.98
39		1195	1.31	3.44	7.55	N.D.	1.19	1.85
40§§		1206	0.50	1.87	2.11	1.97	3.05	32.5
48		1247	N.D.	0.40	1.56	N.D.	N.D.	N.D.
50		1253	3.81	4.59	12.1	3.37	4.52	7.59
52		1272	N.D.	N.D.	N.D.	N.D.	0.42	0.91
56§§		1291	N.D.	N.D.	N.D.	N.D.	1.85	11.9
56A		1292	N.D.	1.47	5.28	N.D.	N.D.	2.18
59		1298	4.82	4.98	8.41	4.09	5.95	6.78
59A		1305	N.D.	N.D.	5.17	N.D.	0.47	1.04
62§§		1309	N.D.	1.61	5.29	N.D.	1.56	9.67

(Continued on p. 10)

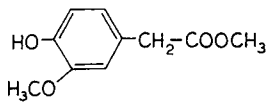
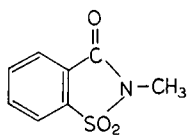
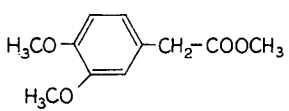
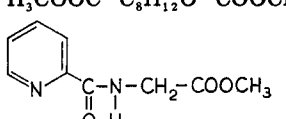
TABLE II (continued)

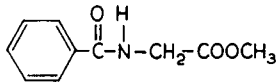
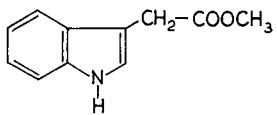
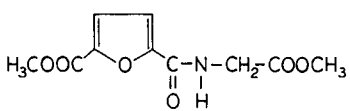
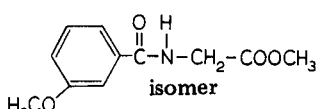
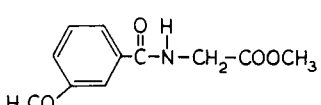
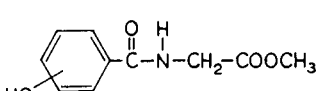
Compound number*	Structural formula** (or partial)	R.I.***	Normalized peak areas§					
			Hemofiltrate ($n=5$)			Urine ($n=4$)		
			Minimum	Median	Maximum	Minimum	Median	Maximum
71§§		1346	4.48	10.8	24.1	N.D.	6.19	16.8
78		1380	N.D.	N.D.	N.D.	N.D.	0.91	2.98
80		1386	N.D.	N.D.	0.62	N.D.	N.D.	3.82
83		1393	N.D.	N.D.	2.71	3.13	3.36	4.98
83A		1395	N.D.	7.24	26.9	N.D.	N.D.	N.D.
86§§		1410	N.D.	N.D.	1.70	N.D.	1.08	12.2
88		1414	1.07	1.30	2.06	N.D.	N.D.	N.D.
89		1416	N.D.	N.D.	3.55	N.D.	N.D.	N.D.
91		1424	N.D.	N.D.	N.D.	N.D.	1.47	4.56

92		1428	N.D.	1.98	5.09	N.D.	0.96	4.31
94		1435	N.D.	N.D.	N.D.	N.D.	0.90	3.97
95	$H_3COOC-C_8H_{16}-COOCH_3$	1435	N.D.	N.D.	2.44	N.D.	0.28	0.87
98	(see 92)	1440	N.D.	N.D.	1.82	N.D.	N.D.	0.96
99	 ?	1441	2.31	2.85	4.50	1.06	1.67	2.90
102		1446	N.D.	1.49	10.4	N.D.	1.23	2.78
109§§	$H_3COOC-C_7H_{14}-COOCH_3$	1469	3.67	5.72	14.3	2.32	9.82	12.2
110		1471	1.46	6.59	11.2	N.D.	1.78	2.06
114§§	$H_3COOC-C_7H_{14}-COOCH_3$	1476	N.D.	5.33	11.3	5.02	7.54	13.3
115	$C_{10}H_{16}O_7$	1483	N.D.	N.D.	N.D.	N.D.	0.65	9.69
118	$H_3COOC-C_7H_{12}-COOCH_3$	1490	N.D.	N.D.	N.D.	0.94	2.28	3.73
125§§	$H_3COOC-C_7H_{14}-COOCH_3$	1514	N.D.	1.13	2.56	0.92	1.60	13.3
133		1537	0.76	1.66	2.65	N.D.	0.28	0.88
134	 ?	1539	N.D.	N.D.	N.D.	N.D.	0.52	1.11

(Continued on p. 12)

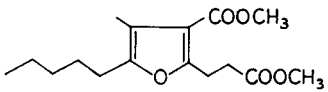
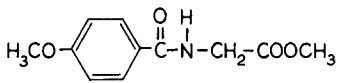
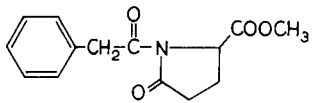
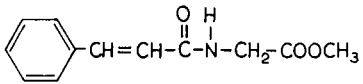
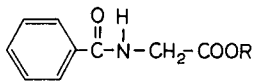
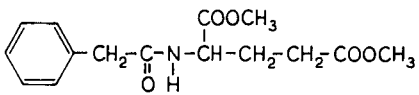
TABLE II (continued)

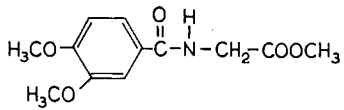
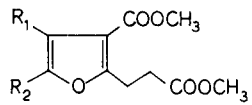
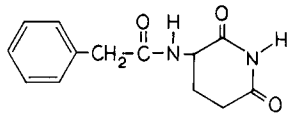
Compound number*	Structural formula** (or partial)	R.I.***	Normalized peak areas§					
			Hemofiltrate ($n=5$)			Urine ($n=4$)		
			Minimum	Median	Maximum	Minimum	Median	Maximum
135		1540	N.D.	N.D.	8.99	1.23	2.80	3.45
139	$H_3COOC-C_{8.}I_{14}-COOCH_3$	1564	N.D.	0.61	1.75	N.D.	N.D.	N.D.
141		1574	N.D.	N.D.	4.24	N.D.	N.D.	3.26
142		1579	N.D.	N.D.	N.D.	0.89	1.06	2.70
142A	UK 5	1580	N.D.	N.D.	0.68	N.D.	N.D.	N.D.
144	$H_3COOC-C_8H_{12}-COOCH_3$	1587	N.D.	N.D.	5.60	N.D.	N.D.	0.50
144A	UK 6	1596	N.D.	N.D.	2.31	N.D.	N.D.	N.D.
150	$H_3COOC-(CH_2)_8-COOCH_3$	1612	N.D.	N.D.	0.85	N.D.	0.59	2.35
153	$C-(CH_2)_3-COOCH_3$ $\|\|$ $C-(CH_2)_3-COOCH_3$	1620	N.D.	4.59	5.04	N.D.	N.D.	N.D.
155	$H_3COOC-C_8H_{12}O-COOCH_3$	1625	N.D.	N.D.	0.32	N.D.	0.31	0.75
157		1634	N.D.	N.D.	N.D.	N.D.	3.16	6.38

162§§		1650	116	359	1188	178	322	1049
162A	UK 7	1722	N.D.	N.D.	23.3	N.D.	N.D.	N.D.
179	$H_3COOC-C_7H_9(OH)-COOCH_3$	1752	N.D.	N.D.	12.9	N.D.	1.35	4.48
183§§		1767	0.95	1.77	4.01	2.76	5.32	12.8
185	Caffeine	1797	N.D.	5.03	12.5	1.96	6.86	20.1
191		1845	N.D.	1.52	3.93	N.D.	1.65	5.85
191A	 isomer	1846	N.D.	N.D.	0.87	N.D.	N.D.	1.39
191B	$H_3C-C_{14}H_{26}-COOCH_3$	1873	N.D.	1.86	2.99	N.D.	N.D.	N.D.
197		1893	N.D.	N.D.	8.08	1.10	5.25	11.7
199	$H_3C-(CH_2)_{14}-COOCH_3$	1910	N.D.	1.66	2.00	N.D.	0.86	1.36
199A		1912	N.D.	N.D.	3.56	N.D.	N.D.	N.D.

(Continued on p. 14)

TABLE II (continued)

Compound number*	Structural formula** (or partial)	R.I.***	Normalized peak areas§					
			Hemofiltrate ($n=5$)			Urine ($n=4$)		
			Minimum	Median	Maximum	Minimum	Median	Maximum
203		1938	N.D.	N.D.	N.D.	N.D.	0.86	2.20
205§§		1940	N.D.	1.54	4.93	5.24	5.54	11.4
208§§		1988	5.25	11.3	21.6	1.28	1.70	7.23
208A		2003	N.D.	N.D.	N.D.	N.D.	1.01	5.48
210A		2027	N.D.	N.D.	5.66	N.D.	N.D.	N.D.
210B	UK 8	2049	N.D.	N.D.	N.D.	N.D.	0.35	0.77
210C	UK 9	2058	N.D.	N.D.	N.D.	N.D.	0.35	0.87
219	$CH-(CH_2)_7-CH_3$ $\|\|$ $CH-(CH_2)_7-COOCH_3$	2101	N.D.	0.73	1.41	N.D.	0.72	0.86
219A	$H_3C-(CH_2)_{16}-COOCH_3$	2102	N.D.	N.D.	0.73	N.D.	0.33	1.15
220		2110	7.34	8.68	16.3	N.D.	1.50	3.51

223		2121	N.D.	N.D.	N.D.	N.D.	0.58	1.99
226	Tetramethyl uric acid	2183	N.D.	N.D.	N.D.	N.D.	1.29	3.32
231		2241	N.D.	N.D.	N.D.	N.D.	0.06	0.91
231A§§		2275	57.3	86.3	100	N.D.	0.29	1.97

*Compound numbers refer to compounds listed in refs. 8 and 13.
**Unknown compounds are labeled UK followed by a number. See Table I for MS data of these compounds.
***Kovats' retention indices.
§See text for discussion of normalization process. N.D. indicates that peak area was lower than integrator minimal area.
§§Compound not indicated in summation (see text).

(4-phenylbutyric acid) are listed in Table III. Peak areas are uncorrected for flame-ionization detector response, yet the table allows comparison of compound concentration in the samples. As can be noted in Table III, peak area ratios for most compounds decrease significantly during the course of hemofiltration, indicating efficient elimination of these compounds.

Contrary to expectations, graphical analysis of the data shows that the dialysate concentration of many larger molecules (see compounds 162, 183, 220, and 231A in Table III) decreases as rapidly as that of many smaller molecules (see compounds 16, 32, and 34) within the time and compound ranges studied. This suggests that clearance efficiency of these larger molecules is comparable to that of the smaller molecules, in agreement with results of Schoots et al. [2] who analysed uremic serum before and after dialysis. They proposed a "dialysis ratio" for a number of compounds to indicate how well the compound was removed from the blood during dialysis (a higher ratio indicating a greater rate of removal). The dialysis ratio mainly depended on initial compound concentration. Their results indicate that compound size does not play as great a role as might be expected. It was noted that the concentration of N-phenylacetyl-α-aminoglutarimide (compound 231A, Table III) decreases rapidly in hemodialysate during the dialysis time range studied, indicating very efficient removal. This could possibly be significant in the study of uremia, since this compound constitutes one of the few truly significant differences between the acid patterns of hemodialysate and urine.

Certain compounds of lower peak area ratio (see compounds 20B, 24, 89, 109, 185, 208, and 229) do not seem to follow the typical pattern but seem to "jump around", indicating that these compounds may form "steady-states" early in the course of the dialysis.

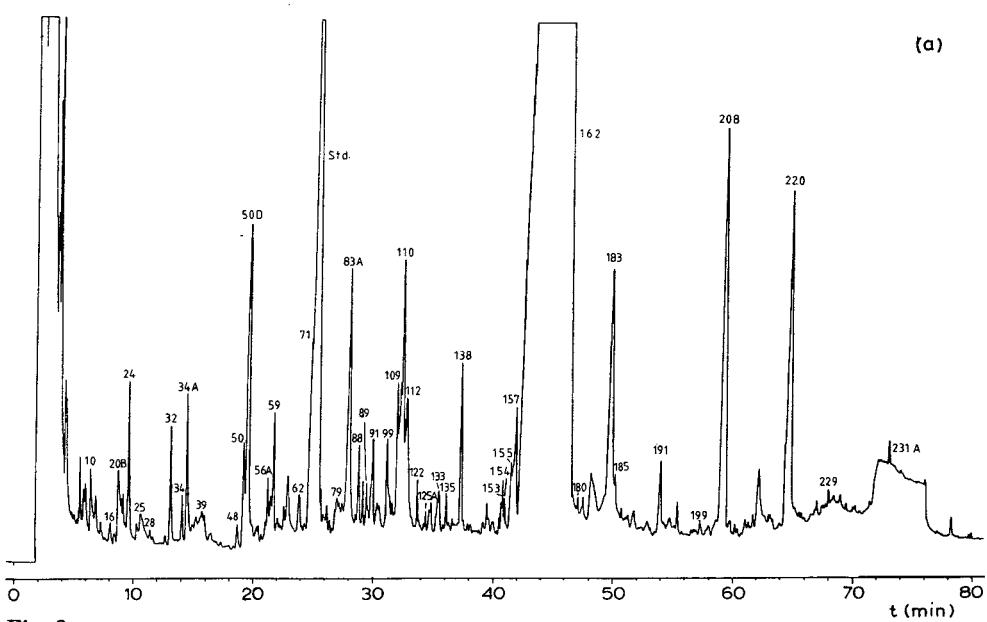


Fig. 3.

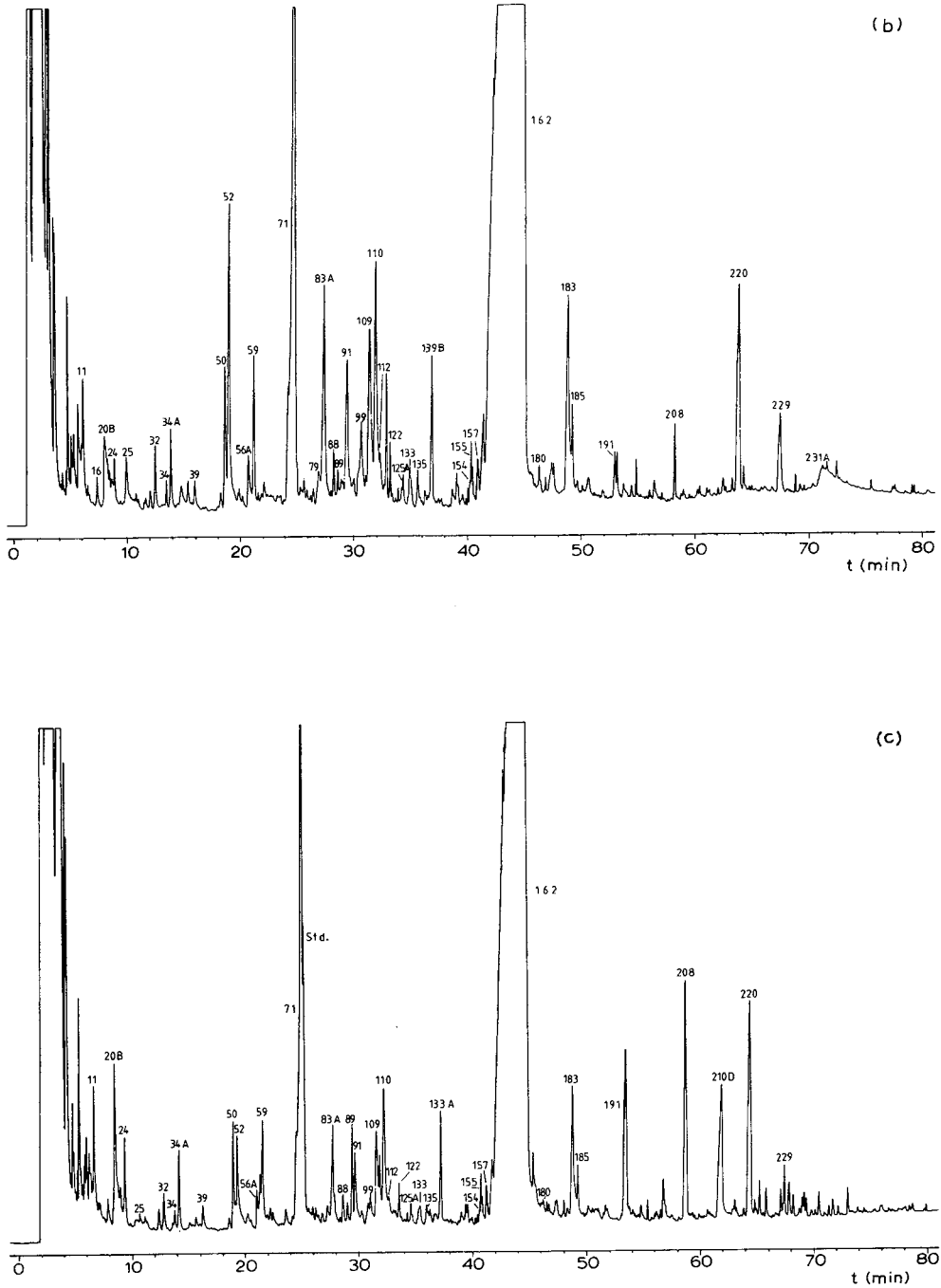


Fig. 3. Glass capillary GC profile of the organic methylates from hemodialysate samples obtained approximately 1 h (a), 4 h (b) and 7 h (c) after the beginning of a routine 8-h dialysis procedure of a 62-year-old uremic man.

TABLE III

RATIOS OF PEAK AREAS TO STANDARD PEAK AREA

Samples were taken approximately 1 h (start), 4 h (middle), and 7 h (end) after the start of an 8-h dialysis procedure.

Compound number*	Ratios of peak areas to standard peak area**		
	Start of dialysis	Middle of dialysis	End of dialysis
11	0.0726	0.1940	N.D.
16	0.0628	0.0564	0.0388
20B	0.3914	0.2844	0.4555
24	0.5204	0.0196	0.1405
25	0.2648	0.1768	N.D.
32	0.3508	0.1180	0.0597
34	0.1258	N.D.	0.0316
34A	0.4607	0.1463	0.1342
39	0.0607	0.0652	0.0419
50	0.3803	0.3873	0.1829
56A	0.2018	0.1943	0.0764
59	0.4075	0.3801	0.2234
62	0.3437	N.D.	N.D.
71	7.1568	3.1236	1.9180
79	0.1796	0.1324	N.D.
83A	1.6079	0.7927	0.2063
88	0.3148	0.0844	0.0432
89	0.1999	N.D.	0.1519
91	0.4762	0.4913	0.1607
99	0.4920	0.2966	0.0385
109	0.5428	0.7092	0.2472
110	1.0485	0.9016	0.4110
112	1.0280	0.0908	0.0507
122	0.1076	0.0825	0.0393
125A	0.0713	N.D.	0.0325
133	0.2470	0.1536	0.0619
135	0.1072	0.1022	0.0365
153	0.1266	0.1008	0.0521
154	0.0817	N.D.	0.0355
155	0.1188	0.1089	0.0668
157	0.3410	0.1701	0.1110
162	80.712	38.741	22.094
180	0.1795	0.0592	N.D.
183	1.9987	0.8365	0.4023
185	0.1327	0.1904	0.0825
191	0.3375	0.1179	0.2079
199A	0.1034	N.D.	N.D.
208	2.9998	0.1424	0.6656
220	2.5774	0.8324	0.4682
229	0.0910	0.3428	0.0751
231A	7.4725	0.3812	N.D.

*Compound numbers refer to compounds listed in Table II.
**Peak areas are not corrected for flame-ionization detector response.

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STEROID PROFILES OF HEALTHY INDIVIDUALS

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SUMMARY

Urine steroid profiles of healthy individuals can be divided into two groups according to greatly different excretion rates of dehydroepiandrosterone (DHEA). About 80% of the population show an excretion of DHEA in urine of just above the detection limit or less of the main androgens androsterone (A) and etiocholanolone (E). This excretion is only enhanced in psychological stress situations. The remaining 20% excrete DHEA in roughly equal amounts as A and E.

While the relation of excreted steroids is rather constant, the absolute amounts may vary greatly. In contrast to the behaviour of all other steroids DHEA excretion is not in relation to other steroids. The group of "high DHEA" producing individuals in particular shows drastic changes in the excretion during a day: the DHEA excretion rapidly rises from morning until afternoon and then drops to rather low values in the resting period during the night. A recognizable DHEA production seems to be closely related to the waking period.

INTRODUCTION

Steroid profiles [1,2] obtained by a gas chromatographic separation of the steroid fraction from body fluids — after appropriate derivatisation to enhance volatility and thermal stability — allow an insight into steroid metabolism. In the course of investigation of the urine steroid profiles of hirsute women we observed in many cases a great (10—30-fold) increase of the amount of dehydroepiandrosterone (DHEA) excreted compared with the excretion of normal individuals [3]. A further study revealed that psychological stress situations stimulate DHEA production by one order of magnitude or more [4]. In the widespread literature, mainly related to 17-ketosteroids, very different and sometimes contradictory reports on DHEA excretion are found [5—7].

"Normal" profiles published in literature show very different DHEA excretions, in most cases not mentioned explicitly. All these results initiated a detailed study of the excretion of steroids in our laboratory — especially of

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DHEA. Samples were taken in 4-h intervals lasting for 3 days to one month to obtain a detailed insight into individual changes in steroid excretion during these periods in order to be able to detect the reasons for the differences in DHEA excretion and to be able to recognize abnormal and possibly pathological changes more easily.

MATERIALS AND METHODS

Collection of samples

Urine samples were collected from healthy individuals 20—40 years' old. During the collection period lasting between 3 and 28 days the individuals lived on normal diet (usually "mensa" food). Coffee drinking was not allowed since this causes interference to the profiles by peaks arising from cauran acids occurring in coffee beans [8]. Samples were collected every 4 h and stored at —18°C. Although only small (10 ml) samples were used for analysis, the whole daily urine production was collected in order to quantify the daily rates of excretion products.

Work-up procedure

The neutral steroids were obtained according to a somewhat modified procedure introduced by Horning et al. [9]. Samples showing high DHEA levels were subjected, after the enzymatic hydrolysis, to solvolysis to cleave the sulfates — especially those of DHEA — completely. The values of DHEA obtained by this procedure are 35—40% higher compared to those obtained by enzymatic hydrolysis alone.

To reduce the danger of unexpected additional peaks interfering with a single added standard compound, we used various standards: eicosanol, hexadecanediol and epi-etiocholanolone [10]. If the standards were not in the correct relation, the composition of the sample was checked by gas chromatography—mass spectrometry (GC—MS) for by-products hidden under the peak of a standard compound.

To a 10-ml aliquot of urine 20 μg of each internal standard compound (see above) were added.

Preparation of derivatives

In general, two methods of derivatisation are used: either trimethylsilylmethoxime (TMS-MO) derivatives [11] are prepared or the steroids are transformed into enol-trimethylsilyl (enol-TMS) ethers [12]. We prefer the preparation of enol-TMS ethers, since they can be prepared in one step and elute from the column much earlier than the corresponding MO-TMS derivatives. The disadvantage that the reaction is sometimes incomplete can be overcome by heating the samples for another couple of hours if incomplete reaction is observed.

Both derivatisation methods afford reproducible and exact values for most of the steroids. Only the determination of 11-oxygenated steroids remains problematic. The 11-oxygen function is rather sensitive to absorption, so that (depending on the column) sometimes great losses must be taken into account. This problem has been studied in this laboratory in detail, since changes in

11-oxygenated steroid concentrations seem to be of diagnostic value [13].

The residue obtained by working up 10 ml of urine was dissolved in 2 ml of methanol. Two-fifths of this solution were transferred to a reaction vessel (1 ml) with a Teflon screw cap and brought to dryness in vacuum at 40—50°C. Five microlitres of pyridine (free of water), 5 μl of tetrahydrofuran and 20 μl of N-trimethylsilyl-N-methyl-trifluoroacetamide (MSTFA) were added; 10 μl of the clear solution were transferred to a small glass tube (ca. 1 mm I.D., ca. 7.5 cm long) containing a crystal of anhydrous sodium acetate. The contents of the tube were air-tight melted and heated to 70°C for 5 h. After cooling 0.5—1.0 μl of the solution was analyzed by GC.

Assignment of structures

The correctness of structure assignment in the gas chromatograms was confirmed using a GC—MS—computer combination.

Calculation of excretion rates

Since it was very difficult in practice to obtain urine samples collected in an exact 4-h rhythm, the excretion rates were calculated on the basis of μg/h for each collection time unit (about 2—4 h).

Usually quantification relies on the peak area of added epi-etiocholanolone (epi-E) [10]. The use of this internal standard requires the use of glass capillary columns of high quality to avoid interference with the peaks of androsterone (A) which elute directly after epi-E in the chromatogram (Δ RI = 8—9).

The calculation of excretion rates of the steroid metabolites is based on the following formula [14]:

$$m_{\text{steroid}} = \frac{\text{peak area}_{\text{steroid}} \times n \, V_{\Delta t}}{\text{peak area}_{\text{I.S.}} \times f \, V_{\text{urine aliquot}} \times \Delta t}$$

where m_{steroid} = excretion rate of the steroid to be calculated (in μg/h), f = GC response factor for the steroid to be calculated, n = mass of internal standard (I.S.) added (in μg), $V_{\Delta t}$ = volume (in ml) of urine collected during Δt hours, Δt = period of time (in h) during which the urine was collected.

INSTRUMENTS AND CHEMICALS

Gas chromatography

Gas chromatograms were taken on a Siemens-L 402 gas chromatograph equipped with a flame-ionization detector. The column was a 25-m open tubular glass capillary (0.3 mm I.D.) wall-coated with OV-101 [15]. Hydrogen carrier gas pressure was 0.8 bar. Temperature program was 2°C/min from 150 to 300°C. Detector temperature was 280°C while the injection port was kept at 260°C. Split ratio was 1:10. Peak area integration was performed by an Autolab System 1 (Spectra-Physics) computing integrator.

Kováts retention indices were found using a standard mixture of even carbon number hydrocarbons from C-16 to C-34.

Gas chromatography—mass spectrometry

Mass spectra were obtained with an LKB 2091 instrument. The electron ion

TABLE I

IDENTIFICATION OF GC PEAKS IN URINARY STEROID PROFILES OF FIGS. 1—4, 7

GC peak No.	Systematic name	Retention index (OV-101)	Molecular weight	Mass-spectra ions [*m/e* (%)]
1	3α-Hydroxy-5α-androst-16-ene, TMS	2186	346	75(68), 94(68), 131(29), 148(42), 241(100), 256(36), 331(15), 346(24)
St 1	1,16-Dihydroxy-hexadecane (hexadecanediol); di-TMS: internal standard	2321	402	73(85), 75(100), 83(90), 97(68), 103(52), 147(74), 149(70), 297(5)
St 2	Eicosanol, TMS: internal standard	2360	370	69(20), 73(34), 75(76), 83(20), 97(18), 103(27), 111(8), 355(100)
St 3	Enol ether of 3β-hydroxy-5β-androstan-17-one (epi-etiocholanolone); di-TMS: internal standard	2519	434	73(100), 143(36), 169(40), 182(22), 239(20), 329(43), 344(13), 419(90), 434(80)
4	Enol ether of 3α-hydroxy-5α-androstan-17-one (androsterone), di-TMS	2527	434	73(100), 143(36), 169(40), 182(22), 239(20), 329(43), 344(13), 419(90), 434(80)
6	Enol ether of 3α-hydroxy-5β-androstan-17-one (etiocholanolone), di-TMS	2534	434	73(100), 143(36), 169(40), 182(22), 239(20), 329(43), 344(13), 419(90), 434(80)
7	3α,17β-Dihydroxy-5α-androstane, di-TMS	2546	436	73(100), 129(55), 215(32), 241(46), 256(54), 331(9), 346(23), 421(5), 436(7)
8	Enol ether of 3β-hydroxy-androst-5-en-17-one (dehydroepiandrosterone), di-TMS	2600	432	129(18), 147(15), 169(10), 303(8), 327(11), 342(3), 417(18), 432(23)
9	3β,17β-Dihydroxy-5-androstene, di-TMS	2620	434	129(90), 213(35), 215(56), 239(44), 254(40), 305(38), 329(25), 344(50)
10	Enol ether of 3α-hydroxy-5β-androstane-11,17-dione (11-keto-etiocholanolone), di-TMS	2640	448	168(21), 182(10), 304(53), 343(68), 358(50), 433(12), 448(22)

12	3α,17α-Dihydroxy-5β-pregnan-20-one, di-TMS	2666	478	143(20), 215(35), 255(90), 345(20), 435(58), 478(2)
13	Enol ether of 3α,11β-dihydroxy-5α-androstan-17-one/3α,11β-dihydroxy-5β-androstan-17-one, di-TMS	2693	450	73(100), 75(78), 169(65), 327(78), 417(36), 432(20), 435(39), 450(100)
14	3α,20α-Dihydroxy-5β-pregnane (pregnanediol), di-TMS	2786	464	73(20), 75(14), 117(100), 269(2), 284(3), 359(1), 449(2), 464(1)
15	3α,16β,17β-Trihydroxy-5β-androstane, tri-TMS	2807	524	73(100), 147(20), 169(14), 191(25), 254(10), 344(9), 419(5), 434(12), 524(7)
16	3α,17α,20α-Trihydroxy-5β-pregnane (pregnanetriol), tri-TMS; 16′ = pregnanetriol, di-TMS	2818	552	73(48), 117(12), 147(18), 215(12), 255(100), 345(20), 435(58), 552(2)
17	3β,16α,17β-Trihydroxy-androst-5-ene, tri-TMS	2858	522	73(100), 129(24), 169(9), 191(11), 239(21), 252(8), 303(6), 329(18), 342(6), 417(9), 432(22), 522(5)
18	3β,16α,20α-Trihydroxy-pregn-5-ene, tri-TMS	2874	550	73(100), 117(80), 147(10), 157(32), 183(8), 370(4), 445(15), 460(48)
19	3α,17α,20α-21-Tetrahydroxy-5β-pregnan-11-one (α-cortolone), tetra-TMS	3066	654	117(10), 147(8), 191(11), 269(30), 359(40), 449(100), 551(20), 654(3)
20	3α,17α,20β-21-Tetrahydroxy-5β-pregnan-11-one (β-cortolone), tetra-TMS	3096	654	117(10), 147(8), 191(11), 269(30), 359(40), 449(100), 551(20), 654(3)
21	Enol ether of 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione (THE = tetrahydrocortisone), tetra-TMS	3112	652	73(100), 75(50), 147(38), 169(30), 243(21), 305(10), 318(6), 331(90), 562(21), 652(60)
23	Enol ether of 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one (THF = tetrahydrocortisol), tetra-TMS	3141	654	73(100), 75(50), 147(35), 169(25), 318(7), 331(47), 564(12), 654(16)
24	Enol ether of 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (α-THF = α-tetrahydrocortisol), tetra-TMS	3168	654	73(100), 75(50), 147(35), 169(25), 318(7), 331(47), 564(12), 654(16)
25	3α,11β,17α,20α,21-Pentahydroxy-5β-pregnane, tetra-TMS	3198	656	129(25), 147(32), 191(20), 243(65), 253(48), 343(18), 451(28), 553(30), 656(5)

source temperature was 250°C, the electron energy 70 eV. The total ion current signal was registered at 20 eV. The GC column and temperature program were identical to those given above.

Chemicals

The chemicals used (and their source) were as follows: hexadecanediol (Aldrich, Milwaukee, WI, U.S.A.); 3β-hydroxy-5β-androstan-17-one (E. Merck, Darmstadt, G.F.R.); MSTFA (Macherey, Nagel & Co., Düren, G.F.R.); helicase (Boehringer, Mannheim, G.F.R.).

RESULTS AND DISCUSSION

Samples were taken from 15 healthy female and 30 male subjects. From every subject 18—20 urine samples were collected in 4-h intervals. To confirm the results, the collection was repeated after a six-week interval.

Urine steroid profiles of healthy individuals are characterized by two main peaks in the region of the androgens — androsterone (A) and etiocholanolone (E) — and one main peak in the region of the corticoids — tetrahydrocortisone (THE) (Fig. 1). Usually these three peaks are of comparable size. A much lower intensity of the peak corresponding to THE is a strong indication that the derivatisation was not properly done and corticosteroids were lost to a large extent [4,10].

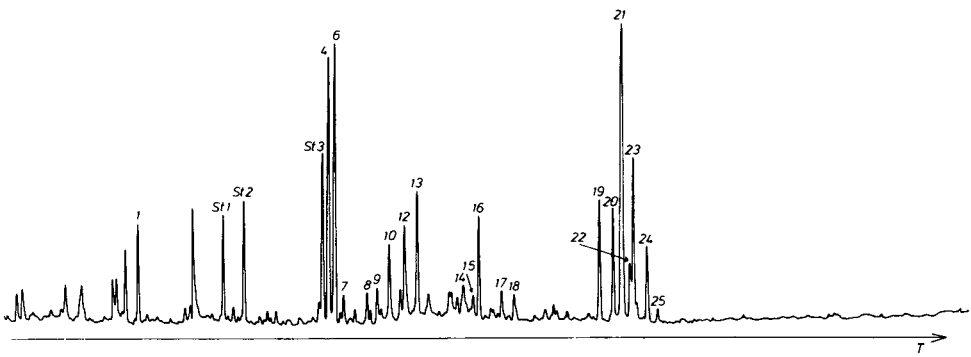

Fig. 1. Urinary steroid profile (TMS-enol-TMS ether) of a healthy 30-year-old male; steroids are identified in Table I.

Earlier it was reported [16] that male subjects always show a much higher excretion of A than of E. Although this is true in many cases, we found several male subjects with an equal or even higher ratio of E/A. An unambiguous distinction between the sexes by an inspection of these ratios therefore seems not to be possible.

The ratio between A and E differs from individual to individual, but the A/E ratio of urine samples of a single individual taken at different times does not differ greatly (Fig. 2).

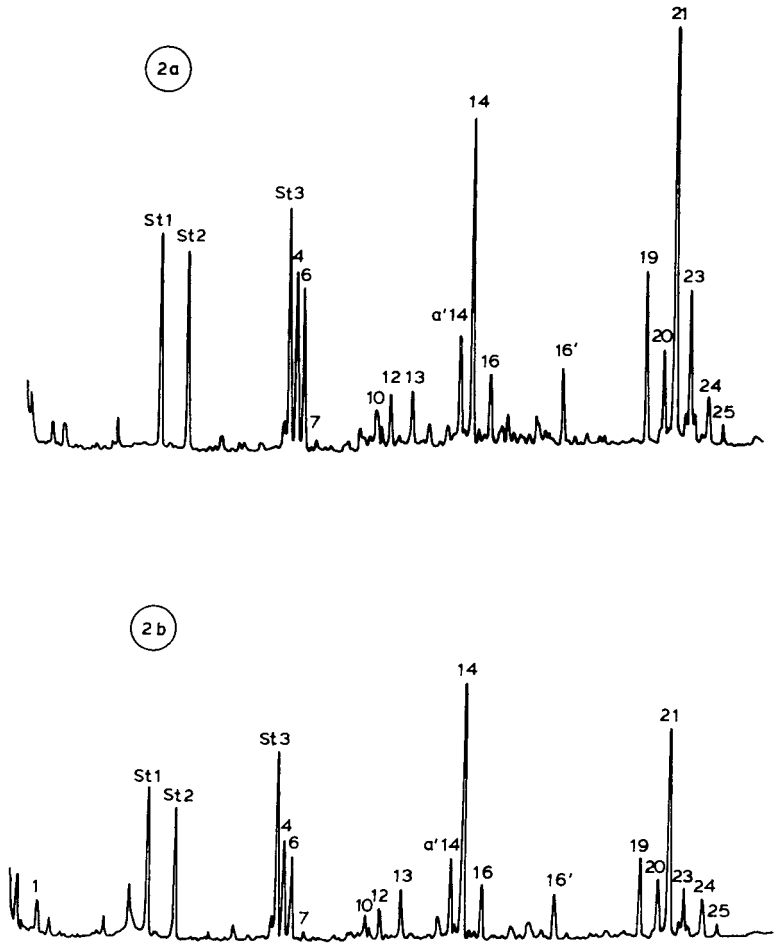

Fig. 2. Comparison of urinary steroid profiles (TMS-enol-TMS ether) of a female (30 years' old, luteal phase of cycle), taken from a 24-h urine specimen (2a) and at 1700 on the same day (2b); steroids are identified in Table I.

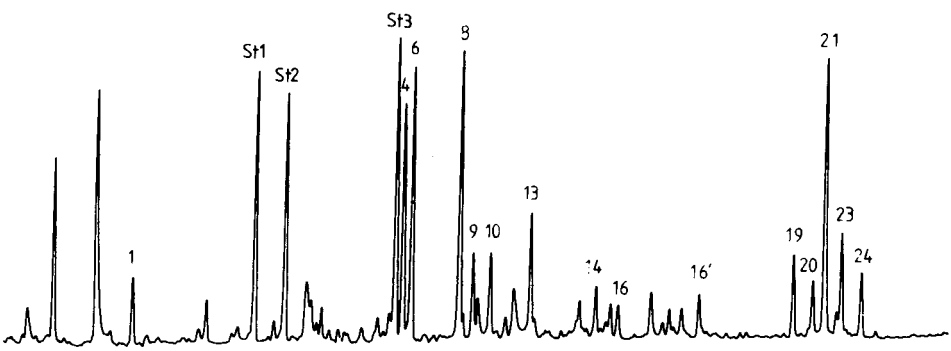

Fig. 3. Urinary steroid profile (TMS-enol-TMS ether) of a "high" DHEA excreting female (25 years' old); steroids are identified in Table I.

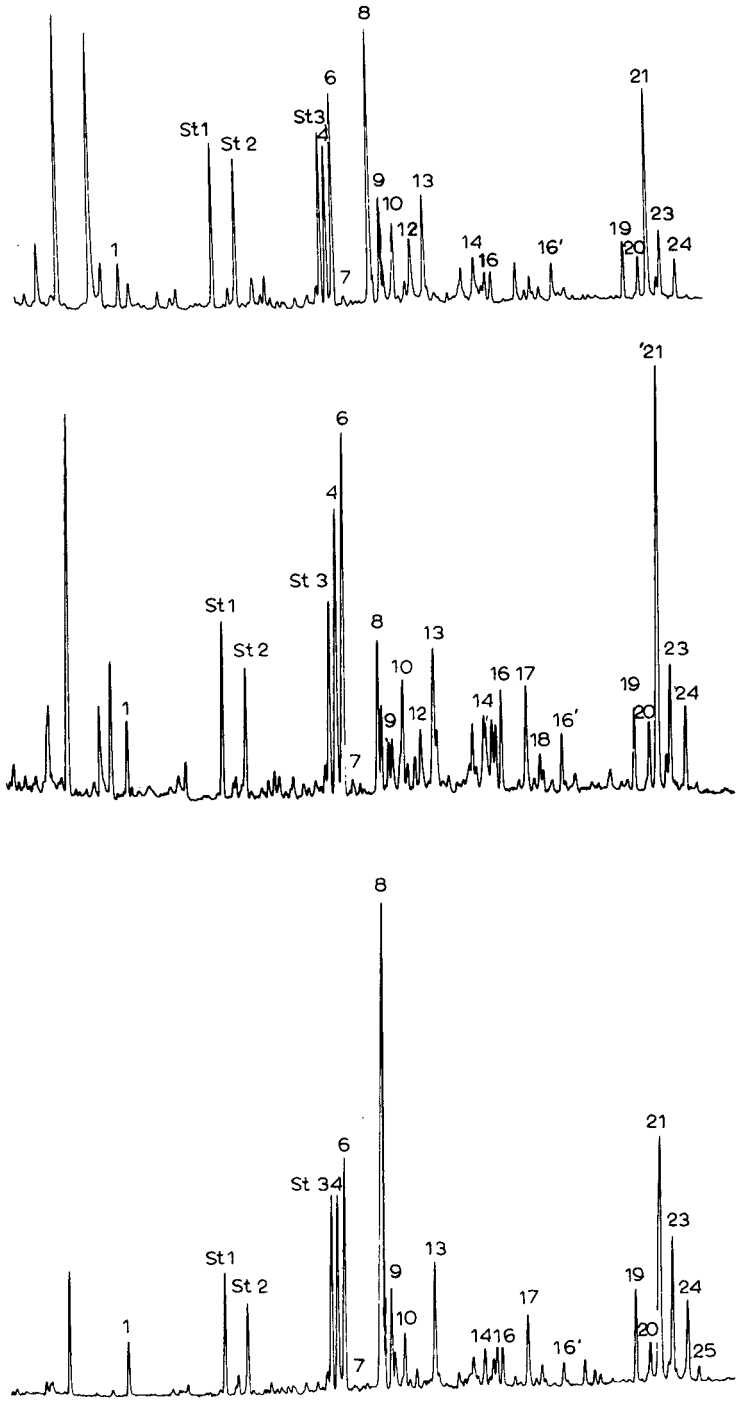

Fig. 4. Urinary steroid profiles (TMS-enol-TMS ether) of a "high" DHEA excreting female (25 years' old) taken from urine samples at different times of day: 2200 (top profile), 0600 (centre profile), 1000 (bottom profile); steroids are identified in Table I.

While the relative excretion of nearly all steroids varies in healthy individuals within certain limits (about 1:5), that of DHEA is subject to large changes (1:50). Usually the excretion of DHEA in a normal individual is just above the detection limit. Only in psychological stress situations is its excretion enhanced in most individuals to amounts comparable to those of A and E, i.e. 10—50-fold.

In this study about every tenth individual showed an excretion rate of DHEA in the same ratio as normal individuals do in a severe psychological stress situation (Fig. 3).

The excretion of DHEA in these persons changes a lot during one day, reaching a maximum about noon or in the afternoon and being low in the resting period (Fig. 4). Even in the resting period the DHEA excretion is about ten times higher than in a "normal" person. To exemplify this the ratio between DHEA and E of a "normal" DHEA excreting person and that of a high DHEA excreting person were compared (Table II).

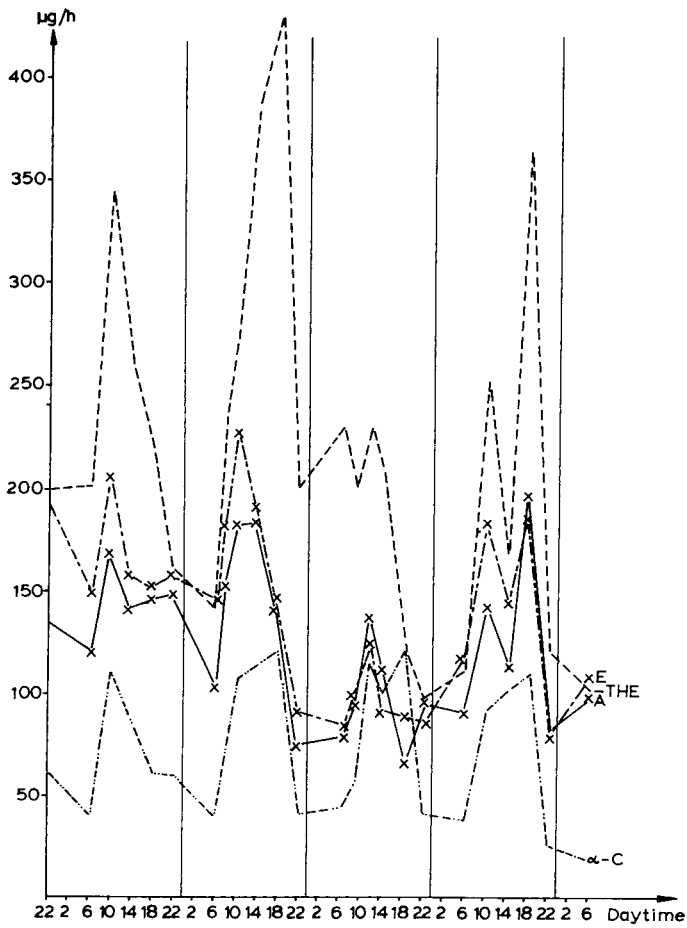

Fig. 5. Comparison of the excretion rates of androsterone (A), etiocholanolone (E), tetrahydrocortisone (THE) and α-cortolone (α-C) of a male (28 years' old) over a 4-day collection period; values expressed in µg/h per collection time unit.

TABLE II

A/E AND DHEA/E RATIOS OF A "NORMAL" DHEA EXCRETING PERSON (I) COMPARED WITH A "HIGH" DHEA EXCRETING PERSON (II) FROM URINE SAMPLES COLLECTED IN A 3—4-h RHYTHM

	2200—0600		0600—1000		1000—1300		1300—1700		1700—2200	
	I	II	I	II	I	II	I	II	I	II
A/E	0.97	0.63	1.09	0.98	1.12	0.89	1.00	0.77	0.91	0.74
DHEA/E	N.D.*	0.36	0.04	3.10	0.10	3.13	0.24	2.22	N.D.*	0.82

*Not detectable.

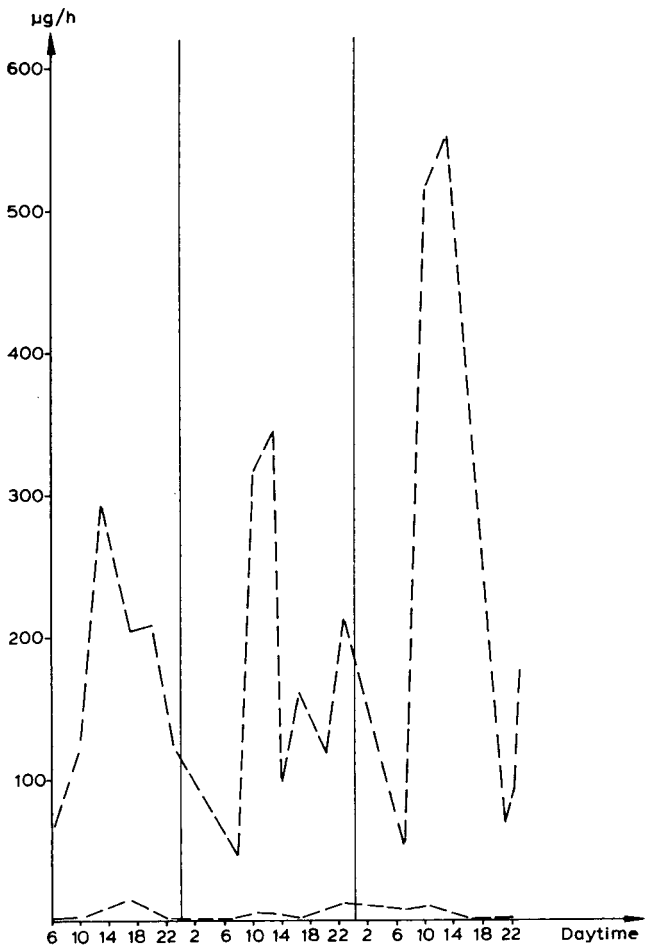

Fig. 6. Drastic differences in DHEA excretion rates between a female (25 years' old) typical of a "high" DHEA excreting person, and a female (30 years' old) typical for a "normal" DHEA excreting person.

It is therefore necessary to distinguish between two types of individuals: those who are living with a low excretion level of DHEA (about 80—90%) and those with a high excretion level of DHEA. This finding explains the great differences in the DHEA levels published [17].

Interestingly, high DHEA excretion is very common in women suffering from idiopathic hirsutism. About 60% of them are high DHEA excretors, a much higher percentage compared to other individuals. This is probably not due to altered adrenal enzyme activity, since there is no evidence for an abnormality of 3β-hydroxysteroid dehydrogenase in any of the hirsute subjects examined [18].

So far we only compared relative rates directly deducible from the profiles. The addition of an internal standard and knowledge of the volume of urine produced within a certain period enable us to calculate the quantitative steroid excretion at different times.

Although the relative rates of steroids excreted are rather constant (Fig. 5) the actual excretion rates change a good deal during the daytime, reaching a maximum about noon (Fig. 5). This effect, well known from group determinations [19—21], can be masked in clinical analysis by taking 24-h specimens.

Moreover, the excretion of urinary steroids also changes from day to day. These changes are not considered in clinical analysis although they are of great importance. Especially striking is the change in the daily excretion of DHEA in "high" DHEA excreting individuals compared to the low excreting individ-

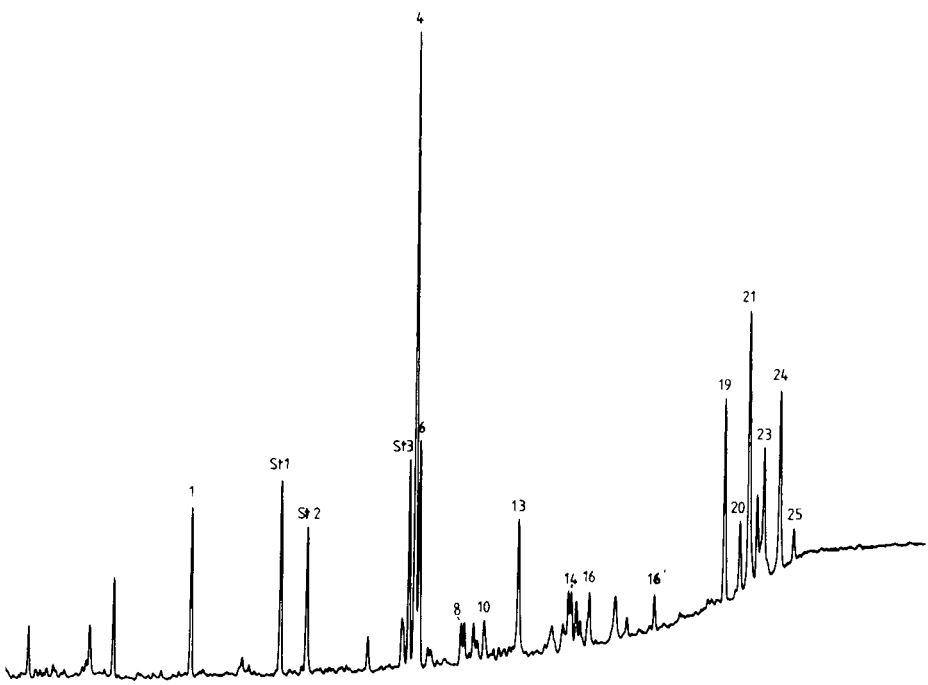

Fig. 7. Urinary steroid profile (TMS-enol-TMS ether) of a male (25 years' old) showing an extremely high A/E ratio; steroids are identified in Table I.

uals (Fig. 6), which is evident even by inspecting the profiles (Fig. 4) without reference to the standard.

One additional observation deserves to be mentioned. The profiles of 29 male subjects showed an A/E ratio of 2:1 or less; only one male subject developed a profile with a 3:1 ratio (Fig. 7). A similarly extreme ratio of the corticoids α-THF/THF > 1 was observed. Quantification revealed a normal E excretion but an increase in A excretion by a factor of 3—4 and in 3α-hydroxy-5α-androst-16-ene by a factor of similar magnitude. This individual obviously has a much higher activity of 5α-reductase than usual. Shackleton et al. [22] described a case of hypertension in which an extreme activity of this enzyme was found.

It must be assumed that there exist even more types of people distinguishable according to their metabolism. It would be of interest to investigate if these subjects also show an alternative rate of drug metabolism.

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CHROMBIO. 792

STUDIES ON STEROIDS

CLXV. DETERMINATION OF ISOMERIC CATECHOL ESTROGENS IN PREGNANCY URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received September 10th, 1980; revised manuscript received November 13th, 1980)

SUMMARY

A method for the determination of 2- and 4-hydroxylated estrone and estradiol in pregnancy urine by high-performance liquid chromatography with electrochemical detection (HPLC—ECD) is described. The urine catechol estrogens were deconjugated, purified by adsorption on alumina, and subjected to HPLC—ECD. Two pairs of isomeric catechol estrogens were distinctly separated on a μBondapak C_{18} column with acetonitrile—0.5% ammonium dihydrogen phosphate (pH 3.0). The amounts of these four compounds were satisfactorily determined with a quantitation limit of 1 ng using 4-hydroxy-16-oxoestradiol 17-acetate as an internal standard. The validity of the present method for the determination of urine catechol estrogens was verified by the recovery test.

INTRODUCTION

Recently considerable attention has been directed to the biological function of catechol estrogens in living animals [1—3]. In a previous paper of this series we reported the separation of catechol estrogens and their related compounds by means of high-performance liquid chromatography (HPLC) [4]. The present paper deals with the separation and determination of 2- and 4-hydroxylated estrone and estradiol in pregnancy urine by HPLC with electrochemical detection (ECD).

MATERIALS AND METHODS

Reagents

2-Hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestradiol and 4-hydroxy-

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estradiol were prepared by the known methods. 4-Hydroxy-16-oxoestradiol 17-acetate (internal standard; m.p. 292°C) was synthesized from 16-oxoestradiol 17-acetate by oxidation with Fremy's salt. Acid-washed alumina 150 (Type T; E. Merck, Darmstadt, G.F.R.) was used as an adsorbent for clean-up of catechol estrogens. All reagents and chemicals were of analytical-reagent grade. Solvents were purified by distillation prior to use.

Sample collection

The pregnancy (16—39 weeks) urine specimens were kindly supplied by Dr. K. Muraguchi, School of Medicine, Tohoku University.

High-performance liquid chromatography

The apparatus used was a Waters Model ALC/GPC 202 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), equipped with a Yanagimoto Model VMD-101 electrochemical detector (Yanagimoto Co., Kyoto, Japan). The potential of the detector was set at +0.8 V vs. the Ag/AgCl reference electrode. The samples were introduced by means of a Model U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. A μBondapak C_{18} (8—10 μm) column (30 cm × 3.9 mm I.D.) (Waters Assoc.) was used under ambient conditions. Acetonitrile—0.5% $NH_4H_2PO_4$ (pH 3.0) (1:2.1) was employed as a mobile phase at a flow-rate of 1 ml/min.

Assay procedure

The hot acid hydrolysis of pregnancy urine (0.2 ml) was carried out according to the procedure described by Gelbke et al. [5]. To the hydrolyzed urine sample was added a known amount of internal standard (ca. 100 ng), and the solution was extracted three times with 2 ml each of ethyl acetate. The organic layers were combined, washed successively each three times with 0.5 ml each of 0.05 *M* Tris·HCl buffer (pH 8.5) and water and then evaporated down under reduced pressure at room temperature. The residue was dissolved in 0.05 *M* Tris·HCl buffer (pH 8.5) (0.1 ml) and stirred with acid-washed alumina (500 mg) at pH 8.5 under ice-cooling for 10 min. After washing five times with 3 ml each of chilled 0.05 *M* Tris·HCl buffer (pH 8.5), the desired catechol estrogen fraction was obtained by elution with 1 *N* HCl. The effluent was extracted with ethyl acetate (10 ml), and the organic layer was washed with 0.5 ml of 0.05 *M* Tris·HCl buffer (pH 8.5), three times with 0.5 ml each of water and then evaporated down under reduced pressure. The residue was dissolved in methanol (0.1 ml), an aliquot of which was injected into the high-performance liquid chromatography.

Gas chromatography—mass spectrometry

A JEOL JGC-20K (GC)-JMS-01SG-2 (MS) combined instrument (JEOL, Tokyo, Japan) equipped with a JMA-2000 data-processing system was used. The coiled glass column (1 m × 0.3 cm I.D.) was packed with 3% OV-1 on Chromosorb W (100—120 mesh), and helium was used as a carrier gas. The temperature of the column was 230°C, and the injection port and ion source were kept at 260°C and 300°C, respectively. The accelerating voltage, ionization voltage and trap current were 8 kV, 70 eV and 200 μA, respectively.

Preparation of trimethylsilyl ether derivatives

The trimethylsilyl (TMS) derivatives were prepared by adding 0.1 ml each of pyridine, hexamethyldisilazane and trimethylchlorosilane and incubating at 60°C for 30 min. After removal of the excess reagents under a nitrogen gas stream, the residue was dissolved in hexane and subjected to gas chromatography—mass spectrometry.

RESULTS AND DISCUSSION

Separation of the four isomeric catechol estrogens has been previously established by HPLC on a μBondapak C_{18} column using acetonitrile—0.5% ammonium dihydrogen phosphate (pH 3.0) (1:2.1) as a mobile phase [4]. In the present study, the separation and determination of catechol estrogens in human pregnancy urine was undertaken employing this technique.

The urine specimen containing labile catechol estrogens was successfully collected and preserved, oxidative decomposition being prevented by the addition of ascorbic acid. The deconjugated estrogen fraction was obtained by hot acid hydrolysis followed by extraction with ethyl acetate. The clean-up procedure for urine catecholamines appeared to be similarly effective for urine catechol estrogens. The catecholic compounds were selectively adsorbed on acid-washed alumina and then recovered by eluting with hydrochloric acid. An aliquot of the eluate was subjected to HPLC—ECD according to the procedure previously established. As shown in Fig. 1, the four isomeric catechol estrogens in pregnancy urine were distinctly separated and identified on the chromatogram by comparison with the authentic samples.

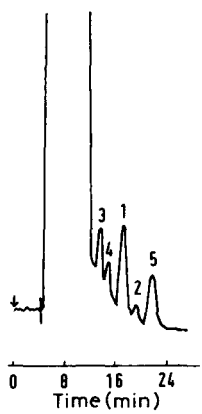

Fig.1. Separation of catechol estrogens in pregnancy urine by HPLC—ECD. 1 = 2-OHE_1 (3.4 ng); 2 = 4-OHE_1 (< 1 ng); 3 = 2-OHE_2 (1.2 ng); 4 = 4-OHE_2 (1.0 ng); 5 = 4-hydroxy-16-oxoestradiol 17-acetate (internal standard) (5.0 ng). Conditions: column, μBondapak C_{18}; mobile phase, acetonitrile—0.5% $NH_4H_2PO_4$ (pH 3.0) (1:2.1), 1 ml/min; detection, Yanagimoto Model VMD-101 electrochemical detector. Abbreviations as in Table I.

The structures of these four peaks were unequivocally characterized by means of gas chromatography—mass spectrometry. The urine catechol estrogens were transformed into the TMS ether derivatives in the usual manner and then analyzed using selected ion monitoring of parent ions at *m/z* 430

for 2- and 4-hydroxyestrone and *m/z* 504 for 2- and 4-hydroxyestradiol. A typical mass chromatogram is illustrated in Fig. 2. Two pairs of peaks obtained by single ion monitoring of each characteristic ion corresponded well with four peaks observed on reconstructed ion chromatography (RIC). In addition, the mass spectrum of each peak on RIC was identical to that of the respective authentic sample. The presence of 2- and 4-hydroxyestrogens in normal late pregnancy urine was thus definitely established. To the best of our knowledge this is the first reported occurrence of 4-hydroxyestrone and 4-hydroxyestradiol in man, although identification of 4-hydroxyestriol in pregnancy urine has recently been demonstrated by Fotsis et al. [6].

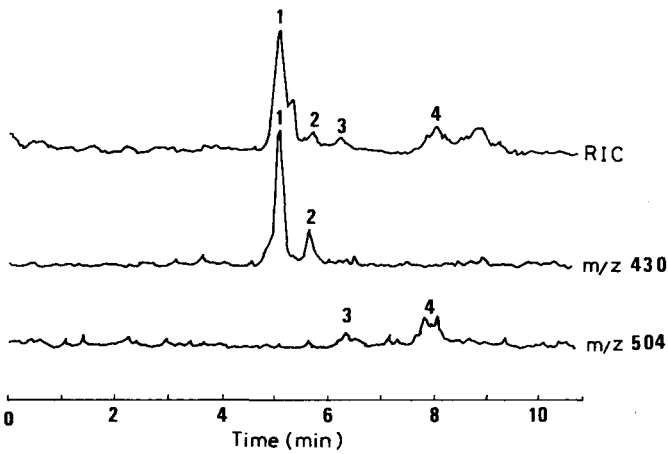

Fig. 2. Mass chromatogram of catechol estrogens in pregnancy urine. 1 = 2-OHE$_1$; 2 = 4-OHE$_1$; 3 = 2-OHE$_2$; 4 = 4-OHE$_2$. Abbreviations as in Table I.

Development of a method for the quantitation of urine catechol estrogens was then undertaken. The calibration graphs were constructed by plotting the peak height of each catechol estrogen to the internal standard (that is 4-hydroxy-16-oxoestradiol 17-acetate) against the amount of the former; satisfactory linearity was observed in the range 1—6 ng of catechol estrogens (Fig. 3).

The hydrolyzed urine was extracted with ethyl acetate and the extract was in turn subjected to the clean-up procedure used for the analysis of urine catecholamines. The use of selective adsorption on acid-washed alumina provided the catechol fraction where the desired catechol estrogens were expected to be present.

In order to confirm the validity of the present method for the determination of urine catechol estrogens, the recovery test was carried out using the authentic samples. First, catechol estrogens recovered through the whole clean-up procedure involving extraction with organic solvents and adsorption on alumina were determined. It is evident from the data in Table I that the four catechol estrogens were all recovered at satisfactory rates. Subsequently, the recovery of catechol estrogens in spiked pregnancy urine was tested. A mixture of known amounts of the four catechol estrogens was added to the urine hydrolysate, and the assay procedure involving extraction and clean-up

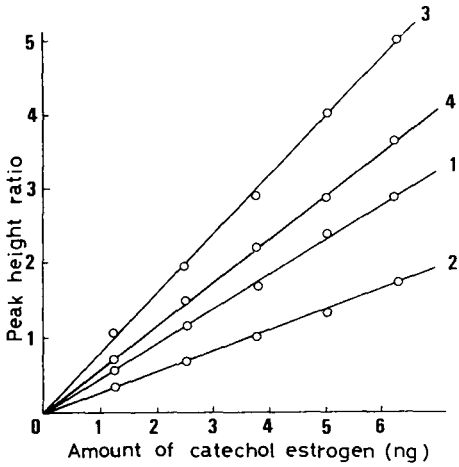

Fig. 3. Calibration graphs for catechol estrogens. 1 = 2-OHE_1 ; 2 = 4-OHE_1 ; 3 = 2-OHE_2 ; 4 = 4-OHE_2 . Abbreviations as in Table I.

TABLE I

RECOVERIES OF CATECHOL ESTROGENS* TAKEN THROUGH THE WHOLE CLEAN-UP

An aqueous solution (0.2 ml) containing 50 ng each of the four catechol estrogens was used for the recovery test.

	Recovery (%)			
	2-OHE_1	2-OHE_2	4-OHE_1	4-OHE_2
Mean ± S.D. (n=6)	74.8 ± 3.8	73.9 ± 2.4	74.7 ± 1.2	75.1 ± 2.2

*2-OHE_1 = 2-hydroxyestrone = 2,3-dihydroxy-1,3,5(10)-estratrien-17-one; 4-OHE_1 = 4-hydroxyestrone = 3,4-dihydroxy-1,3,5(10)-estratrien-17-one; 2-OHE_2 = 2-hydroxyestradiol = 1,3,5(10)-estratriene-2,3,17β-triol; 4-OHE_2 = 4-hydroxyestradiol = 1,3,5(10)-estratriene-3,4,17β-triol.

followed by HPLC was carried out. The mean recovery values of the four catechol estrogens in spiked pregnancy urine were found in the range 95.4—106.0% (Table II).

The present method was then applied to quantitation of the four catechol estrogens excreted in pregnancy urine (Table III). In almost all cases 2-hydroxyestrone showed the highest value of these four. The amount of 4-hydroxyestrogens excreted was less than that of 2-hydroxyestrogens. It is to be noted that biologically potent catechol estrogens, in particular 4-hydroxyestrogens, can be determined with satisfactory accuracy and precision by the newly developed procedure.

The availability of a simple and sensitive method may serve to clarify the biological function and metabolic significance of catechol estrogens. Further studies on the development of a more sensitive method for determination of catechol estrogens in tissues are being conducted in these laboratories and the details will be reported in the near future.

TABLE II

DETERMINATION OF CATECHOL ESTROGENS* IN SPIKED URINE

A pregnancy urine specimen containing 2-OHE_1 (34 ng/ml), 2-OHE_2 (24 ng/ml), 4-OHE_1 (27 ng/ml) and 4-OHE_2 (21 ng/ml) was used.

Amount of each spiked (ng/ml)	Determined (ng/ml)			
	2-OHE_1	2-OHE_2	4-OHE_1	4-OHE_2
100	97	96	103	106
250	252	246	266	258
500	513	477	519	505
1000	972	966	1026	962
Relative recovery rate (%) (Mean ± S.D.; n=5)	99.1 ± 2.4	98.2 ± 3.1	103.8 ± 1.7	102.0 ± 3.6

*For abbreviations see footnote to Table I.

TABLE III

URINE LEVELS OF CATECHOL ESTROGENS* IN NORMAL PREGNANCY

Subject	Determined (ng/ml)			
	2-OHE_1	2-OHE_2	4-OHE_1	4-OHE_2
A (16 w)	125	103	51	44
B (16 w)	138	36	n.d.**	21
C (25 w)	128	138	n.d.	58
D (29 w)	276	59	n.d.	104
E (34 w)	145	305	n.d.	95
F (37 w)	382	130	36	39
G (38 w)	203	385	116	n.d.
H (38 w)	264	104	22	136
I (38 w)	749	109	73	72
J (39 w)	109	168	65	n.d.

*For abbreviations see footnote to Table I.
**n.d. = not detectable.

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CHROMBIO. 774

DETERMINATION OF CATECHOLAMINES BY RADIOENZYMATIC ASSAY USING ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

A simple radioenzymatic method for the simultaneous determination of norepinephrine, epinephrine and dopamine in human serum was developed. Catecholamines were converted to normetanephrine (NMN), metanephrine (MN), 3-methoxytyramine (3-MT) and their 4-O-methyl isomers by catechol O-methyl transferase with S-adenosylmethionine-(^{3}H-methyl) ([^{3}H]SAM). After addition of unlabelled NMN, MN and 3-MT as carriers, the methyl derivatives were isolated from the reaction mixture by passing through a small boric acid gel column and separated from each other by ion-pair liquid chromatography. Interference by tailing of radioactivity of [^{3}H]SAM and its degradation products eluted earlier was minimized by the use of a precolumn and a branched flow path.

INTRODUCTION

A radioenzymatic assay of catecholamines has been developed by combining the selectivity of the methylation reaction of catechol O-methyl transferase (COMT) and the sensitivity of radioactivity determination. Generally tritium-labeled normetanephrine (NMN), metanephrine (MN) and 3-methoxytyramine (3-MT) produced in the enzyme reaction, are separated from unreacted S-adenosylmethionine-(^{3}H-methyl) ([^{3}H]SAM) and its degradation products by either solvent extraction [1,2] or precipitation with phosphotungstic acid [3] and isolated separately by thin-layer [1,2] or paper chromatography [4,5] followed by

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determination of the radioactivity. Recently, separation of 3-O-methyl derivatives was performed by high-performance liquid chromatography [6, 7]. However, in the procedures previously reported, formation of 4-O-methyl derivatives was not described, in spite of the possible formation of 4-O-methyl isomers during the enzyme reaction [8, 9].

In this paper we have used a boric acid gel column to adsorb unreacted [^{3}H]-SAM and [^{3}H] methylthioadenosine (MTA), demonstrated the formation of 4-O-methyl isomers and separated three pairs of methyl derivatives of catecholamines by high-performance liquid chromatography for the accurate determination of catecholamines in biological materials.

EXPERIMENTAL

Reagents

S-Adenosylmethionine-(^{3}H-methyl) (spec. act. 11.0 and 85.0 Ci/mmol), ^{14}C-dopamine·HBr (spec. act. 54.0 mCi/mmol) and Aquasol-II scintillation cocktail were obtained from New England Nuclear (Boston, MA, U.S.A.). COMT (2250 units/mg protein) purified from porcine liver was obtained from Sigma (St. Louis, MO, U.S.A.). A radioenzymatic assay kit of catecholamines, KAT-A-KIT^{TM}, which uses COMT from rat liver, was obtained from Upjohn (Kalamazoo, MI, U.S.A.). Boric acid gel was purchased from Aldrich (Milwaukee, WI, U.S.A.). 3,4-Dihydroxybenzylamine (DHBA)·HCl and vanillylamine·HCl were synthesized according to the method of Nelson [10]. All other chemicals were highest grade reagents.

Preparation of deproteinized serum

Blood collected by vein puncture was left for 15 min at 4°C and centrifuged to separate the serum. Three microliters of 5 *N* perchloric acid were added to 60 μl of serum and precipitated protein was removed by centrifugation at 10,000 *g* for 30 min. Supernatant (50 μl) was used for the enzyme reaction.

Preparation of a boric acid gel column

Boric acid gel was allowed to swell in 5% aqueous acetone overnight and activated by washing with 1 *N* HCl, water, 0.1 *N* NaOH and water successively and stored in water. Columns (17 mm × 3 mm I.D.) were prepared with fresh gel for each determination.

Enzyme reaction and sample preparation for chromatography

The buffer solution for the enzyme reaction was prepared to contain 2.5 *M* Tris, 50 m*M* EGTA, 400 m*M* $MgCl_2$ and 1.3 *M* mercaptoethanol. The pH was adjusted to 8.8 using HCl. Fifty microliters of a standard mixture of catecholamines or deproteinized serum were placed in 5-ml glass-stoppered centrifuge tubes cooled on ice. The buffer solution of enzyme reaction (10 μl), [^{3}H] SAM (1 μCi), porcine COMT (30 units) and DHBA (10 pg) were added and the total volume was adjusted to 100 μl with water. The solution was mixed with a vortex mixer for 5 sec and incubated for 45 min at 37°C. The reaction was stopped by the addition of 7 *N* NaOH (5 μl). Carrier solution (5 μl) containing 75 μg of NMN, MN and 3-MT was added. The solution was mixed with a vortex

mixer for 10 sec and centrifuged. Supernatant (100 μl) was applied to the boric acid gel column and O-methylated derivatives were eluted with 3 × 100 μl of 250 m*M* Tris—NaOH solution (pH 13.0). Eluate was collected in a tube containing 18% HCl (16 μl); a quarter of the eluate was subjected to ion-pair liquid chromatography.

Chromatography

A high-performance liquid chromatograph was assembled from a pump (Kyowa Seimitsu KHU-52H, Tokyo, Japan), a short column (10 cm × 4 mm I.D.), a 254-nm UV detector (Shimadzu UVD-2, Kyoto, Japan) and a fraction collector (Tokyo Rikakikai DC-180, Tokyo, Japan). Columns were packed with LiChrosorb RP-18, 10 μm, by the balanced density method. The mobile phase was acetonitrile—water (7:93, v/v) containing 0.5 g of sodium heptanesulfonate and 1.4 or 4.0 ml of acetic acid per liter and was adjusted to pH 4.0 with 15% NaOH [11]. The flow-rate was 1.0 ml/min.

Branched flow path

The flow system (Fig. 1) was essentially the same as the one devised for large-volume sample injection in gas chromatography [12]. After sample injection, the outlet of the precolumn was connected directly to the detector and elution was monitored by UV absorption. After elution of the peak (shown by an arrow in Fig. 2A) originating from mercaptoethanol in the enzyme reaction mixture, the main column was connected as in Fig. 1. Essentially all the radioactivity of degradation products and [^{3}H] SAM was eluted from the precolumn before this stage and thus contamination of the main column was avoided. Radioactivity was determined on every 0.5-ml fraction with 5 ml Aquasol-II using an Aloka liquid scintillation spectrograph (Aloka LSC-903, Tokyo, Japan).

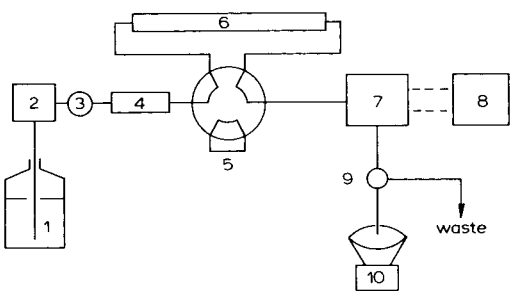

Fig. 1. Schematic diagram of the flow system. 1 = Eluent reservoir; 2 = pump; 3 = line sample injector; 4 = precolumn; 5 = six-way valve; 6 = analytical column; 7 = detector; 8 = recorder; 9 = three-way valve; 10 = fraction collector.

RESULTS

Chromatographic separation of methyl derivatives

The chromatogram of the reaction mixture of dopamine (DA) displayed four peaks by radioactivity determination [Fig. 2B]. The peak appearing at the front was a mixture of [^{3}H] SAM and its degradation products. Degradation of SAM

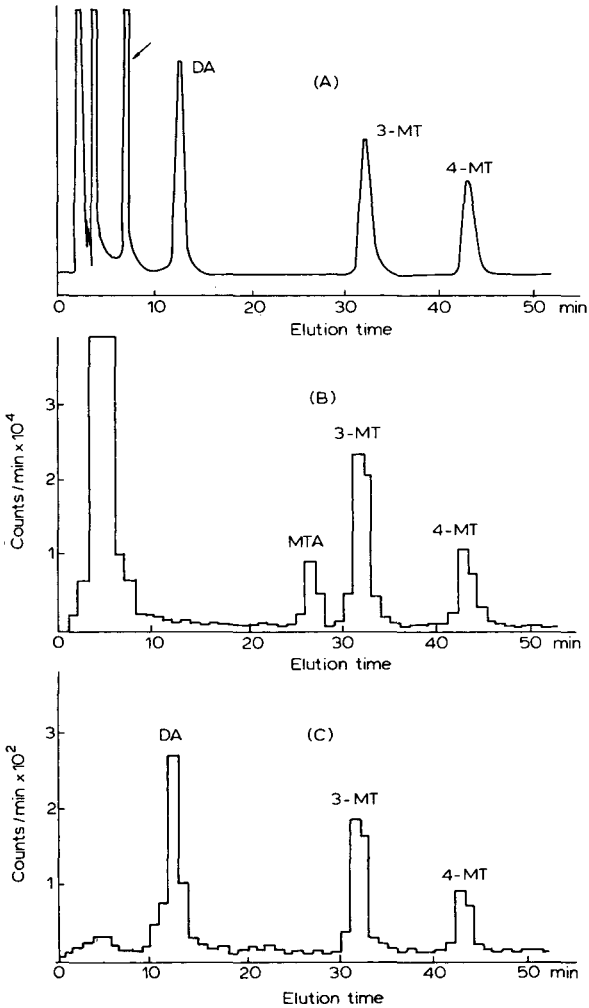

Fig. 2. Chromatogram of dopamine and its O-methylated derivatives using UV and radioactivity detection (A, absorbance at 254 nm; B, ^{3}H radioactivity; C, ^{14}C radioactivity). Sample was a quarter of a 100-µl reaction mixture of [^{14}C]dopamine (15 ng, 4 µCi), porcine COMT (30 units) and [^{3}H]SAM (1 µCi, spec. act. 11.0 Ci/mmol). DA, 3-MT and 4-MT (75 µg each) were added as carriers after enzyme reaction. Column was LiChrosorb RP-18, 10 µm, 250 × 4 mm I.D. Eluent was acetonitrile—water (7 : 93, v/v) containing 0.5 g of sodium heptanesulfonate and 4.0 ml of acetic acid (adjusted to pH 4.4 with NaOH). Flow-rate was 1.0 ml/min; temperature, ambient.

to MTA was also observed. Two peaks appeared from the enzyme reaction of dopamine and their elution times corresponded to those of authentic 3-MT and 4-MT (Fig. 2A and B). The peaks of dopamine derivatives were further examined by methylating [^{14}C]dopamine under the same conditions (Fig. 2C). The ratio of ^{14}C to ^{3}H in each peak was identical and essentially the same as the ratio of specific activity of [^{14}C]dopamine and [^{3}H]SAM. The relative quantity of 3-MT to 4-MT was not significantly changed when rat liver COMT in the KAT-A-KIT was used in place of porcine liver COMT.

The enzymatic methylation of norepinephrine or epinephrine also yielded two peaks. The peaks eluted earlier had retention times corresponding to authentic NMN and MN. Peaks eluting later were assumed to be 4-O-methyl derivatives. In these chromatographic conditions, six peaks from three catecholamines and MTA were resolved and no other distinct peak was observed except for [^{3}H] SAM and its degradation products which eluted at the front. However, a high level of radioactivity, 250—300 cpm, was observed between peaks (Fig. 3).

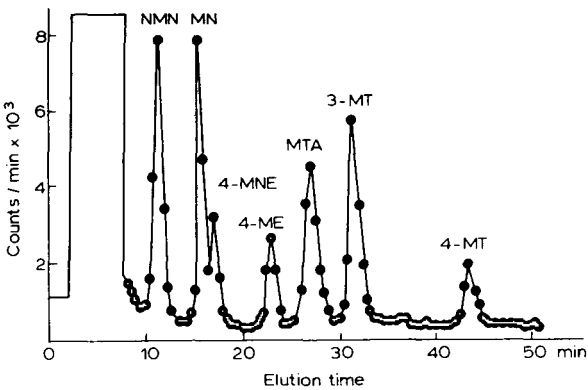

Fig. 3. Chromatogram of O-methylated catecholamines. Sample was a quarter of a 100-μl reaction mixture of catecholamines (4 ng each). Other conditions as in Fig. 2.

Boric acid gel treatment and branched flow with a precolumn

Extraction of the methyl derivatives with a mixed solvent of isoamyl alcohol and toluene is a usual procedure to separate them from [^{3}H] SAM and its degradation products. In place of solvent extraction, a combination of two simple procedures was examined in the present investigation. A gel which has dihydroxyboronyl groups chemically bonded on to polystyrene—dinivylbenzene copolymer is available and has been used to adsorb nucleosides [13], saccharides [14] and catecholamines [15]. Compounds with vicinal diol groups in the reaction mixture, such as SAM and MTA, were expected to be adsorbed by the boric acid gel from alkaline solution.

Elution of the methyl derivatives from the boric acid gel column was examined (Table I). The methyl derivatives were quantitatively eluted with 0.25 *M* Tris—NaOH solution (pH 13), while almost all of the SAM and MTA was retained by the column. However, an excessive amount of radioactivity from degradation products, appearing at the front of the chromatogram in Fig. 3, was not adsorbed by the boric acid gel.

Because the radioactivity of baseline fractions decreased as elution was continued and reached the level of natural background after about 2 h, small quantities of radioactive compounds seemed to be adsorbed strongly on the column and eluted gradually, resulting in prolonged tailing. To minimize contamination of the column with radioactivity from the degradation products, the flow system in Fig. 1 was used.

By employing the boric acid gel treatment and branched flow system, radioactivity of the baseline fractions was reduced to ca. 50 cpm, roughly twice the natural background.

TABLE I

EFFECT OF pH OF ELUENT ON RECOVERY (%) OF 3-O-METHYL CATECHOLAMINES, SAM AND MTA FROM BORIC ACID GEL COLUMN

Sample solution was 3-O-methyl catecholamines (75 μg) or SAM and MTA (2 μg) in the buffer solution (100 μl). The sample solution was made alkaline and passed through a boric acid gel column (17 × 3 mm I.D.). Elution was carried out with 3 × 100 μl of 250 m*M* Tris—NaOH solution of various pH values.

Compound	pH of eluent				
	10.0	11.0	12.0	13.0	13.7
NMN	78	95	100	100	85
MN	52	76	87	100	85
3-MT	18	35	41	98	88
SAM				5	52
MTA				5	40

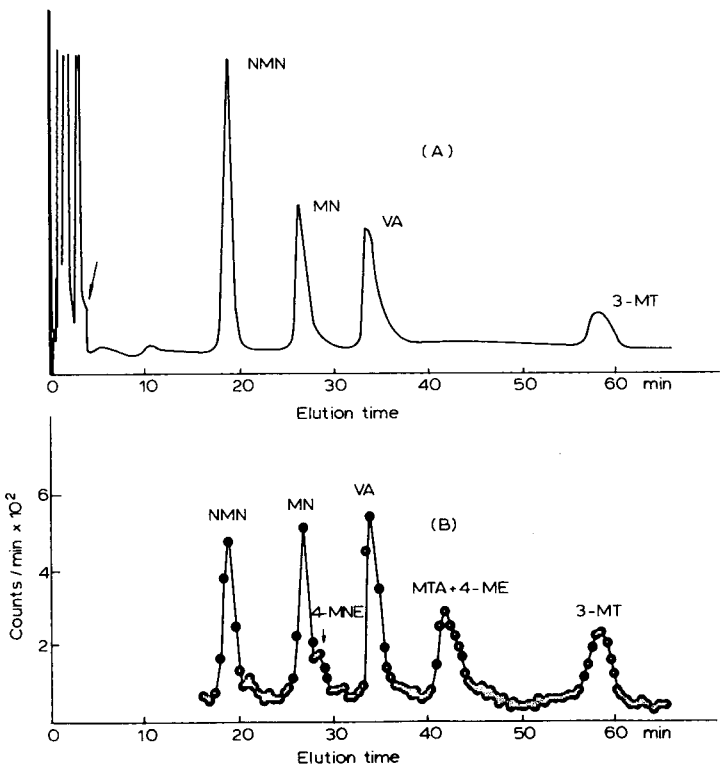

Fig. 4. Chromatograms of O-methylated catecholamines and vanillylamine using the branched flow system (A, absorbance at 254 nm; B, ^3H radioactivity). Sample was a quarter of a reaction mixture of catecholamines (15 pg each), porcine COMT (30 units) and [^3H]-SAM (1 μCi, spec. act. 85.0 Ci/mmol) with carriers after boric acid gel treatment. Precolumn was LiChrosorb RP-18, 10 μm, 100 × 4 mm I.D. Main column was LiChrosorb RP-18, 10 μm, 250 × 4 mm I.D. Eluent was acetonitrile—water (7:93, v/v) containing 0.5 g of sodium heptanesulfonate and 1.4 ml of acetic acid (pH adjusted to 4.4 with NaOH) per liter. Flow-rate was 1.0 ml/min; temperature, ambient. Main column is connected at the time shown by the arrow (chromatogram A).

A chromatogram of a standard mixture of the three catecholamines was obtained using the above procedure (Fig. 4). The amounts of the methyl derivatives injected corresponded to ca. 4 pg of catecholamines in the enzyme reaction mixture. The time required for elution of the methyl derivatives was increased by the use of the precolumn and reduction of the acetic acid concentration in the eluent. 4-Methylnorepinephrine (4-MNE) was eluted as a shoulder of the MN peak and a peak of 4-methylepinephrine (4-ME) overlapped that of MTA leaked from the boric acid gel column.

DHBA was also methylated by COMT and was added to the reaction mixture to detect accidental or unexpected failure of the enzyme reaction. Under the reaction conditions used, approximately 70% of DHBA was converted to vanillylamine, while no significant amounts of isovanillylamine, the 4-methyl derivative of DHBA, which should elute between MTA and 3-MT, were formed.

Determination of catecholamines in serum

Catecholamines were determined from the radioactivity of the 3-methyl derivatives, since 3-O-methylation proceeds preferentially under the reaction conditions used. The radioactivity was corrected by the recoveries of methyl derivatives added as carriers. Standard curves of norepinephrine, epinephrine and dopamine were obtained between 4 pg and 48 pg per 50 μl (Fig. 5). Rates of formation of 3-methyl derivatives were calculated from specific activity of

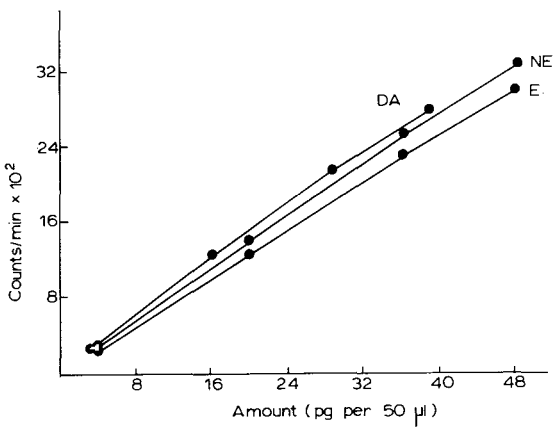

Fig. 5. Standard curves of catecholamines. NE = Norepinephrine; E = epinephrine; DA = dopamine.

TABLE II

RECOVERY OF NE, E AND DA ADDED TO DEPROTEINIZED SERUM

Amount added (pg per 50 μl)	Found (pg per 50 μl)*		
	NE	E	DA
8	8	8	7
16	15	14	16
24	25	26	24
40	38	41	40

*Endogenous catecholamines are subtracted.

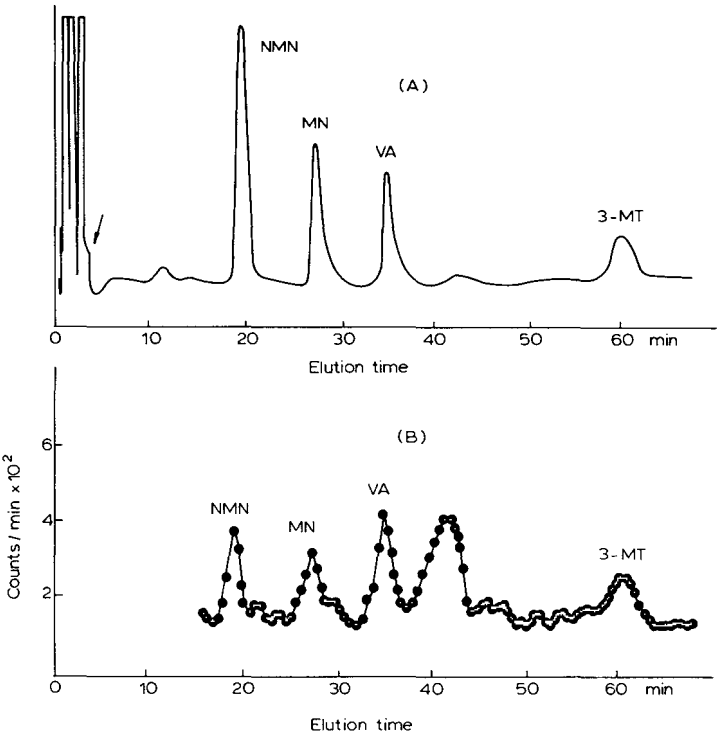

Fig. 6. Chromatograms of O-methylated serum catecholamines (A, absorbance at 254 nm; B, ^3H radioactivity). Sample was a quarter of the methylated products from 50 μl of deproteinized serum. Other conditions as in Fig. 4.

TABLE III

DETERMINATION OF SERUM CATECHOLAMINES

Serum sample	Amount (pg/ml)		
	NE	E	DA
A	516	177	244
B	236	147	144
C	436	154	177
	404	185	170
	423	170	165

[^3H] SAM, and were 60, 60 and 57% for norepinephrine, epinephrine and dopamine, respectively, at a concentration of 20 pg per 50 μl. The rates decreased slightly with increase in catecholamine concentration.

The procedure was applied to the analysis of serum catecholamines (Fig. 6). Although the radioactivity of the baseline fractions increased more than two times, distinct peaks corresponding to three catecholamine derivatives were observed. The peak eluting after VA has approximately the same retention time as MTA and 4-ME; however, it also contains an unidentified component from serum. The recoveries of catecholamines added to 50 μl of deproteinized

serum were practically quantitative between 8 and 40 pg (Table II). This indicated that standard curves for artificial mixtures were applicable to the analysis of serum. Results of analysis of sera from three healthy adults are listed in Table III. Serum C was analysed three times and the relative average deviations were between 3 and 6%. The values in Table III were in the range of catecholamine concentrations reported for healthy adults [7, 16, 17].

DISCUSSION

Since this procedure was designed for routine analysis of catecholamines, chemical reactions like periodate oxidation [6] and cumbersome manipulations like solvent extraction and thin-layer chromatographic separation [12] were avoided. Instead, we used boric acid gel column and liquid chromatography for isolation of the methyl derivatives.

Catecholamines are known to be methylated by COMT at both the 3 and the 4 positions on the catechol ring and the relative quantity of the two isomers depends on the reaction conditions and the nature of the enzyme [18, 19]. However, little consideration has been given to this in radioenzymatic assay procedures previously reported. In the present procedure, substantial amounts of 4-methyl derivatives were produced. The relative quantity of 4-methyl derivatives was not significantly reduced by using rat liver COMT in a commercially available catecholamine assay kit, KAT-A-KIT. The rate of formation of 4-methyl derivatives in the enzyme reaction was reported to be independent of catecholamine concentration [20], but it seems to decrease at extremely low concentrations, although they were always detectable. From these observations it can be seen that the isomers should be separated from the methyl derivatives for accurate determination of each catecholamine, as in the present procedure.

The sensitivity of radioenzymatic assay depends on several factors. These are the specific activities of isotope derivatives, rate of enzyme reaction, recovery of products during isolation and signal-to-noise ratio in the determination. In the present procedure, we used [^{3}H] SAM, tritiated about 90%, for the determination of picogram levels of catecholamines; we obtained about 60% of catecholamines as 3-methyl derivatives and reduced the contaminating radioactivity to ca. 120 cpm including natural background. Consequently, it is still possible to increase the sensitivity by finding the reaction conditions under which 3-methyl derivatives are produced quantitatively and by removing all the contaminating radioactivity from reaction products.

In conclusion, an accurate and sensitive procedure for the determination of catecholamines has been developed. The procedure requires little expertise by the use of boric acid gel column and ion-pair liquid chromatography with a branched flow path.

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CHROMBIO. 790

MEASUREMENTS OF POLYAMINES AND THEIR ACETYLATED DERIVATIVES IN CELL EXTRACTS AND PHYSIOLOGICAL FLUIDS BY USE OF AN AMINO ACID ANALYZER

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SUMMARY

A fast and sensitive method for the determination of free polyamines and their acetylated derivatives is presented. The separation is carried out on a Durrum DC-6A cation-exchange resin with an automated amino acid analyzer. The determination is based on a stepwise elution with a sodium chloride—sodium citrate buffer system. Detection is done by fluorescence of the *o*-phthaldialdehyde—polyamine conjugates. The sensitivity is in the picomole range. No prior purification step is needed. The method has been applied to cell extracts and urine samples.

INTRODUCTION

The significance of polyamines in biochemical and physiological processes has stimulated the development of a large number of methods for their rapid and sensitive determination [1]. Ion-exchange column chromatography with automated instruments is one of the most suitable methods for routine analysis of the polyamines in cell extracts and body fluids. The methods described to date have been exclusively concerned with the separation of free polyamines [2, 3]. However, recently, evidence has been accumulating that points to the importance of acetyl derivatives of polyamines in cell metabolism. The monoacetyl derivative of putrescine has been found in the human brain [4] and in various other tissues [5]; spermidine is excreted in human urine, almost exclusively as acetylspermidine both in normals and cancer patients [6, 7].

A method of separating both the conjugates and the free polyamines has been described recently [8]. This method is based on pre-separation on Dowex resin, derivatisation by dansylation and subsequent thin-layer chromatography.

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For a rapid quantitative determination of the conjugates and the free polyamines no routine method is as yet available. We describe here the separation of free and conjugated polyamines by ion-exchange chromatography in a single step using the automatic amino acid analyzer. The application of this method to cell extracts and human urine is shown.

EXPERIMENTAL

Chemicals

Chemicals for buffers and reagents were obtained from Merck (Darmstadt, G.F.R.) and were of analytical grade quality. The non conjugated polyamine hydrochlorides were from Sigma (Munich, G.F.R.). Acetylputrescine and N^1-acetylspermidine were kindly donated by Dr. N. Seiler, Centre de Recherche Merell International (Strasbourg, France).

Sample preparations

Cell extracts were obtained from the gram-positive *Bacillus subtilis* or from the slime mould *Dictyostelium discoideum.*

B. subtilis, strain W 23, was grown in Tris-buffered glucose salt medium [9] to a density of 0.5 E_{578}. An aliquot of 5 ml from a 20-ml shaking culture was centrifuged without chilling and the cells were washed twice using 5 ml of distilled water in each case. Polyamines were twice extracted from the cells with 500 μl 10% trichloroacetic acid (TCA) for 1 h at 4°C with shaking.

The slime mould *Dictyostelium discoideum* was grown in axenic medium [10] to a density of $3 \cdot 10^6$ cells per ml. Cells were harvested by centrifugation, washed twice with buffer (2 m*M* Na_2HPO_4–14.7 m*M* KH_2PO_4, pH 6.7) and twice extracted for 1 h at 4°C with 100 μl of 10% TCA per ml cell suspension. One-milliliter samples of human urine were filtered through a Millipore filter (0.22 μm) and diluted with 500 μl 0.1 *N* hydrochloric acid, 250 μl ethanol and 250 μl buffer 1 (see Table I) specially adjusted to pH 1.9 (urine dilution A). A 100-μl aliquot of urine sample A was diluted further with 100 μl buffer 1 (urine dilution B). Various amounts of these urine samples were used for analysis.

TABLE I

COMPOSITION OF BUFFER AND CONDITIONS OF ELUTION

Number of buffer	Sodium chloride*	Sodium citrate dihydrate*	Ethanol (%)	Final pH	Temperature (°C)	Time (min) of programmed buffer change
1	–	0.04	5	2.90	73	35
2	0.2	0.3	5	6.20	68	20
3	0.9	0.3	5	5.55	68	22
4	3.0	0.3	5	5.55	68	29

*Molarity of Na^+.

Conditions of polyamine analysis

All analyses were run on a Biotronik LC 6000 E instrument. The 10 × 0.5 cm column was filled with Durrum DC-6A resin (11 μm) up to a bed height of 7.5 cm. Conditions of the analysis: temperature, elution time and the composition of eluting buffers were as indicated in Table I. The back pressure during a complete analysis did not exceed 43 kP/cm^2 in the buffer pump and 13 kP/cm^2 in the reagent pump. The flow-rates were 42 ml/h for the buffers and 28 ml/h for the reagent. Buffers were made with water that had been deionised, double distilled and cleaned through a LOBAR B column (Merck) or a Milli Q apparatus. The buffers were adjusted to a pH of about 0.5 units above the final value with 6 *N* hydrochloric acid. After filtration of the buffer through a Millipore filter (0.22 μm) the final pH was adjusted and the ethanol added.

The reagent was prepared by dissolving 400 μg of *o*-phthalaldehyde in 20 ml ethanol, and adding this solution to a nitrogen-saturated solution of 74.2 g boric acid, 60.0 g potassium hydroxide, 5 ml Brij 35 (30%, w/v) and 5 ml β-mercaptoethanol in 2 l water.

The column was regenerated with 0.6 *N* sodium hydroxide for 15 min and subsequently equilibrated with buffer 1 for 20 min. After each run the resin was allowed to expand for 5 min.

RESULTS

Standardisation of the procedure

Marton and Lee [3] have described the basic procedure for the separation of the non-conjugated polyamines by use of an amino acid analyzer. They have shown that the reaction product of polyamines with *o*-phthalaldehyde gives a linear absorption curve over a wide concentration range. The relationship between sample concentration and relative peak area is also linear for acetylputrescine and acetylspermidine in the 100—2000 pmol range.

The coefficients of variation (C.V.) of the integrated areas were calculated from five runs of a standard mixture of the free and the acetylated polyamines (500 pmol each) (Table II).

TABLE II

COEFFICIENTS OF VARIATION

In each case $n = 5$. A standard mixture containing 500 pmol each of the free and the acetylated polyamines was used for each run.

Peak*	Integrated area ($\bar{X}$)	S.D.	C.V. (%)
Ac-Put	252 033	8 265	3.2
Ac-Spd	244 895	3 934	1.6
Put	182 209	1 910	1.0
Spd	480 048	10 156	2.1
Sp	307 236	7 279	2.3

*For abbreviations, see legend to Fig. 1.

An integrator is coupled to the fluorimeter and quantifies the peaks. The integrator was calibrated every day with a standard mixture of 500 pmol of each polyamine.

Recovery was measured by adding 500 pmol of each polyamine to a test sample, and was found to be 92—105%.

From a standard solution containing 500 pmol each of free and acetylated polyamines, 3 nmol of an amino acid standard (Hamilton) containing 19 amino acids and about 10 nmol ammonia, all the polyamines and their derivatives can be separated (Fig. 1). From 5—10 pmol of spermidine and 15—20 pmol of the other polyamines up to 2000 pmol can be measured accurately.

Buffer 1 elutes the acidic and neutral amino acids in two peaks and separates the basic amino acids. The most important prerequisite is the sufficient separation of ammonia and acetylputrescine, since in human urine ammonia is in large excess over acetylputrescine. Buffer 2 elutes some basic amines, which have not been characterised further. Buffer 3 separates acetylspermidine, 1,3-diaminopropane and putrescine, whereas buffer 4 elutes spermidine and spermine.

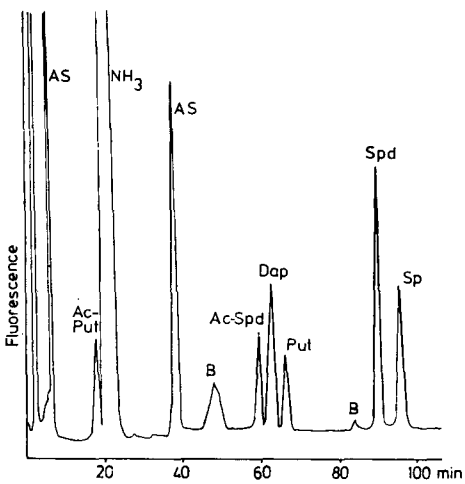


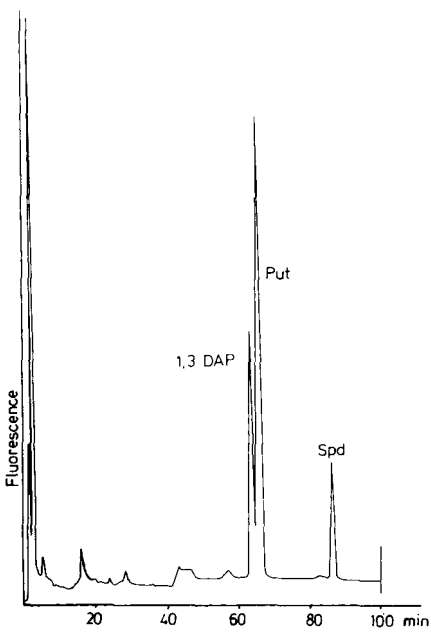
Fig. 1. Chromatogram of a standard mixture of polyamines (500 pmol each), amino-acid mixture (3 nmol) and ammonia (10 nmol). Peaks: AS = amino acid mixture; Ac-Put = monoacetylputrescine; NH_3 = ammonia; Ac-Spd = N^1-monoacetylspermidine; Dap = 1,3-diaminopropane; Put = putrescine; Spd = spermidine; Sp = spermine; B = peaks occurring occasionally after buffer change. (N^8-monoacetylspermidine has exactly the same retention time as N^1-monoacetylspermidine.)

Application of polyamine analysis

The polyamine content was estimated in cell extracts of *Bacillus subtilis*. The chromatographic profile of an extract of *B. subtilis*, grown in a minimal medium shows that spermidine synthesis predominates in these cells whereas putrescine is found only in trace amounts. Acetylated polyamines are not present in the extracts.

The slime mould *Dictyostelium discoideum* represents a good system for the study of cell development. The cells can be grown vegetatively in a maximal medium and development can be induced by starvation. The chromatogram of a sample of an extract of *Dictyostelium discoideum* is shown in Fig. 2. This lower eukaryote contains spermidine, putrescine and high amounts of 1,3-diaminopropane, but no spermine.

The analysis of free polyamines and their conjugates in human urine needs two separate runs. Because of the large excess of amino acids and ammonia, the simultaneous determination of acetylputrescine with all the other polyamines is not possible. In the first run we determined the concentration of acetylputrescine in a 30-min chromatogram with buffer 1 only. Normally a 10-μl sample of urine dilution B is sufficient to permit quantification and good separation from amino acids and ammonia (Fig. 3). The complete disappearance of the acetylputrescine peak after hydrolysis shows that no other compound interferes with this peak. The other polyamines were determined with 150 μl of urine dilution A; buffer 2 is used as the starting buffer (Fig. 4).



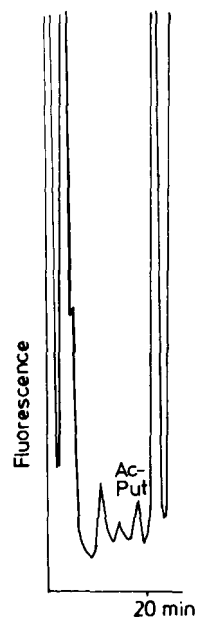


Fig. 2. Chromatogram of trichloroacetic acid extracted polyamines from *Dictyostelium discoideum* (20-μl sample). Peaks: 1,3 DAP = 1,3-diaminopropane; Put = putrescine; Spd = spermidine.

Fig. 3. Chromatogram of a sample of human urine (normal male individual); 10 μl urine dilution B to determine acetylputrescine (Ac-Put).

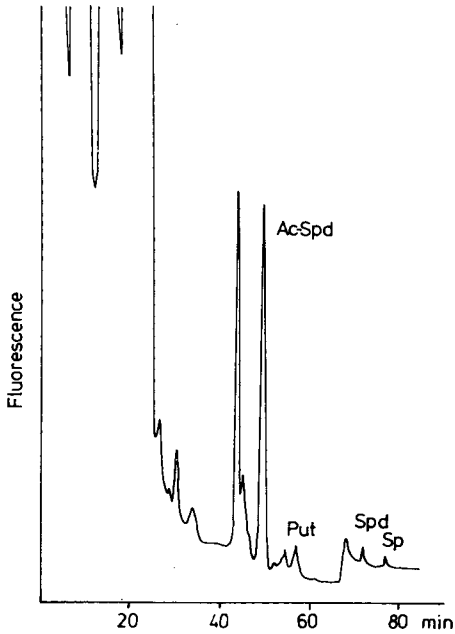


Fig. 4. Chromatogram of a sample of human urine, 150-μl sample of urine dilution A, buffer 2 is used as the starting buffer. Peaks: Ac-Spd = N^1-monoacetylspermidine; Put = putrescine; Spd = spermidine; Sp = spermine.

CONCLUSION

In the present work we describe a method which permits the separation and the quantitative determination of the free polyamines as well as their acetylated derivatives, on a Durrum DC-6A resin using an automatic amino acid analyzer. The advantages are that prior purification of the polyamines is not necessary. This prior purification step is often time-consuming and entails the risk of losing compounds present in trace amounts. Reproducibility and sensitivity of the method are excellent. The reason for the peaks occasionally occurring after buffer change has not yet be discovered — most probably they are due to contaminations in the buffer chemicals. The method is now being used in our laboratory to study the influence of polyamines on cell development. In a clinical study we are using it to investigate the excretion of polyamines in the urine of cancer patients undergoing chemotherapy.

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CHROMBIO. 793

PURIFICATION AND ASSAY OF BOVINE PARATHYROID HORMONE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) has been used to purify a crude extract of bovine parathyroid glands, in a single run on an analytical column, to give a high yield of homogeneous material with full bioactivity in in vivo bioassay. Bovine parathyroid hormone (bPTH) prepared and purified by conventional procedures has been rapidly and quantitatively separated from its oxidation and other degradation products, from hormone fragments and from non-hormonal contaminants. Recovery of bPTH, monitored by region-specific immunoassays, in vivo bioassay and re-chromatography on HPLC was > 93%. The detection limit of the HPLC system, using endogenous tryptophan fluorescence, was 20 ng bPTH.

INTRODUCTION

Conventional methods for the purification and assay of extracted bovine parathyroid hormone (bPTH), reviewed in ref. 1, are associated with cumulative loss of up to 40% of the hormone during lengthy gel and ion-exchange

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chromatography and with costly, laborious and insensitive bioassay. We here report the use of a reversed-phase high-performance liquid chromatographic (HPLC) system, already exploited for the study of many other peptides in complex biological materials [2—5], to purify a crude bPTH extract in a single preparative run on an analytical column to give a high yield of homogeneous material with in vivo biological activity of 2300 IU/mg.

MATERIALS AND METHODS

Preparations

Eight bPTH materials studied by HPLC included two bulk preparations — crude trichloroacetic acid precipitated bPTH (TCA bPTH, batch 280178), and highly purified bPTH (bPTH, batch 100375), both prepared at the National Institute for Biological Standards and Control; four ampouled preparations — the WHO International Reference Preparation of Parathyroid Hormone, Bovine, for Immunoassay (IRP ibPTH, ampoule code 71/324), IRP bPTH for Bioassay (IRP bioPTH, ampoule code 67/342), Medical Research Council Clinical Reagent (MRC bPTH, code 72/286), and NIBSC Clinical Reagent (NIBSC bPTH, code 77/533). Ampouling was carried out according to procedures used for international biological standards [6]. Other materials included in the study were oxidized bPTH (batch 100375) containing less than 10% residual bioactivity by in vivo bioassay, a common contaminant believed to be bovine haemoglobin α-chain [7], synthetic bPTH fragment 1—34 and enzymatically cleaved fragment 53—84 (kindly supplied by Dr. J. Tregear and Dr. H. Keutmann).

HPLC methods

Gradient conditions and equipment used have been described previously [3, 4] and are indicated in the legend to Fig. 1. The system used ODS-Hypersil (Shandon Southern, Runcorn, Great Britain) in 150 × 4.6 mm I.D. stainless-steel columns. The primary solvent (in which samples were injected) was 0.155 *M* sodium chloride (pH 2.1) and components were separated at 35°C using a gradient of three linear segments, with acetonitrile as the secondary solvent, at a flow-rate of 1 ml/min. The eluate was monitored sequentially by UV absorbance at 215 nm and by endogenous tryptophan fluorescence (activation wavelength 225 nm, emission filter 340 nm). Isocratic separations were carried out at ambient temperature on Nucleosil 5 C8 (Macherey, Nagel & Co., Düren, G.F.R.) in 100 × 5 mm stainless-steel columns. The mobile phase was 0.155 *M* sodium chloride (pH 2.1)—acetonitrile (70:30) at a flow-rate of 0.5 ml/min [5]. Fractions were collected in narrow glass or polypropylene tubes containing 0.1 ml of 0.1% bovine serum albumin, snap-frozen, lyophilized and stored at −40°C until reconstituted in the appropriate buffer for immuno- or bioassay.

Radioimmunoassay

An overnight radioimmunoassay (RIA) system was used with ^{125}I-bPTH (batch 100375), and antisera Burroughs Wellcome 211/32 (NIBSC Code 76/507) (final dilution of 1/300,000 for intact and amino-region bPTH) and NIBSC/Wellcome 1127/21 (final dilution 1/300,000 for intact and carboxyl-region bPTH). Separation of bound and free fractions was achieved in 4 h using

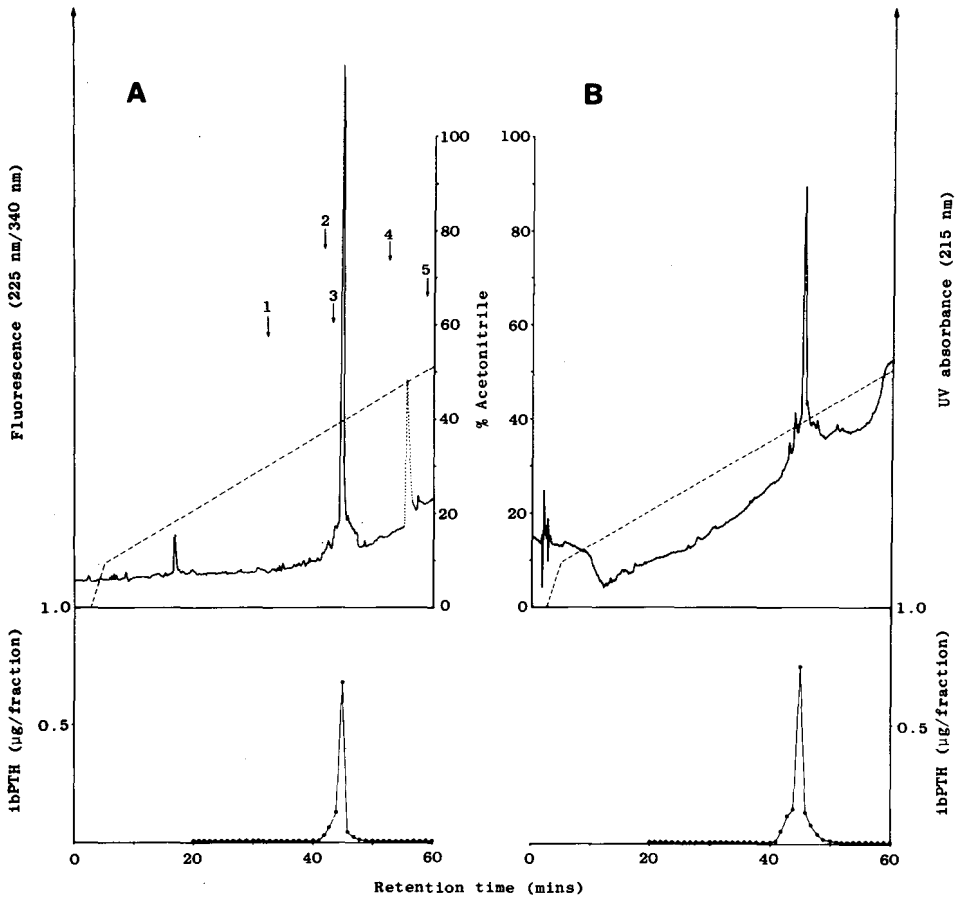

Fig. 1. Gradient elution HPLC of bovine parathyroid hormone on ODS-Hypersil. Separations were carried out at 35°C and constant flow-rate of 1 ml/min, with 0.155 *M* sodium chloride (pH 2.1) as primary solvent and acetonitrile as the organic modifier (see dashed line for gradient profile). (A) Fluorescence trace from highly purified bulk bPTH (100375) (the dotted peak represents a solvent impurity); (B) UV absorbance of an ampouled preparation (77/533). The corresponding profiles of immunoreactivity (ibPTH) in 1-ml eluent fractions are shown below each trace. The arrows on (A) indicate the retention times of (1) $bPTH_{53-84}$, (2) oxidised bPTH (100375), (3) $bPTH_{1-34}$, (4) human serum albumin and (5) bovine haemoglobin α-chain.

a polyethylene-glycol-accelerated second antibody precipitation. The detection limit of intact bPTH in both RIA systems, using IRP ibPTH as standard and assuming 1 µg bPTH per ampoule was 0.9 ng/ml; 50% displacement was 9 ng/ml. An aliquot of the solution loaded on to the HPLC columns was included in serial dilution in the RIA as the standard curve for estimation of recovery. HPLC fractions were assayed at 2—4 dilutions to screen for immunochemical heterogeneity.

In vivo bioassay

The intact chick, 1-h acute hypercalcaemia [8] assay was carried out, and the

doses were injected intravenously. The bioassay house standard was highly purified ampouled MRC Clinical Reagent (code 72/286) and results expressed in International Units defined by the IRP bio-PTH standard. It is generally accepted that bulk highly purified bPTH can be expected to have an in vivo biological activity of approximately 2500 IU/mg [1].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in 6 *M* urea was carried out according to the procedure of Panyim and Chalkley [9]. Samples from HPLC were loaded in up to 50 μl of eluent after evaporation of acetonitrile.

RESULTS AND DISCUSSION

Chromatograms for carrier-free highly purified bPTH and an ampouled preparation of highly purified bPTH (code 77/533) together with profiles of immunoreactivity (obtained with the RIA for intact and amino-region bPTH) are shown in Fig. 1A and B. The retention times for oxidized bPTH, haemoglobin α-chain and the 1—34 and 53—84 bPTH fragments are marked on Fig. 1A for reference. In all preparations there were indications of a shoulder at each side of the main peak. Isohormones have been described [10] and it is possible that the HPLC systems used may partially resolve these. A consistent pattern of small peaks eluting close to the main component, present in bulk and ampouled preparations (77/533 and 72/286) of highly purified bPTH, was seen in both UV and fluorescence traces. Relative proportions of the peaks varied but no single minor peak contained more than 10% of total UV absorbance and 5% total immunoreactivity. More than 90% of UV absorbance and tryptophan fluorescence was associated with the major peak for the IRP ibPTH (code 71/324), previously characterized as bPTH isohormone I [10]. Immediate reinjection of the major HPLC peak fraction of highly purified bPTH 100375, after dilution in primary solvent, yielded only a single peak of identical size and gave a single band on polyacrylamide gel electrophoresis in 6 *M* urea (Fig. 2). Minor components in bPTH ampoule 77/533 were more clearly seen under isocratic conditions (Fig. 3). Rechromatography of the main peak fraction after it had been lyophilized under laboratory conditions showed about 12% of the total UV absorbance appearing as an earlier peak. Controlled oxidation of highly purified bPTH with hydrogen peroxide yielded a series of peaks whose retention times corresponded to some of the minor components seen in the preparations tested. However, more vigorously oxidized bPTH reference material was not retained under the isocratic conditions, although eluting only 3 min before bPTH on gradient systems (Fig. 1A).

Analysis of the IRP bioPTH (ampoule code 67/342), which had been prepared by TCA precipitation and which was known to give anomalous results in some in vivo and in vitro bioassay systems, showed an atypical gradient elution chromatogram (Fig. 4) when compared with other bPTH preparations. The peak identified as bPTH by its retention time and immunoreactivity was superimposed on a broad fluorescence and UV absorbing background peak. It is not known whether this was an atypical batch of TCA bPTH (the bulk TCA bPTH, prepared in 1966, is no longer available) or whether artifacts had arisen at one or more stages of the ampouling procedure.

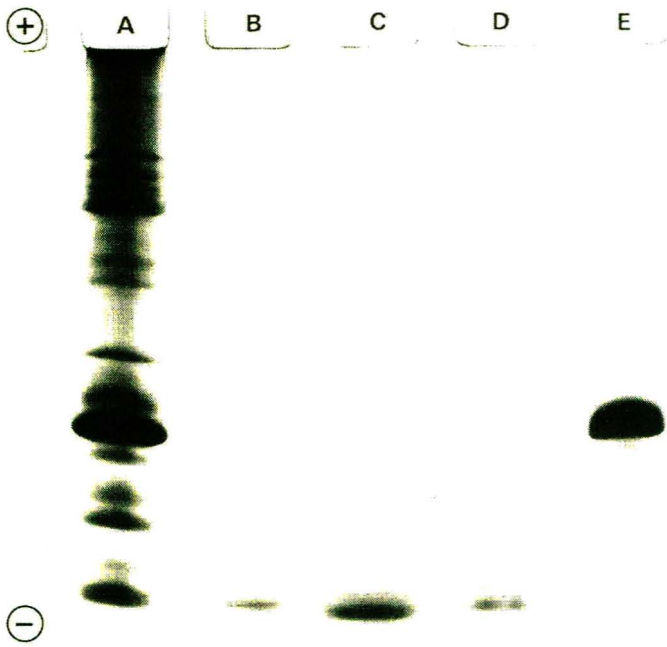

Fig. 2. Polyacrylamide gel electrophoresis of bulk and HPLC-purified bPTH preparations. Separations were carried out on 15% gels with 6 *M* urea using 0.9 *N* acetic acid (pH 2.5). (A) TCA precipitate of parathyroid glands (280178) containing approximately 2—3% bPTH; (B) bPTH purified from TCA precipitate (280178) by HPLC; (C) highly purified bPTH (100375) before HPLC; (D) bPTH (100375) after HPLC; (E) bovine haemoglobin α-chain from parathyroid glands.

A chromatogram and an immunoreactivity profile for bulk crude TCA bPTH (280178) are shown in Fig. 5. More than 79% of the total immunoreactivity corresponded to the fluorescent peak at 45 min and comparison of peak height with that given by highly purified bPTH (bulk batch 100375 used as HPLC reference) indicated a bPTH content of 30 μg/mg, in good agreement with the original bioassay estimate of potency of 50 IU/mg (approximately 2% pure), and also with measurement of total recovered immunoreactivity (35 μg). The practical detection limit based on tryptophan fluorescence was 20 ng with a linear response between 20 ng and at least 12.5 μg, and a precision for replicate assays of 2.2% (C.V., n = 7). After preparative HPLC (10-mg load), which gave an identical profile to that shown in Fig. 5, the bPTH peak yielded material on which a bioassay estimate of 2300 IU/mg (95% confidence limits 1700—3100 IU/mg) was obtained, and which gave a single band on PAGE (Fig. 2).

There was no significant indication from results of the two RIA systems of immunochemical heterogeneity in the major (or minor) peaks after HPLC of two crude and three highly purified preparations. As, furthermore, none of the minor HPLC peaks resolved by isocratic chromatography (Fig. 3) had retention times equal to those of fragments of known structure, it seems likely that heterogeneity revealed by this HPLC system was not due to the presence of hormonal fragments.

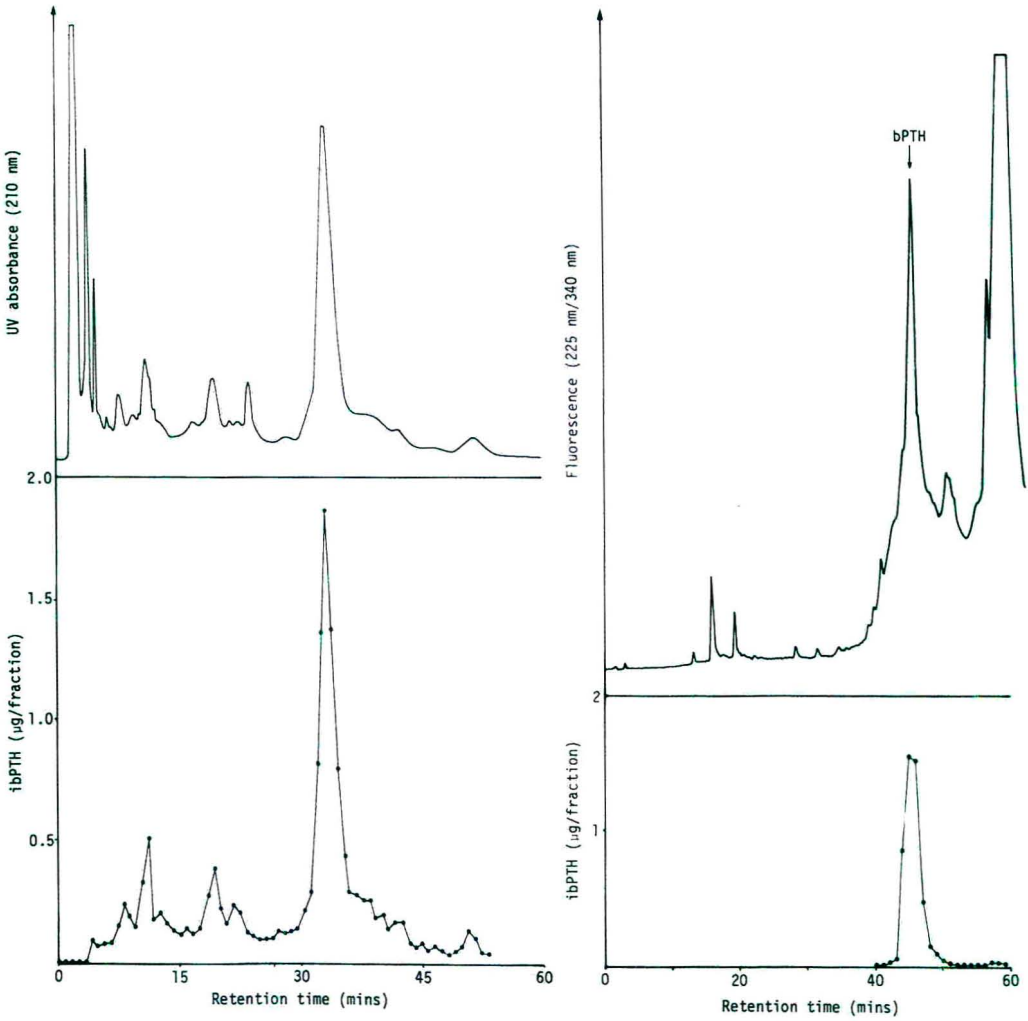

Fig. 3. Isocratic HPLC of an ampouled bovine parathyroid hormone preparation (77/533) on Nucleosil 5 C8. Separations were obtained at ambient temperature and a flow-rate of 0.5 ml/min using a mobile phase of 0.155 *M* sodium chloride (pH 2.1)—acetonitrile (70:30). The upper trace shows the UV absorbance (210 nm) (cf. Fig. 1B) with the corresponding immunoreactivity profile in 200-µl eluent aliquots below.

Fig. 4. Gradient elution HPLC of ampouled International Reference Preparation for Bioassay (bioPTH 67/342) prepared by TCA precipitation. Chromatographic conditions were as given in Fig. 1. The fluorescence chromatograms and the corresponding immunoreactivity in 1-min eluent fractions are shown. The retention time of a bPTH reference standard (100375) is indicated for comparison (cf. Fig. 1). Note that the bPTH 67/342 gave a relatively broad fluorescent/immunoreactive peak compared with other preparations using gradient elution.

Retention of bPTH on the reversed-phase supports used under the conditions described is expected to depend on the content of hydrophobic residues; thus reversed-phase HPLC is complementary to conventional evaluation of bPTH preparations by molecular size or charge. Oxidation of methionine residues will reduce hydrophobicity and oxidation of bPTH results in the appearance of

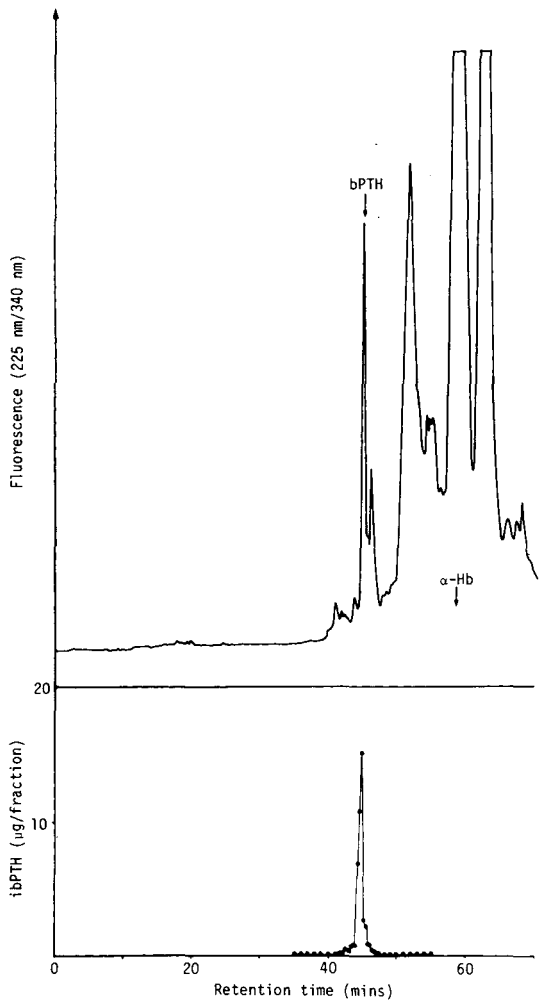

Fig. 5. Gradient elution HPLC of 1 mg of bulk crude TCA precipitated bPTH (280178). Chromatographic conditions were as given in Fig. 1. The fluorescence profile of this material is shown, together with the associated immunoreactivity in 18-sec eluent fractions. The retention times of highly purified bPTH (100375) and of haemoglobin α-chain are also indicated. The total amount of immunoreactive material recovered (35 μg) corresponded well with the relative peak height on the fluorescence trace (equivalent to 30 μg bPTH 100375).

peaks with decreased retention times. Some minor peaks eluting before the main peak in bPTH are probably attributable to different oxidation products arising during isolation and purification and, possibly, during ampouling procedures. The nature of the minor peaks eluting after the main peak is unknown. However, it has been suggested that deamidation of glutamine and asparagine residues may occur during the acid conditions of extraction and conventional purification of bPTH [1, 11]. Such deamidation products might be expected to have longer retention times than the native molecule, as found for insulin [12, 13].

In summary, reversed-phase HPLC under gradient conditions, complemented by isocratic chromatography for detailed examination of closely associated components has been shown to be (1) a rapid, reproducible, specific, sensitive and quantitative system for the assay of bPTH in crude and highly purified bulk materials and in preparations ampouled with carrier substances; (2) a high-resolution system for the assessment of heterogeneity of highly purified bPTH by the separation of bPTH from its oxidation and other degradation products, small peptide fragments, a common contaminant (bovine haemoglobin α-chain) and from added carrier proteins (serum albumins); (3) a rapid and complete system for high-yield purification of homogeneous bPTH with high biological activity from a crude extract. Since individual degradation products and the enzymatically cleaved 53—84 fragment are readily resolved under appropriate conditions, their recovery in high yield in highly purified form is also possible. Reversed-phase HPLC systems such as those described in this report are likely to have immediate application to the resolution and recovery (for analysis in other systems) of other highly purified hormonal components synthesized and released into culture medium by parathyroid cells maintained in vitro [14] or of specific bPTH enzymatic cleavage products, such as those produced by parathyroid and liver cathepsin B, an enzyme postulated to play a major role in peripheral inactivation as well as in the glandular regulation of secretion of the parathyroid hormone [15].

Reversed-phase HPLC methods are also being used to study human PTH (hPTH) [16]. We are now using the method described herein, in conjunction with an ultra-sensitive cytochemical bioassay for hPTH [17] and region-specific hPTH immunoassays, to separate components in partially and highly purified extracts of human parathyroid adenomata, and in biological fluids derived from such tissue maintained in vitro [18].

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ACTION PATTERN OF HUMAN PANCREATIC α-AMYLASE ON MALTOHEPTAOSE, A SUBSTRATE FOR DETERMINING α-AMYLASE IN SERUM

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SUMMARY

An enzymatic assay for the determination of α-amylase in serum was developed which employed a soluble substrate, maltoheptaose, and a coupled enzymatic indicator reaction consisting of α-glucosidase and the hexokinase—glucose-6-phosphate dehydrogenase system. We used high-performance liquid chromatography (HPLC) to establish the action pattern of maltoheptaose under the test conditions: (A) the action pattern of α-amylase alone, (B) that of the combined action of α-amylase and α-glucosidase. Conducive to this effort was: the availability of pure maltoheptaose and human pancreatic α-amylase; the development of an adequate procedure for sample pretreatment (partition chromatography on a mixed-bed ion exchanger) and of an HPLC system for separation of substrate and reaction products without interference from by-products of the assay (partition chromatography on a cation-exchange column with acetonitrile—water); and the use of a new, very sensitive refractometric detector revealing sugar amounts as low as 40 ng.

We derived the following stoichiometric equations:

$$\text{maltoheptaose} \xrightarrow{\alpha\text{-amylase}} 0.10 \text{ maltopentaose} + 0.79 \text{ maltotetraose} + 0.87 \text{ maltotriose} + 0.29 \text{ maltose}$$

$$\text{maltoheptaose} \xrightarrow[\alpha\text{-glucosidase}]{\alpha\text{-amylase}} 0.10 \text{ maltopentaose} + 0.77 \text{ maltotetraose} + 0.06 \text{ maltotriose} + 0.07 \text{ maltose} + 2.94 \text{ glucose}$$

The standard deviation of the rate coefficients is about 5%.

INTRODUCTION

The determination of serum α-amylase (1,4-α-D-glucaglucanohydrolase, EC

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3.2.1.1), a hydrolase which cleaves 1,4-α-glycosidic bonds, became a very important tool for the diagnosis of pancreatitis.

The basis of the most current procedures for the measurement of the enzymatic activity is the hydrolysis of starch or of chromogenic derivatives thereof [1, 2]. The reactions are followed by determining the formation of reducing groups or the release of the dye component. All these methods exhibit several drawbacks: (1) there is no exact stoichiometric relation between the reducing groups formed or the dye released and the number of hydrolyzed bonds; (2) the reactions are non-linear with time; (3) the rates observed are very much dependent on the kind and the quality of the polysaccharide used. We therefore developed a new method to determine α-amylase in serum by employing maltoheptaose as substrate (seven glucose residues in a linear 1,4-α-linkage) and a three-step NADH-forming indicator reaction: α-amylase acts on maltoheptaose yielding lower oligosaccharides which are partly hydrolyzed by α-glucosidase. The liberated glucose is determined by the hexokinase—glucose-6-phosphate dehydrogenase (NAD-dependent) method.

Since it was undecided what kind of reaction products originate from maltoheptaose and which of them enter the indicator reaction, the issue was to evaluate the action pattern of human pancreatic α-amylase on maltoheptaose under the reaction conditions envisaged for the enzymatic test. We had to establish the stoichiometry of the reaction, which is the basis for the calculation of amylase activity from the formation of NADH, i.e. from the photometric extinction in the enzymatic test. For this purpose two kinds of action patterns had to be investigated: that created by α-amylase alone and also the action pattern of the coupled α-amylase—α-glucosidase system to see how many mols of glucose result from 1 mol of degraded maltoheptaose.

EXPERIMENTAL

Chemicals

KH_2PO_4, NaCl, KOH, NaOH, methanol, conc. HCl, and acetonitrile were all reagent grade and purchased from E. Merck, Darmstadt, G.F.R. Bovine serum albumin and α-glucosidase were from Boehringer Mannheim GmbH, Mannheim, G.F.R. Maltoheptaose was synthesized by one of us (E. Schaich), and α-amylase was purified from human pancreas by H. Lenz (both Boehringer Mannheim GmbH, Biochemica Werk Tutzing, Tutzing, G.F.R.). The α-amylase had a specific activity of 230 units/mg lyophilisate, according to the amylochrome test (Hoffmann-La Roche, Basel, Switzerland).

Dowex cation-exchange resin 50W-X8 (H^+), 50—100 mesh, was reagent grade and the anion exchanger MWA-1 (OH^-), 20—50 mesh, was practical grade. Both were obtained from Serva, Heidelberg, G.F.R. The cation exchanger was preconditioned by washing with 1 *N* HCl, then with water, until the eluate was free from chloride, then with methanol, and again with water. The anion exchanger was treated with 1 *N* NaOH, then with water, until the eluate was neutral, then with methanol and again with water.

Chromatographic system

We used a Model 931 HSRI liquid chromatograph from Optilab, Stockholm,

Sweden. The system includes a thermostated column box, a Thermostir 941 to accommodate and thermostat the solvent reservoir, a refractometer Multiref 902 equipped with a measuring cell of 10 mm path length, a constant flow pump, Model 600/200, from Gynkotek, Munich, G.F.R., and a pulse dampener for Touzart-Matignon, Vitry-sur-Seine, France.

The detector works according to the interferometric principle [3]. Its sensitivity is about 100 times higher than that of conventional differential refractometers. However, utilization of this sensitivity requires a highly stable temperature and eluent flow-rate in the chromatographic system, since the smallest changes in these parameters become apparent as signal noise. To provide a temperature constancy of 0.01°C during each chromatographic run we employed a thermostat device consisting of two water-baths connected in series. The first bath was set at 27°C and countercooled with tap water. It tempered a second bath which thermostated the measuring cell of the detector, the column box and the solvent reservoir. This arrangement served to keep the heating pulses generated in the first water-bath from being passed on to the thermostated circuit.

Enzymatic procedure and subsequent sample treatment

The steps of sample preparation are listed in Fig. 1. The enzymatic assays were carried out in phosphate buffer (pH 7.0) at 30°C, with maltoheptaose as substrate. After certain time intervals aliquots were removed from the assay mixture and diluted in a slurry of the mixed-bed ion exchanger, thereby stopping the enzymatic reaction. The ion exchanger was poured into a column and washed with 300 ml of water. This amount was sufficient for quantitative elution of the oligosaccharides. The eluates were partly evaporated and lyophilized. The pre-column step was necessary to remove the protein and most of the buffer ions which would have interfered with the HPLC separation of the oligosaccharides.

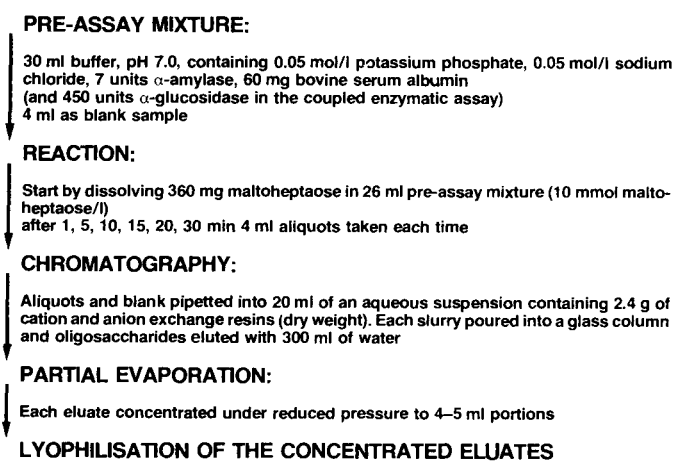

Fig. 1. Conditions of the kinetic assay and sample treatment.

Chromatographic analysis

Each lyophilized sample was dissolved in acetonitrile—water (1:1) at a concentration of 10 mg/ml for the quantification of the lower oligosacharides and at a concentration of 2 mg/ml for quantification of maltoheptaose. The lyophilized blank was dissolved in 5 ml of sample solvent. Twenty microlitres of each sample solution (containing 0.2 and 0.04 mg of oligosaccharides, respectively) were injected into the chromatographic system. For automatic sample injection we used the LC 7713 sampler from Kipp Analytica, Solingen, G.F.R.

The oligosaccharides were separated by partition chromatography on the cation-exchange column Nucleosil 10 SA (250 × 4 mm) from Macherey, Nagel & Co., Düren, G.F.R., with an eluent containing 72.5% acetonitrile in water, and at a flow-rate of 0.7 ml/min. The column back pressure was 5.1 MPa.

The column effluent was monitored by the Multiref differential refractometer and then routed back to the solvent reservoir. The Multiref was set at attenuation 20, i.e. full recorder scale was equivalent to 2×10^{-6} RI units.

A Hewlett-Packard (Böblingen, G.F.R.) automatic integrator, Type 3385A, recorded peak areas electronically from the detector signal.

Calculation

The areas of glucose to maltohexaose were taken from the chromatograms of the concentrated sample solutions. The maltoheptaose areas were obtained from the runs of the diluted sample solutions, since the amount of maltoheptaose contained in the concentrated sample solutions was above the linear detection range.

The factors relating peak area to weight are the same for all oligosaccharides. Therefore the weight per cent value of each oligosaccharide was equated to its area per cent value (100% being equal to the area sum of all oligosaccharide peaks in the sample). Dividing by the molecular weight and norming again to 100% gave the mol per cent values.

With α-amylase alone, four parallel kinetic assays were carried out; with the α-amylase—α-glucosidase system eight parallel assays were prepared and treated statistically.

RESULTS AND DISCUSSION

To investigate the action pattern of human pancreatic α-amylase we had to overcome two problems:

(1) To remove the so-called "buffer peak" in the chromatogram. This peak is produced by assay components that are not retained on the mixed-bed column. In conventional oligosaccharide separation by high-performance liquid chromatography (HPLC) on NH_2 columns with an acetonitrile—water eluent the buffer peak is eluted between maltose and maltotriose and interferes with the quantification of these two compounds.

(2) To achieve the sensitivity of detection necessary to quantify 0.2 mol % maltoheptaose fragments formed by the enzymatic hydrolysis, in the presence of a large excess of undegraded maltoheptaose. In preliminary experiments we had found that under assay conditions only about 20% of the substrate is

cleaved during an incubation period of 30 min. This surplus is necessary in order to keep a state of substrate saturation during the enzymatic assay.

First we tried to solve both problems by derivatizing the oligosaccharides with UV-absorbing tags. We worked with dansylhydrazine [4], dinitrophenylhydrazine [5], phenylhydrazine [6], and with 4-nitrobenzoyl chloride [7]. All those attempts to get a complete reaction and pure derivatives failed, since the higher oligosaccharides reacted sluggishly and isomers and side-products were formed.

Because we could not improve the procedure for the purification of the samples, we concentrated our efforts on developing an HPLC system in which the buffer peak was shifted to another part of the chromatogram where no interference with the oligosaccharide peaks did occur. Of the many methods we tested, chromatography on the Nucleosil SA cation-exchange column was the only one to yield the desired results. All oligosaccharides were separated in this system and the buffer peak was eluted together with the "solvent peak" at the beginning of the chromatogram.

The required sensitivity of detection was achieved by the Multiref differential refractometer. It had a quantitative detection limit of about 40 ng for glucose at a signal-to-noise ratio of 2:1, i.e. 0.02% when 0.2 mg of sample was injected. This enabled us to perform the amylase assay and to establish the action pattern under conditions close to the assay system employed in clinical chemistry. The amylase activity we used equalled about fifteen times the physiological α-amylase activity in human serum (7 units/30 ml according to the amylochrome test). To establish the action pattern the amylase concentration could not be lowered further, since during the elaborate sample preparation some spontaneous hydrolysis of maltoheptaose took place which interfered with the enzymatic hydrolysis.

Action pattern of α-amylase (without α-glucosidase)

Chromatograms C—H in Fig. 2 show the progress of enzymatic maltoheptaose hydrolysis in the amylase assay mixture after the indicated periods of incubation. Fig. 2A shows the chromatogram of the maltoheptaose lot which we used for our investigations. It is seen that it contains minor amounts of maltohexaose, maltopentaose, maltotetraose and of an unidentified impurity ("compound X"). Fig. 2B shows the pattern of the blank sample taken from the assay mixture before the start by addition of maltoheptaose. Since the eluate was routed back to the solvent reservoir, the solvent became enriched in maltoheptaose. The lack of maltoheptaose in the blank sample caused a negative peak, but because the peak area of maltoheptaose in the assay sample runs was more than 100 times higher, the error caused in the quantitative determination could be neglected.

The quantitative analysis of the chromatograms is presented in Table I. The concentrations of the oligosaccharides were calculated in mol per cent to display the relative interdependencies between the production of the respective oligosaccharides (100 mol % equals the total oligosaccharide content in each sample). As shown in Fig. 2E—H, maltopentaose and "compound X" were not fully separated in those samples that were incubated for 10 min or more. In

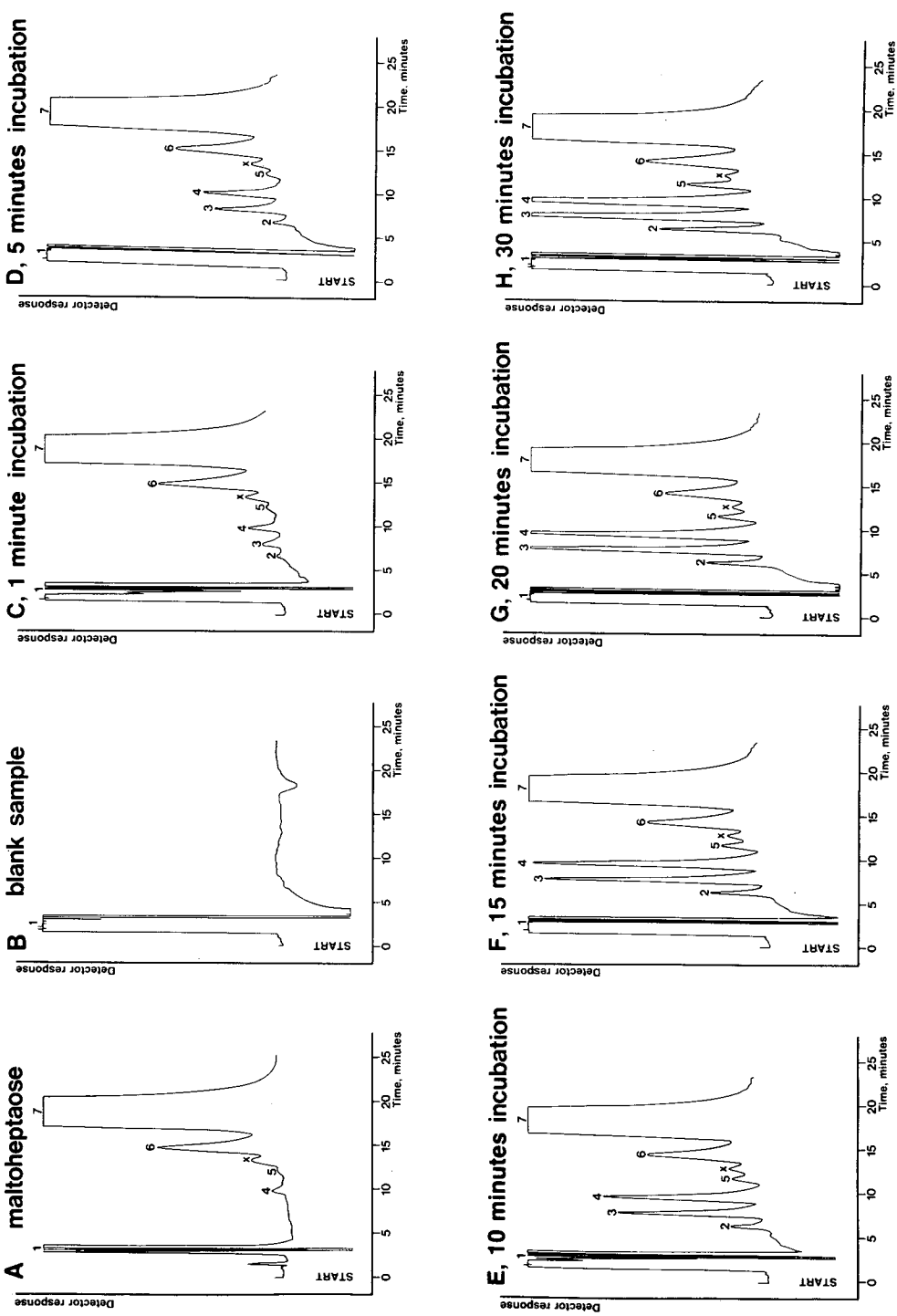
A maltoheptaose
B blank sample
C, 1 minute incubation
D, 5 minutes incubation
E, 10 minutes incubation
F, 15 minutes incubation
G, 20 minutes incubation
H, 30 minutes incubation
Detector response
START
Time, minutes
0
5
10
15
20
25
1
2
3
4
5
x
6
7

TABLE I

HYDROLYSIS PRODUCTS FROM MALTOHEPTAOSE BY HUMAN PANCREATIC α-AMYLASE

Incubation time (min)	Amount of hydrolysis compounds* in assay mixture (mol %)						
	G_2	G_3	G_4	G_5	Compound X	G_6	G_7
1	0.25	0.66	0.91	0.11	0.31	2.86	94.90
5	0.77	2.48	2.40	0.17	0.29	2.56	91.32
10	1.32	4.58	4.38	0.46		2.45	86.52
15	2.03	6.67	6.28	0.66		2.39	81.66
20	2.61	8.74	8.17	0.75		2.25	77.17
30	4.10	12.34	11.34	1.38		2.56	68.00

*G_2 = maltose; G_3 = maltotriose; G_4 = maltotetraose; G_5 = maltopentaose; G_6 = maltohexaose; G_7 = maltoheptaose.

this case the sum of their areas was determined since "compound X" does not seem to be a substrate of α-amylase and its concentration remains constant throughout the incubation period.

In Fig. 3 these data are presented graphically. The diagram shows that during the 30 min of incubation maltoheptaose decreases linearly to 68 mol % (equal to 80 weight per cent). As main products of the enzymatic action maltotriose and maltotetraose are produced in about equimolar amounts. Both compounds increase linearly up to 20 min. Then their production rates diminish slightly. The concentrations of maltohexaose and "compound X" which are contaminants of maltoheptaose do not change significantly. The amount of glucose remains below 0.15 mol % after 30 min of incubation. So we can safely assume that glucose is not a product of α-amylase action itself. These results can be explained by the reaction scheme in Fig. 4. The main reaction pathway is the cleavage of maltoheptaose into maltotetraose and maltotriose.

Only 10% of maltoheptaose, degraded enzymatically, is hydrolyzed to maltopentaose and maltose. A small amount of maltose is derived from the enzymatic hydrolysis of maltotetraose, since from our experiments it seems very likely that maltopentaose and maltotetraose themselves can act as substrates for α-amylase, competing with maltoheptaose. This explains the steeper increase in maltose compared to maltopentaose in Fig. 3 and the slower increase in maltotetraose compared with maltotriose. The values that are underlined in the reaction scheme were directly calculated from the chromatographic runs, i.e. from the concentration changes during incubation. The values in

Fig. 2. Chromatograms of the reaction products of maltoheptaose after various periods of incubation with α-amylase. Peaks: 1 = solvent front; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; 5 = maltopentaose; x = compound X; 6 = maltohexaose; 7 = maltoheptaose. (A) Pure maltoheptaose as used for the assay. (B) Blank sample before the addition of substrate. (C—H) Chromatograms after 1, 5, 10, 15, 20, and 30 min, respectively, of incubation. Incubation conditions: 7 units of α-amylase in 30 ml of 0.05 mol phosphate buffer (pH 7.0) at 30°C. Chromatographic conditions: column, 10 μm Nucleosil SA (250 × 4 mm); solvent, acetonitrile—water (72.5 : 27.5); flow-rate, 0.7 ml/min; temperature, 27°C; detection, differential refractometer Multiref 902, full scale = 2×10^{-6} RI units.

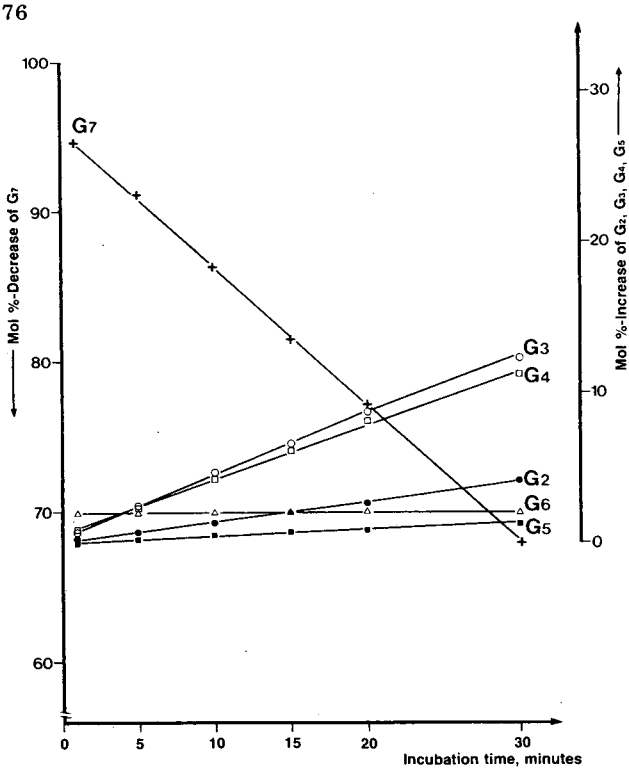

Fig. 3. Kinetics of α-amylase with maltoheptaose as substrate. For abbreviations see Table I.

parentheses relate to unobservable reaction steps and are derived from the underlined values. Since we measured that 0.87 mol of maltotriose (stable end-product) originated from 1 mol of maltoheptaose, also 0.87 mol of maltotetraose must have originated primarily in the assay. From the fact that only 0.79 mol was found it can be concluded that the missing 0.08 mol was hydrolyzed to maltose. The second source of maltose is the side-reaction of maltoheptaose (cleavage to maltopentaose and maltose).

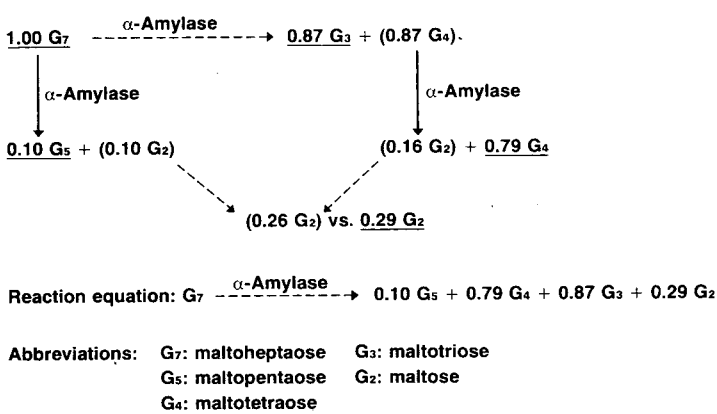

Fig. 4. Reaction scheme for the degradation of maltoheptaose by the action of α-amylase.

The measurable liberation of 0.1 mol of maltopentaose from 1 mol of maltoheptaose must be accompanied by the occurrence of an equimolar amount of maltose. Altogether 0.29 mol of maltose should result from 1 mol of reacted maltoheptaose. This inferred value is in good agreement with the experimental value of 0.26 mol. To show that the α-amylase action pattern does not change with lower enzyme concentrations we performed the kinetic assay with one-tenth of the enzyme activity used for the previous experiments. Fig. 5 presents the resulting patterns in analogy to Fig. 2. It is obvious that there is no qualitative change in the appearance of the patterns although the chromatographic runs cannot be evaluated quantitatively.

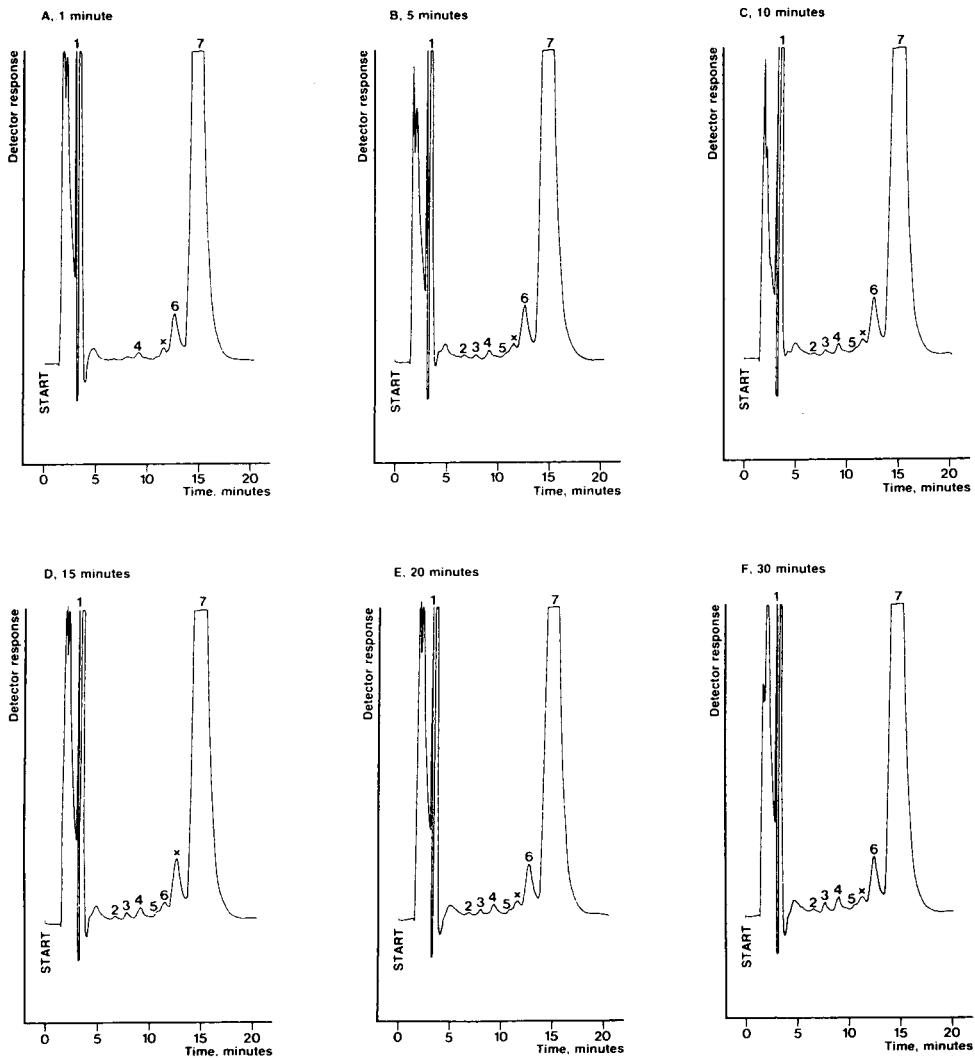

Fig. 5. Chromatograms of the reaction products from maltoheptaose by human pancreatic α-amylase at physiological activity after various periods of incubation. Peak identification and chromatographic conditions as in Fig. 2.

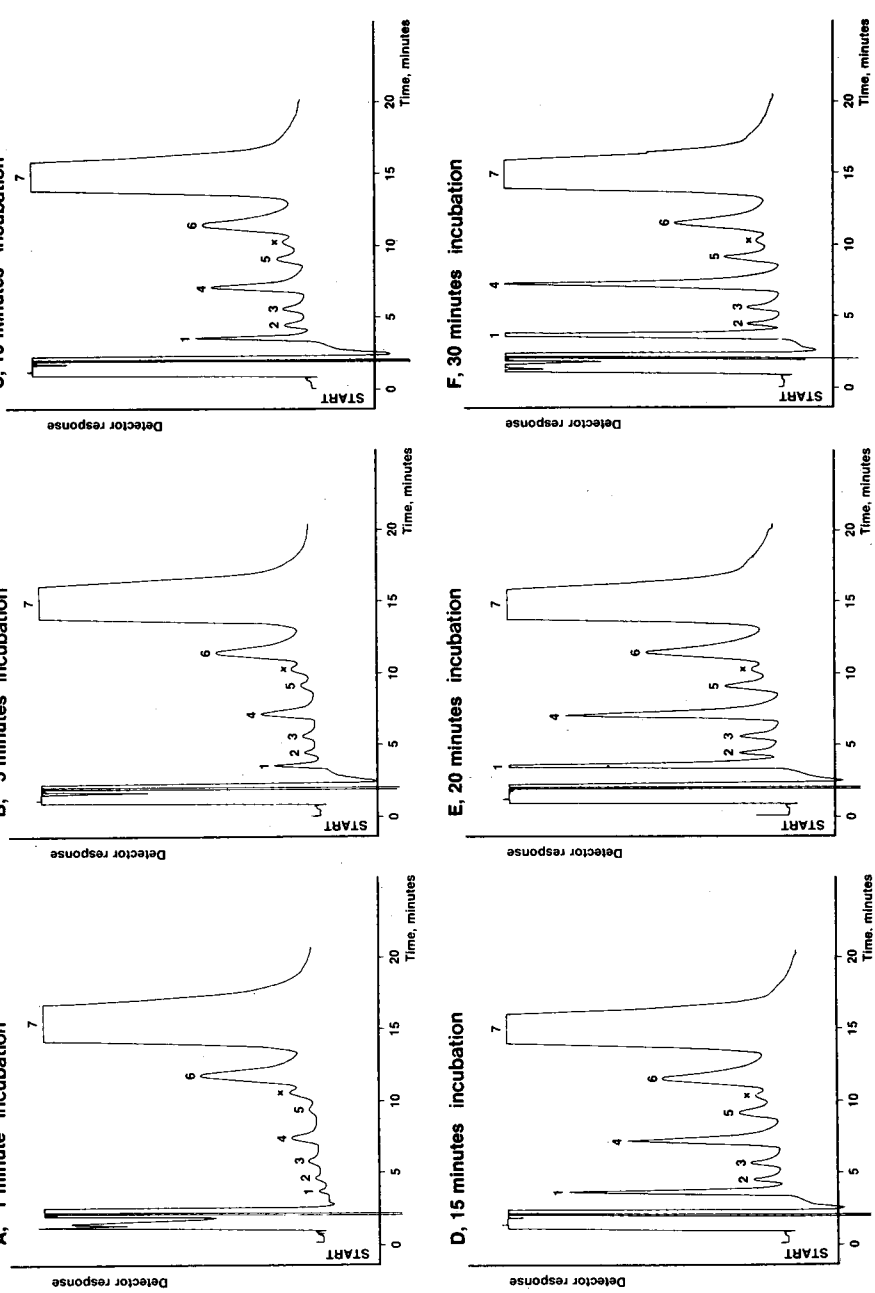

Fig. 6. Chromatograms of the action patterns of maltoheptaose after the indicated periods of incubation with α-amylase and α-gl osidase. Peak identification and conditions as in Fig. 2, except that here peak 1 is the glucose peak.

TABLE II

HYDROLYSIS PRODUCTS FROM MALTOHEPTAOSE BY THE COUPLED ENZYME SYSTEM α-AMYLASE–α-GLUCOSIDASE

Incubation time (min)	Hydrolysis compounds in the assay mixture							
	G_1	G_2	G_3	G_4	G_5	Compound X	G_6	G_7
	Weight per cent (± S.D.)							
1	0.16 (± 0.10)	0.28 (± 0.15)	0.40 (± 0.16)	0.77 (± 0.24)	0.55 (± 0.35)	0.37 (± 0.06)	4.13 (± 0.80)	93.33 (± 1.89)
5	0.83 (± 0.14)	0.42 (± 0.15)	0.54 (± 0.18)	1.62 (± 0.24)	0.79 (± 0.36)	0.37 (± 0.05)	4.34 (± 0.89)	91.12 (± 1.84)
10	1.76 (± 0.23)	0.43 (± 0.13)	0.55 (± 0.15)	2.52 (± 0.28)	0.88 (± 0.28)	0.36 (± 0.07)	4.11 (± 0.75)	89.38 (± 1.61)
15	3.03 (± 0.34)	0.53 (± 0.15)	0.71 (± 0.16)	3.83 (± 0.38)	1.22 (± 0.29)	0.36 (± 0.05)	4.53 (± 0.80)	85.80 (± 1.66)
20	4.09 (± 0.44)	0.56 (± 0.10)	0.74 (± 0.13)	4.84 (± 0.40)	1.34 (± 0.19)	0.36 (± 0.05)	4.34 (± 0.58)	83.73 (± 1.31)
30	6.27 (± 0.75)	0.55 (± 0.16)	0.74 (± 0.16)	6.70 (± 0.75)	1.55 (± 0.26)	0.34 (± 0.06)	3.83 (± 0.66)	80.02 (± 2.36)
	Mol per cent							
1	1.0	0.9	0.9	1.3	1.1	—	4.7	90.4
5	4.9	1.3	1.2	2.6	1.1	—	4.7	84.3
10	9.9	1.3	1.1	3.8	1.1	—	4.2	78.5
15	15.8	1.4	1.3	5.5	1.4	—	4.3	70.2
20	20.3	1.4	1.3	6.5	1.4	—	3.9	65.0
30	28.2	1.3	1.2	8.2	1.5	—	3.2	56.4

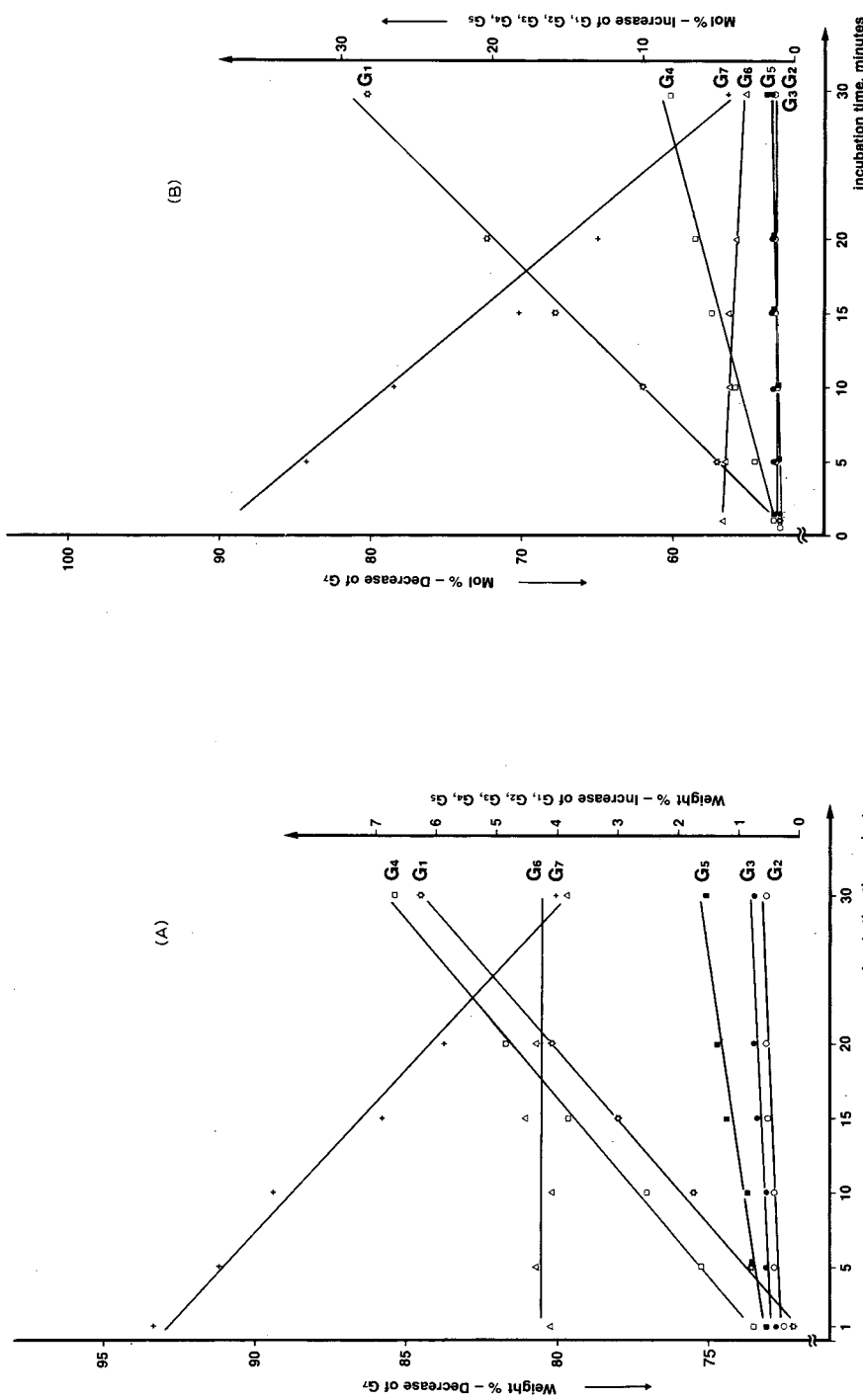

Fig. 7. Kinetics of the coupled enzyme system α-amylase—α-glucosidase with maltoheptaose in weight per cent (A) and in mol per cent (B). For abbreviations see Table I.

Action pattern of α-amylase—α-glucosidase

The second series of incubation assays contained in addition to α-amylase also α-glucosidase with the same enzymatic activity as used in the photometric test for determination of α-amylase activity in body fluids. Enzymatic procedure, sample pre-treatment and chromatographic conditions were the same as before.

Fig. 6 shows the chromatograms of assay samples after consecutive periods of incubation. Table II and Fig. 7A and B give the quantitative evaluation of the chromatographic runs from eight independent assays by weight and mol per cent.

In the combined assay glucose and maltotetraose increase rapidly and linearly. The maltopentaose concentration also increases significantly during the incubation period, whereas maltose and maltotriose reach only small steady state concentrations.

The large standard deviations (10—25%) displayed by the weight per cent mean values of the hydrolysis products can be explained to a large extent by differences in the α-amylase activities among the eight parallel assays (amylase is the rate-limiting enzyme). Whereas the extent of maltoheptaose hydrolysis, i.e. the weight percentages of the hydrolysis products, depends on α-amylase activity in the test, the relative distribution of the hydrolysis products should be independent. Therefore, for example, the quotient weight % (glucose)/ weight % (maltotetraose) should be independent of amylase activity and should have smaller standard deviations than the weight per cent values (see Table III). Since the statistical error is greater for smaller peaks, the coefficient of variation (C.V.) of this quotient becomes lower with increasing time of incubation and is around 5% for incubation periods of 15 min and more. The rate equation was derived from the net change of the weight per cent values for each oligomer during the whole incubation period. From these differences the rate coefficients for each oligosaccharide were calculated (Table IV).

As the stoichiometric equation is independent of the amylase activity in the test, the statistical error of the coefficients should be around 5%. From the stoichiometric coefficients the reaction scheme in Fig. 8 can be constructed. The coefficients of the end-products are calculated from the chromatograms and are underlined. These values and those obtained from the action pattern of amylase alone (Fig. 4) were used to derive the coefficients of the reaction inter-

TABLE III

STATISTICAL ERROR INFLUENCING THE RATE COEFFICIENTS

Period of incubation (min)	C.V. (%) of the quotient weight % (glucose)/weight % (maltotetraose)
1	34
5	12
10	8
15	6
20	4
30	5

TABLE IV

RATE COEFFICIENTS FOR HYDROLYSIS OF THE MALTOHEPTAOSE

Oligomer	Concentration changes during 29 min of incubation	Stoichiometric coefficients
Glucose	+ 6.11	+2.94
Maltose	+ 0.27	+0.07
Maltotriose	+ 0.34	+0.06
Maltotetraose	+ 5.94	+0.77
Maltopentaose	+ 1.00	+0.10
Maltohexaose	+ 0.30	+0.03
Maltoheptaose	−13.31	−1.00

mediates which could not be measured directly. In the scheme they are placed in parentheses. Consideration of both reaction schemes leads to the following interpretation. Again maltotriose and maltotetraose are the most important primary reaction products of the amylase-dependent hydrolysis of maltoheptaose. Whereas maltotriose is cleaved by the auxiliary enzyme α-glucosidase almost quantitatively into three glucose molecules, maltotetraose remains largely stable in the assay. Only 11% is cleaved by amylase into two maltose units which are further hydrolyzed to glucose by α-glucosidase. The third pathway that provides glucose runs via the side-reaction of α-amylase, namely the cleavage of maltoheptaose into maltose and maltopentaose. A test for the consistency of the data is the coefficient of glucose which can be obtained either directly from the chromatograms or calculated from the data pool. Both values differ by 2%, showing that the data pool is self-consistent. The stoichiometric coefficients of comparable reaction steps in both schemes are in agreement. The enzymatic indicator system has therefore no impact on the

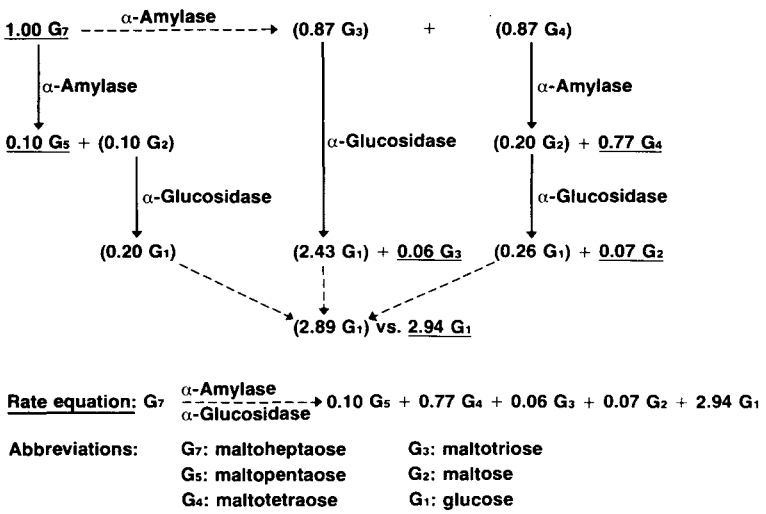

Fig. 8. Reaction scheme for the degradation of maltoheptaose by the combined action of α-amylase and α-glucosidase.

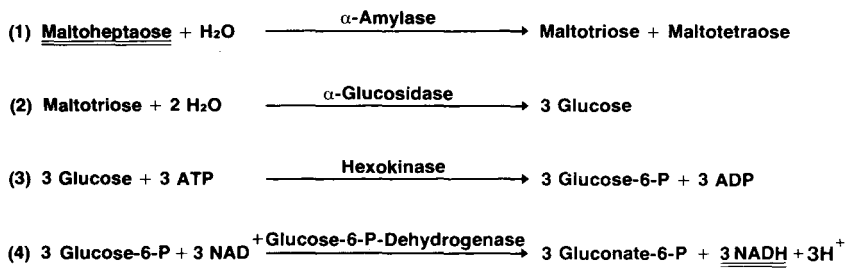

Fig. 9. Assay principle (simplified).

primary amylase-dependent reaction steps, even if there are several common intermediates. Secondly, the results show that the α-glucosidase content in the assay is high enough to cleave nearly all maltose and maltotriose resulting from amylase action on maltoheptaose. Only steady-state concentrations of these two intermediates remain in the assay, reflecting the K_M values of α-glucosidase.

Knowing the mechanism and the stoichiometric coefficients of the enzymatic cleavage of maltoheptaose we could set up the reaction equations pertaining to the α-amylase test (Fig. 9). Each cleavage of maltoheptaose by α-amylase effects the occurrence of three glucose molecules which lead to the formation of three molecules of NADH, i.e. the indicator reaction amplifies the primary reaction by a factor of 3, a feature which adds to the sensitivity of the test.

CONCLUSION

This work shows that HPLC can be used in clinical chemistry to investigate the mechanisms of multiple-step enzymatic reactions. By applying chromatography to the reaction mixtures the concentrations of various reaction intermediates and end-products can be obtained, i.e. more parameters than a photometric test can deliver. In this way complex reaction cascades can be split up into a set of individual steps making clear the stoichiometry of the overall reaction.

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CHROMBIO. 785

THE ASSAY OF FENTANYL AND ITS METABOLITES IN PLASMA OF PATIENTS USING GAS CHROMATOGRAPHY WITH ALKALI FLAME IONISATION DETECTION AND GAS CHROMATOGRAPHY--MASS SPECTROMETRY

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SUMMARY

Fentanyl was determined using gas chromatography (GC) and alkali flame ionisation detection (AFID), in the plasma of patients who had received a high single dose (up to 60 μg/kg body weight). The relative standard deviation is 6% for 11 ng/ml while the calculated detection limit is 3.3 ng of fentanyl per 1 ml of plasma. The concentration of fentanyl in patients ranged from 40 to 3 ng/ml of plasma in the first hour after administration. In the plasma of patients treated with fentanyl two metabolites could be detected and identified using GC—AFID and GC—MS.

INTRODUCTION

The pharmacokinetics of fentanyl, 1-(2-phenethyl)-4-N-(N-propionylanilino)-piperidine (Fig. 1), have been investigated using either radioimmunoassay [1, 2] or an isotopic method with tritium-labelled fentanyl [3]. Re-

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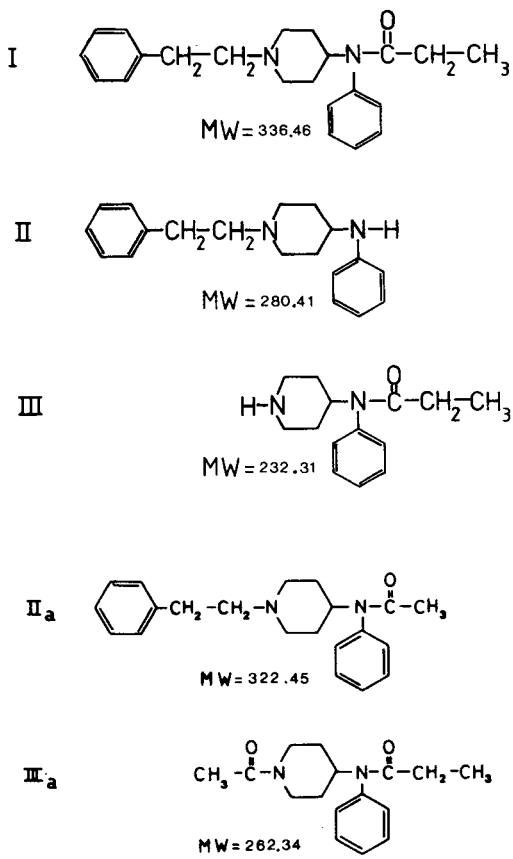

Fig. 1. Structural formulae of fentanyl (I), its metabolites II and III and their acetyl derivatives (IIa and IIIa).

sults indicated that a three-compartment model [1, 2] could be used to fit the data. Other than radioimmunoassay, no sensitive method for fentanyl determination has been described. Previously the metabolism of fentanyl in rats was studied [4, 5] and it was found that in plasma and urine of rats two metabolites can be identified; namely, 1-(2-phenethyl)-4-N-anilino-piperidine (structure II in Fig. 1) [4] and 4-N-(N-propionyl-anilino)-piperidine (structure III in Fig. 1) [5]. However, the presence of such metabolites in human patients has not been confirmed, which may be due to the lack of a sensitive and selective method for determining these metabolites in plasma. Although radioimmunoassay can meet the sensitivity requirements, appropriate assay materials for these potential metabolites are not currently available.

In the present study we report the results of an investigation into the application of gas chromatography (GC) and alkali flame ionisation detection (AFID) for the quantitative determination of fentanyl in plasma of patients who have received intravenous high single doses of fentanyl (up to 60 μg/kg). Moreover, the identification of two metabolites by means of gas chromatography—mass spectrometry (GC—MS) and GC—AFID) is reported.

EXPERIMENTAL

Gas—liquid chromatography

A gas chromatograph (Perkin-Elmer 3920), equipped with an alkali flame ionisation detector and a linear potentiometric recorder (Servogor, Type 541) was used. A glass column (120 cm × 2 mm I.D.) was filled with Chromosorb W HP (80—100 mesh) and loaded with 3% OV-17 (Chrompack, Middelburg, The Netherlands). The temperatures of the injection port, the oven and the interface were 290°C, 250°C and 300°C, respectively. For the assay of metabolite derivatives the oven temperature was set at 230°C. The carrier gas was helium (flow-rate 35 ml/min).

Gas chromatography—mass spectrometry

An LKB 2091—2130 gas chromatograph—mass spectrometer—computer system was used for the identification of the compounds eluted from the GC column. The gas chromatograph had a modified solid injector (Becker, Model 767) and a capillary SCOT column 10 m × 0.5 mm I.D. consisting of a Carbowax 20M stationary phase on a Tullanox support (Cabot Corp., Boston, MA, U.S.A.). Helium was used as the carrier gas (flow-rate 10 ml/min). The temperatures of the injector, column and separator were 260°C, 210°C and 210°C, respectively. The ion source temperature was 210°C. The electron energy was 70 eV, the trap current 50 μA and the accelerating voltage 3.5 kV.

Mass spectra were recorded and stored in a Digital PDP-11/05 computer.

Chemicals and reagents

In all experiments double-distilled water was used. All solvents and chemicals were of analytical grade, except for benzene, which was of pesticide grade (Merck, Darmstadt, G.F.R.). No pretreatment of reagents was carried out. All drugs and metabolites were kindly donated by Janssen Pharmaceutica (Beersse, Belgium).

Sample preparation

Blank pooled plasma and plasma samples containing fentanyl and its metabolites were stored at −20°C. After thawing all samples were ultrasonicated for 3 min. To extract fentanyl and its metabolites 0.1 ml of 4 *N* NaOH and 5.0 ml of benzene were added to 1 ml of plasma in a glass-stoppered centrifuge tube. The tube was shaken for 1 min and then centrifuged for 5 min at 900 *g*. Four millilitres of the upper layer were removed using a Hamilton syringe (Type 1005) and transferred to a glass-stoppered, tapered glass tube. This extraction procedure was repeated three times and the extracts were pooled. In order to derivatize the metabolites 0.5 ml of acetic anhydride and 10 μl of pyridine were added to the tube, which was subsequently heated in a water-bath at 75°C for 2 h. The organic reagents were evaporated to dryness in a water-bath at 75°C using a gentle stream of nitrogen. The residue was dissolved in 50 μl of a solution of the internal standard (papaverine 0.5 mg per 100 ml) in benzene. Ten microlitres of this final solution were injected into the gas chromatograph or evaporated on the tip of the solid injector of the GC—MS system. Peak heights of fentanyl and internal standard were measured and compared with

peak heights obtained after injecting standard amounts of fentanyl and internal standard.

Standard solutions

Standard solutions of fentanyl and its metabolites were prepared in methanol and also in water (2 mg per 100 ml) and were stored at 4°C. Standard solutions of the acetyl derivatives of the potential metabolites were freshly prepared.

RESULTS AND DISCUSSION

Chromatography

Until now the determination of fentanyl and its two potential metabolites (Fig. 1) in biological samples could be accomplished only by radioimmunoassay [6], by radioactivity measurement including an inverse isotope dilution method [5], or by separation using thin-layer chromatography [7].

One of the objectives of this present study was to search for the potential metabolites of fentanyl (II and III, Fig. 1) in man after intravenous administration of a single dose (up to 60 μg/kg) of fentanyl in coadministration with lorazepam and pancuronium bromide to patients undergoing heart surgery. It was decided to use GC in combination with AFID or MS because of the low concentrations expected to be present in the plasma samples. For reasons discussed under recovery and reproducibility of the extraction, it was found necessary to form acetyl derivatives of the metabolites prior to the evaporation of the organic extraction solvent. Therefore all GC measurements were performed with these acetyl derivatives (IIa and IIIa in Fig. 1).

Two stationary phases, OV-17 and Carbowax 20M, were examined for the analysis of fentanyl and its two metabolite derivatives. In order to elute fentanyl in a reasonable time the column temperature had to be 250°C, whereas a lower column temperature was necessary to resolve the metabolite derivatives from any coextracted endogenous substances. On both stationary phases symmetrical peak shapes were obtained. However, OV-17 gave the better selectivity and was used for subsequent experiments using AFID. GC—MS analysis was performed with Carbowax 20M as the stationary phase, because of serious bleeding of OV-17, which interferes with the MS measurements. The use of two stationary phases provides additional information for the identification of the metabolites.

Mass spectrometry

The mass spectra of fentanyl and the acetyl derivatives of its metabolites were recorded using electron impact ionisation at 70 eV and mass fragmentography and are shown in Fig. 2.

For mass fragmentography three *m/z* values were chosen: 245 for fentanyl, 231 for the derivative of metabolite II, and 231 and 274 for the derivative of metabolite III.

Quantitative aspects

The precision and linearity of the GC method were established by injecting

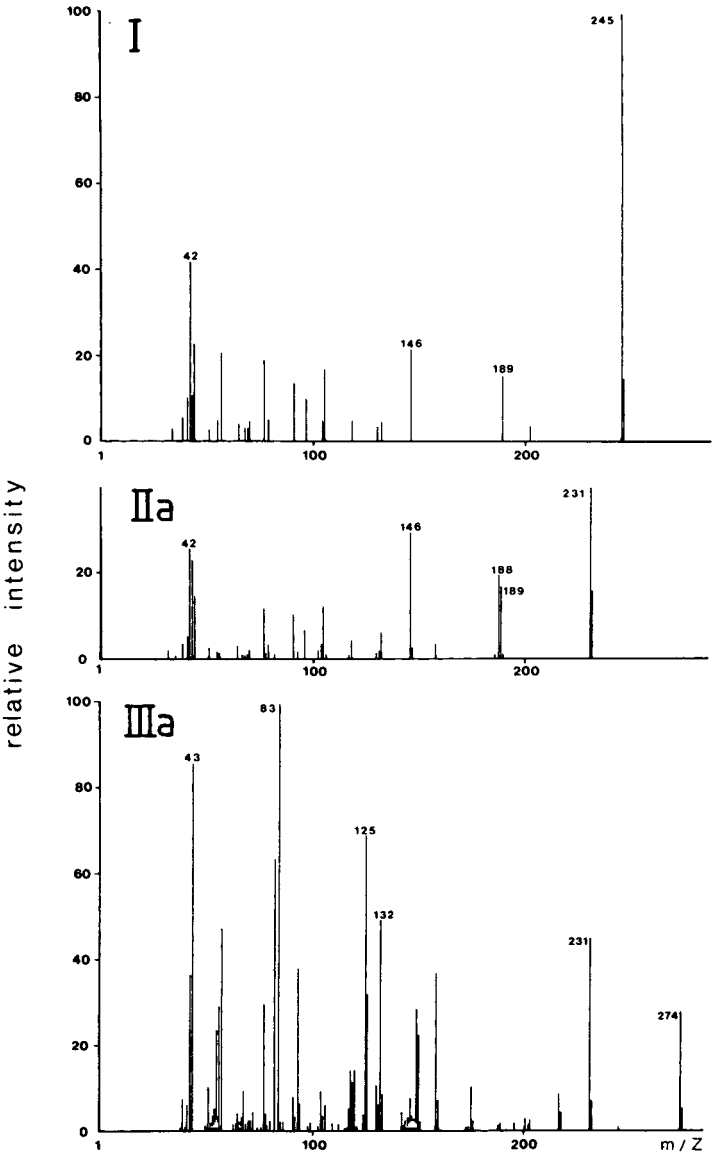

Fig. 2. Mass spectra of fentanyl (I) and the acetyl derivatives of the metabolites (IIa and IIIa).

10 μl of solutions of the solutes under investigation at different concentrations and subsequently measuring the peak heights. The regression analysis of peak heights vs. amount injected is found to be linear up to 8.5 μg/ml for fentanyl, up to 7.5 μg/ml for IIa and up to 8.5 μg/ml for IIIa, with correlation coefficients of 0.9996 and 0.9988, respectively, which indicates an acceptable degree of linearity.

The precision of the method was estimated from repeated injections (n=5) of standard solutions of the solutes in methanol and for 4.3 ng of I, 3.0 ng of IIa, and 6.0 ng of IIIa is 4.7, 6.6 and 5.0%, respectively.

The calculated limits of detection for a signal-to-noise ratio of 3 for the three solutes I, IIa and IIIa are 0.25 ng, 0.82 ng and 0.13 ng, respectively. The corresponding detection limits per ml plasma are 3.3 ng, 5.4 ng and 0.65 ng, respectively.

Recovery and reproducibility of extraction

The recovery and reproducibility of the extraction were determined by spiking blank pooled plasma samples with the solutes. For I (11 ng/ml of plasma) the recovery was 76.1% ± 6.0% (n=7) for II (14 ng/ml of plasma) the recovery was 74.1% ± 6.5% (n=7), and for III (22 ng/ml of plasma) the recovery was 96.2% ± 4.0% (n=7).

Initially, after the solutes were extracted, the organic solvent was evaporated using a gentle stream of nitrogen at 60°C. However, it was found that serious losses of compound III occurred with this method, because of its high volatility. Therefore a derivative-forming step was introduced by which a much less volatile derivative is obtained. Under these conditions no significant losses of the derivative of compound III were found during evaporation of the extraction solvent. It is therefore possible that Maruyama and Hosoya [4], who did not preacetylate the metabolites, could not detect metabolite III because it had disappeared during the evaporation of the extraction solvent (chloroform).

Determination of fentanyl in plasma samples of patients

The method developed for the analysis of fentanyl and its metabolites has been applied to plasma samples of patients receiving high doses of fentanyl. Fig. 3 shows a typical gas chromatogram of a blank pooled plasma extract and of a plasma extract of a patient. From all patients plasma sam-

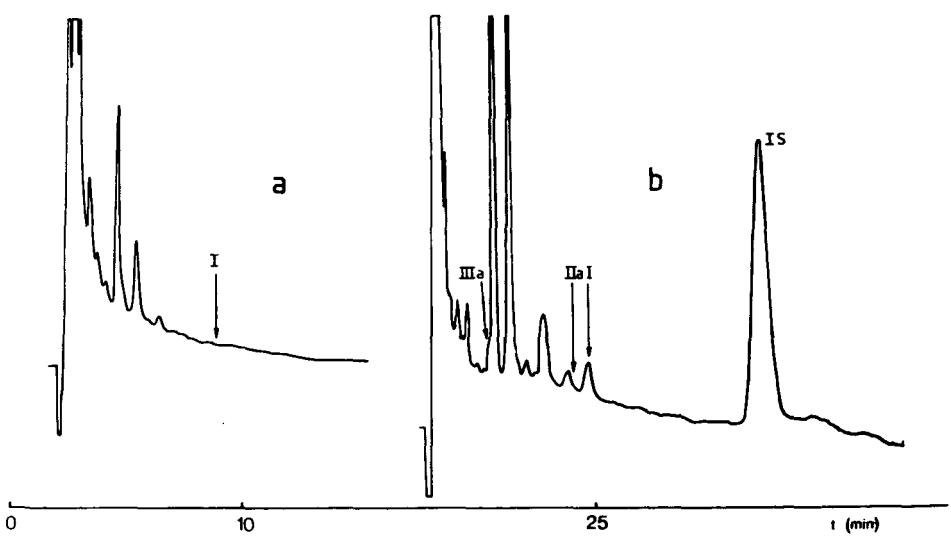

Fig. 3. Gas chromatogram of an extract of a blank pooled plasma sample (a) and of a plasma sample of a patient after the intravenous injection of fentanyl (b). Conditions: column, Chromosorb W HP coated with 3% OV-17; oven temperature, 250°C; AFID detection. IS = internal standard (papaverine).

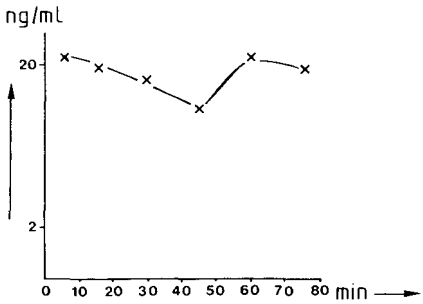

Fig. 4. Plasma concentration of fentanyl vs. time after intravenous administration of 3.5 mg of fentanyl (50 μg/kg) to patient C.

ples were taken at regular fixed time intervals up until 75 min post-administration. The blood levels of fentanyl ranged between 40 and 3 ng/ml of plasma. After this time the blood circulation was connected to a heart—lung machine which prevented more representative samples being taken.

Fig. 4 shows the time course of the plasma concentration of fentanyl in patient C, as determined by the GC method developed. In this patient a significant increase in the fentanyl concentration occurs at 60 min after administration of the drug. Such an increase has been found previously [8] and has been attributed to gastrointestinal recirculation of the drug.

From the time course of fentanyl in four patients, a mean half-life of 20 min is calculated; this is in accordance with earlier reports [1, 2].

The data also indicate that a rapid distribution of fentanyl occurs in the first minutes after administration.

Identification of metabolites

For the identification of the acetyl derivatives of the metabolites of fentanyl the column temperature was set at 230°C.

In the chromatograms of the plasma extracts of patients receiving fentanyl, peaks appear at the elution times of the acetyl derivatives of two potential metabolites. In order to obtain a more specific detection and positive identification, GC—MS analysis has been used. With this technique both metabolites could be identified in all the plasma samples taken from different patients 30 min after fentanyl administration. Typical GC—MS recordings of a test mixture and of plasma extracts of patients A and B are given in Fig. 5.

As can be seen from the ratio of IIIa and IIa in Fig. 5, it appears that significantly different metabolic routes or rates exist in patients. From the results of the GC—AFID measurements a rough estimate of the concentration of the metabolites can be obtained. For both metabolites the concentration ranges from 3 to 8 ng/ml of plasma.

CONCLUSION

GC with AFID has been found to be extremely useful for the determination of fentanyl in the plasma of patients. For the identification of the

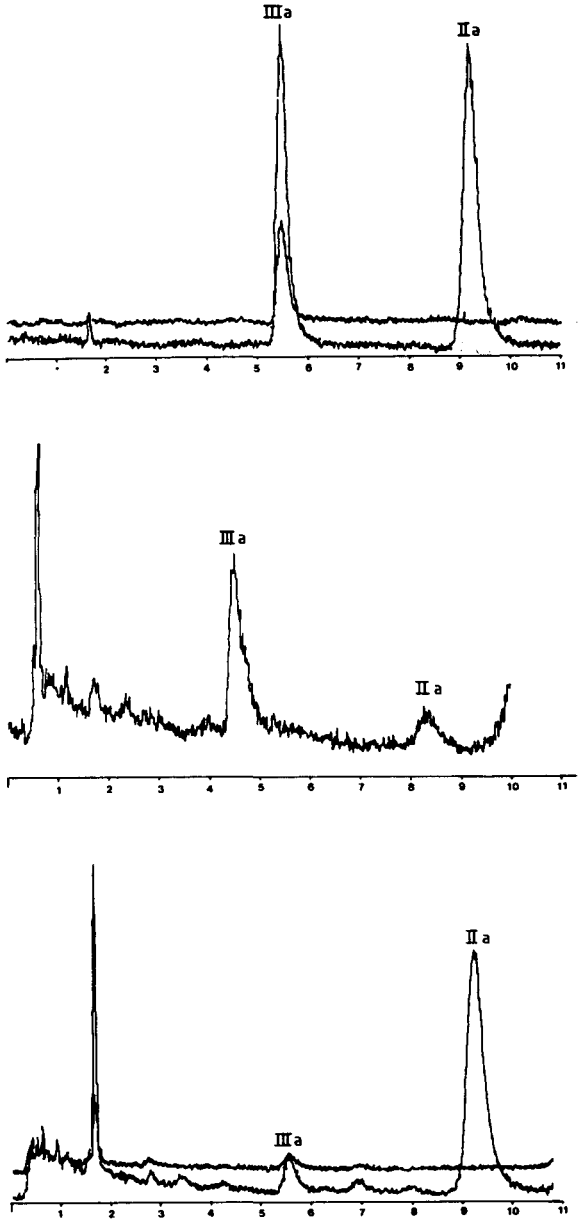

Fig. 5. GC—MS recordings of (top trace) a standard solution containing IIa and IIIa, (middle trace) an extract of a plasma sample of patient A, and (bottom trace) an extract of a plasma sample of patient B after intravenous injection of fentanyl. Conditions: column, capillary SCOT coated with 1% Carbowax 20M; oven temperature, 210°C; MS detection.

metabolites a GC—MS combination has been found necessary. With this combination two metabolites, which had previously been found in rats, could be positively identified in the plasma of patients.

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CHROMBIO. 796

SENSITIVE GAS CHROMATOGRAPHIC DETERMINATION OF TRIFLUOPERAZINE IN HUMAN PLASMA

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SUMMARY

Plasma trifluoperazine levels of patients taking a single 20-mg dose of trifluoperazine were measured by a sensitive and linear method. The low detection limit of 0.1 ng/ml plasma was obtained through use of a highly sensitive nitrogen—phosphorus detector combined with an efficient extraction method. Recovery of trifluoperazine added to human plasma was 96%. Data are presented on the stability of trifluoperazine in refrigerated human plasma.

INTRODUCTION

Trifluoperazine (TFP) and other phenothiazines are widely used in psychiatric therapy, and are usually given on a long-term basis. Not infrequently, do patients fail to improve when administered "therapeutic" doses of a phenothiazine. A probable cause for inadequate improvement in non-responding patients is the failure to obtain an effective plasma concentration of the drug. Indeed, for patients on chlorpromazine therapy, data suggest that a minimum plasma concentration is necessary for clinical improvement, and that an excessively high value is related to toxicity [1].

Trifluoperazine which is about 80% removed from rat hepatic venous blood by the liver [2] would appear to be a logical candidate for therapeutic monitoring. There appears to be a paucity of methods published for quantification of TFP in human plasma. This is probably due to the small recommended adult

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doses of 2—20 mg daily [3], and the likely small plasma concentrations which would be difficult to measure.

A fluorimetric procedure has been used to quantitate TFP and its sulfoxide (TFP-SO) in human urine [4]. While the analysis was relatively rapid, it was necessary to selectively extract the sulfoxide away from the parent drug, and then to convert the parent to the sulfoxide before determinations could be made. With a detection limit in the vicinity of 0.5 μg/10 ml it would not be sufficiently sensitive for human plasma samples.

A thin-layer chromatography—ultraviolet reflectance photometry method was used to quantitate TFP and metabolites in rat tissues [5], but it also is too insensitive for human samples. West and Vogel [6] measured tritiated TFP and TFP-SO in rat plasma and tissues, but this procedure is not routinely applicable with human subjects.

Other phenothiazines have been analyzed at sensitivities of 1 part per billion or less by gas chromatography (GC) with electron-capture detection [7, 8] or nitrogen—phosphorus detection [9], or by high-performance liquid chromatography with electrochemical detection [10]. These methods, while sensitive, either recovered only 50—70% of added drug or were too involved for routine use. Chlorpromazine has been measured by radioimmunoassay [11], but reagents for TFP analysis by this method are not commercially available.

EXPERIMENTAL

Materials

Trifluoperazine HCl and chlorpromazine HCl were obtained from Smith, Kline & French Labs. (Philadelphia, PA, U.S.A.). Kimax 12-ml conical tubes and 15-ml screw-top culture tubes (Owens-Illinois, Toledo, OH, U.S.A.) were silanized prior to use to avoid drug adsorption to glass by rinsing them with a 5% solution of dimethyldichlorosilane (Sigma, St. Louis, MO, U.S.A.) in toluene. Four liters of hexane (Matheson, Coleman & Bell, Norwood, OH, U.S.A.) were passed through a 85 × 3.0 cm glass column filled with Matheson, Coleman & Bell grade 12 silica gel, 28—200 mesh, prior to use to remove chromatographic interferences. Extraction solvent was prepared by mixing 19 volumes hexane with 1 volume U.S.P. alcohol (U.S. Industrial Chemicals, New York, NY, U.S.A.).

Gas chromatography

A Hewlett-Packard 5710A gas chromatograph equipped with a specific nitrogen—phosphorus detector was used for analyses. Separation was attained with a 2 m × 2 mm I.D. silanized glass column packed with 3% OV-17 on Gas-Chrom Q, 80—100 mesh. The injector port and detector were maintained at 300°C, and the column at 260°C. Flow-rates were 30 ml/min for the helium carrier gas, 50 ml/min for air, and 4 ml/min for hydrogen. Electrometer range and attenuation were set at 1 and 8, respectively, and collector voltage was adjusted between 13 and 15 V to obtain adequate sensitivity.

Standards

A 4 ng/ml assay standard was prepared by adding 0.1 ml of a 1 ng TFP per

μl ethanol solution to a 25-ml volumetric flask, adding 0.5 ml distilled water, filling with human plasma, and mixing thoroughly. Assay standards of 2.0 and 0.5 ng/ml were prepared by diluting portions of the 4 ng/ml standard with human plasma.

Analytical procedure

To 2 ml of standards or samples contained in culture tubes were added 0.5 g sodium chloride, 0.5 ml 2.0 *N* sodium hydroxide, 5 ml extraction solvent and 4 ng chlorpromazine (as the internal standard). The tubes were sealed with PTFE-lined caps and mixed for 5 min on a mechanical shaker. After brief centrifugation, the upper hexane phases were transferred to extraction tubes containing 5 ml 0.1 *N* hydrochloric acid. The tubes were then shaken 5 min, centrifuged, and the hexane separated and discarded. After adjusting the pH to 13 with 2.0 *N* sodium hydroxide, the drugs were extracted into 5 ml extraction solvent. Following centrifugation, the hexane was transferred to 12-ml conical tubes and then evaporated to dryness at 40°C with a stream of nitrogen. To remove drugs from the sides of the tubes, 0.2 ml ethanol was added, vortexed briefly, and then evaporated. The extracts were reconstituted with 30 μl ethanol, and 8 μl were injected on the GC column.

Human samples

Four patients were given a single oral dose of 20 mg TFP (Stelazine^{R}, Smith Kline & French). Blood was drawn into heparinized plastic syringes immediately prior to drug administration, and at several time intervals afterwards. Plasma was separated and stored frozen for seven months until analysis. Assay standards of 0, 0.5, 2.0, and 4.0 ng/ml were routinely processed with patient samples according to the procedure described. Linear regression analysis of peak height ratio versus concentration of standards was performed, and TFP concentrations of patient samples were determined from peak height ratios, and the equation for the standard curve.

TFP recovery

To determine recovery of TFP added to drug-free plasma, TFP standards of 0.2 and 3.0 ng/ml plasma were prepared. These were extracted in the usual manner except that no internal standard was used. Chlorpromazine was added to the final extracts as an external standard prior to evaporation. Non-extracted standards corresponding to 100% recovery were prepared, and the extracted standards were compared to these.

Pharmacokinetic analysis

The concentration of TFP in plasma was fitted according to the equation $C = A(e^{-\beta t} - e^{-\alpha t})$, where C is the plasma concentration at time t, A is a constant, α is the apparent absorption rate constant and β is the terminal elimination rate constant. The terminal half-life of TFP in the plasma was calculated according to the equation $t_{1/2}^{\beta} = 0.693/\beta$.

RESULTS AND DISCUSSION

Linearity and sensitivity

The linearity of the method was demonstrated by correlation coefficients of peak height ratio versus TFP concentration of 0.99 or greater. Sensitivity (defined as a signal-to-noise ratio greater than 2) was 0.1 ng TFP per ml plasma when a 2-ml sample was used.

Recovery

The data in Table I illustrate the high efficiency of the extraction by the nearly complete recovery (96%) in the final extract of TFP added to plasma. Efficiency of the extraction was markedly enhanced by addition of sodium chloride to plasma prior to extraction. When the sodium chloride was omitted, recovery of TFP added to human plasma was only 60%. Addition of salt apparently decreases protein binding of TFP, and thus it is not necessary to add salt when extracting TFP from non-proteinaceous samples. Indeed, spectrophotometric measurements showed that more than 98% of TFP in an aqueous alkaline solution was removed by extraction with an equal volume of hexane.

TABLE I

RECOVERY OF TRIFLUOPERAZINE ADDED TO HUMAN PLASMA

No.	Trifluoperazine concentration (ng/ml)	Recovery (%)	Mean recovery (%)
1	3.0	99.9	
2	3.0	98.4	
3	3.0	91.4	96.6
4	0.2	95.9	
5	0.2	94.6	
6	0.2	97.7	96.1

Precision and accuracy

Precision and accuracy of the method were determined by preparing TFP plasma standards of several concentrations for triplicate analysis. The results presented in Table II show that the overall accuracy of the procedure was quite good. As would be expected, relative variation of the measurement increases as plasma TFP decreases.

TABLE II

PRECISION AND ACCURACY OF DETERMINATION OF TRIFLUOPERAZINE ADDED TO HUMAN PLASMA

Actual value (ng/ml)	Observed value (ng/ml)	$\overline{X}$	Coefficient of variation (%)
0.2	0.14, 0.25, 0.24	0.21	29.0
0.6	0.49, 0.49, 0.63	0.54	15.1
1.8	1.72, 1.96, 1.66	1.78	8.9

Selectivity

Evidence for the selectivity of the method was furnished by characteristic GC retention times of the reference compounds and the lack of interfering peaks in plasma extracts from subjects not treated with TFP (Fig. 1). Approximate retention times for chlorpromazine and trifluoperazine under the described conditions were 4.4 and 6.0 min, respectively.

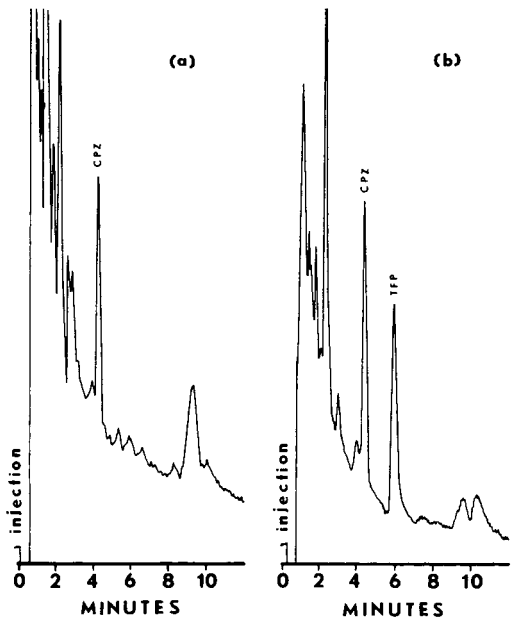

Fig. 1. Chromatograms of trifluoperazine (TFP) and the internal standard chlorpromazine (CPZ) after extraction from plasma. (a) Patient did not receive TFP; (b) patient treated with 20 mg Stelazine^{R} orally.

Patient use of chlorpromazine will interfere with accurate quantitation of TFP. Therefore, it is necessary to ascertain that the patient has not been using chlorpromazine. If this cannot be confirmed, a different internal standard must be selected.

Drugs that might interfere in the assay were tested (Table III). Chlordiazepoxide is not extracted from organic solvents by 0.1 *N* hydrochloric acid, and so would not interfere even though it is not completely resolved from TFP. If diazepam is present, an alternative internal standard should be selected due to its inadequate resolution from chlorpromazine. The other drugs listed do not interfere in the analysis.

Additionally, fluphenazine and perphenazine should not interfere in the assay. Due to their unshielded, highly polar hydroxyl groups, they are strongly adsorbed on the GC column and give very poor response unless derivatized [9, 12].

Even though none of the metabolites of TFP were chromatographed, their interference in this assay does not seem likely. TFP-SO produced by both rats

TABLE III

RETENTION TIMES FOR SOME PSYCHOACTIVE DRUGS RELATIVE TO THAT OF TRIFLUOPERAZINE

Drug	Relative retention time
Trifluoperazine	1.00
Amitriptyline	0.31
Imipramine	0.34
Promazine	0.47
Chlorpromazine	0.74
Diazepam	0.81
Chlordiazepoxide	1.13
Prochlorperazine	2.75
Haloperidol	2.83

[5, 6] and man [4], and 7-hydroxytrifluoperazine produced by rats [2, 5] have higher molecular weights and are more polar than TFP, and so would be expected to elute later than TFP, if present. A mixed function amine oxidase isolated from hog liver microsomes oxidizes the terminal nitrogen atom of the piperazine moiety of TFP, forming the N-oxide [13]. If this metabolite is formed in humans, it is unlikely to interfere in the assay because its higher molecular weight would cause it to elute after the parent drug.

If desmethyltrifluoperazine, and several other dealkylated primary or secondary amine metabolites produced by rats [5] are found in man, they are likely to elute after TFP due to their more polar nature. Also, hydroxylated metabolites would be expected to give very poor chromatographic response [12].

Stability

The stability of TFP in refrigerated (9°C) plasma was tested. A plasma sample containing 1.80 ng TFP per ml was prepared. Portions of this were tested at various intervals after preparation. Data are presented in Table IV. TFP appears to be sufficiently stable in refrigerated human plasma to allow storage in that condition for several days prior to analysis.

TABLE IV

TRIFLUOPERAZINE STABILITY IN REFRIGERATED PLASMA

A plasma sample containing 1.80 ng trifluoperazine per ml was prepared and stored refrigerated. Trifluoperazine concentration was measured at various intervals after preparation.

Days after preparation	Observed value (ng/ml)
0	1.84
4	1.84
7	1.72
10	1.71
14	1.46
16	1.87

CLINICAL APPLICATIONS

Fig. 2 shows the TFP absorption and disposition profile of a subject given a single 20-mg dose of Stelazine^{R}. Additional data presented in Table V on this and other subjects show large variations in peak TFP plasma concentrations and half-lives. These large variations underscore that the monitoring of individual patient TFP levels may improve the desired pharmacological action of TFP and related compounds.

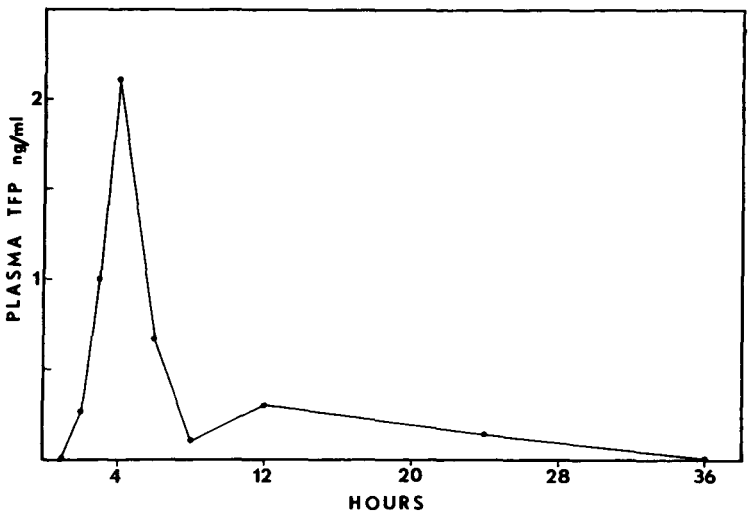

Fig. 2. Representative time course of plasma trifluoperazine (TFP) concentration following oral administration of 20 mg Stelazine^{R} to subject 1.

TABLE V

TFP PEAK PLASMA LEVELS AND HALF-LIVES OF PATIENTS GIVEN 20 mg STELAZINE ORALLY

Numbers in parentheses indicate hour of peak level.

Subject	Peak level		$t^{*}_{½}$
	ng/ml	h	
1	2.12	(4)	8.08
2	0.93	(6)	17.90
3	1.24	(4)	10.88
4	3.97	(3)	7.16

*$t_{½}$ estimated from the β elimination constant which was determined as described in Methods.

The sensitivity of the analysis should permit investigation of the possible relationship between plasma TFP levels and clinical effects. It might also allow one to determine patient compliance. Additionally, the procedure would be useful in comparing bioavailability of different TFP preparations.

It should be noted that when collecting samples for TFP analysis, the blood

should not come into contact with rubber stoppers since plasticizer in some rubber-stoppered blood collection tubes can cause displacement of drug into the red blood cells [11].

CONCLUSIONS

The method described for trifluoperazine quantitation is suitable for measurement of patient plasma levels after a single large dose. It should also be useful for determining levels following administration of multiple lower doses.

Sensitivity in the sub part per billion range was obtained through the use of an extremely sensitive nitrogen—phosphorus detector combined with a highly efficient extraction technique. Specificity was obtained through selective extraction, the selectivity of the nitrogen—phosphorus detector, and chromatographic resolution.

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CHROMBIO. 775

DETERMINATION OF ANTRAFENINE AND ITS MAIN ACID METABOLITE, 2-{[7-(TRIFLUOROMETHYL)-4-QUINOLINYL]AMINO}-BENZOIC ACID, IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LARGE VOLUME AUTOMATIC INJECTION AND GAS—LIQUID CHROMATOGRAPHY WITH DERIVATIVE FORMATION

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SUMMARY

Specific and sensitive analytical methods have been developed for the measurement of antrafenine and its main acid metabolite, 2-{[7-(trifluoromethyl)-4-quinolinyl]amino} benzoic acid (FQB), at therapeutic concentrations in plasma and urine.

Following the addition of internal standards (the methyl ester of FQB and 2-{[8-(trifluoromethyl)-4-quinolinyl]amino}benzoic acid) the parent drug and the metabolite were extracted from biological material with diethyl ether at a weakly acid pH. Drug extracts were evaporated to dryness prior to chromatographic analysis.

Antrafenine was measured by high-performance liquid chromatography using a Spherisorb 5-μm ODS column with acetonitrile—0.1 *M* sodium acetate as the mobile phase. Samples were injected automatically using a 500-μl injection loop. The detector wavelength was 353 nm corresponding to the maximum UV absorption of both drug and internal standard. The coefficient of variation (C.V.) for the determination of antrafenine concentrations between 5 and 250 ng/ml ranged between 24 and 3%, respectively.

The acid metabolite of antrafenine was measured by gas—liquid chromatography with electron-capture detection using a 1-m column packed with 3% OV-225 on Gas-Chrom Q (100—120 mesh) at 240°C and on-column methylation with trimethylphenyl ammonium hydroxide. The C.V. of the method for the analysis of metabolite concentrations between 10 and 500 ng/ml ranged between 3 and 9%, respectively.

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INTRODUCTION

Antrafenine (Stakane®) is an ester type non-narcotic analgesic and anti-inflammatory drug (Fig. 1) which is hydrolyzed in vivo to the corresponding acid and alcohol metabolites, which are also pharmacologically active [1–3].

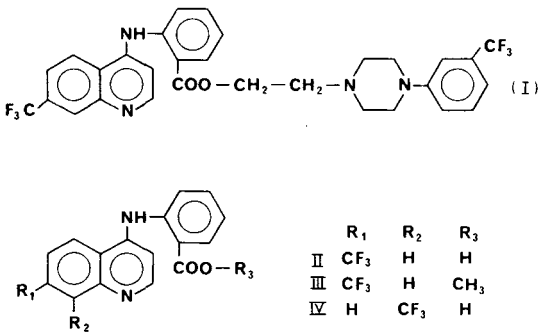

Fig. 1. Structure of antrafenine (I), the acid metabolite, FQB (II); the internal standard of antrafenine, EFQB (III); and the internal standard of FQB, AFQB (IV).

After the oral administration of antrafenine, the parent drug and its acid metabolite are detectable in plasma for at least 48 h, whereas the alcohol metabolite is only detectable in low concentrations in the form of a conjugate. Almost no unchanged antrafenine is excreted in urine. Following oral administration of [^{14}C]antrafenine labelled in the quinolyl and piperazine parts of the molecule, approximately 40% of the dose was recovered in the urine and 60% in the faeces. Most of the drug was found to be excreted as conjugated acid and alcohol metabolites.

In an earlier method (unpublished findings) antrafenine was hydrolyzed to the corresponding acid and alcohol products, both compounds being separated by thin-layer chromatography. These were then scraped off the thin-layer plate and following derivative formation, quantified by gas chromatography. This technique was found to be very laborious and unsuitable for pharmacokinetic studies.

In this paper, we describe an analytical procedure for the measurement of antrafenine and its main acid metabolite in plasma and urine using high-performance liquid chromatography (HPLC) with automatic injection (antrafenine) and gas–liquid chromatography (GLC) with on-column methylation (metabolite).

MATERIALS AND METHODS

Hydrochloric acid, distilled diethyl ether, methanol (analytical grade) and acetonitrile (LiChrosolv) used in this study were purchased from Merck (Darmstadt, G.F.R.). Trimethylphenyl ammonium hydroxide (TMPAH) 0.002 *M* (methanolic solution) was obtained from Pierce (Rockford, IL, U.S.A.).

Pure standards of antrafenine, its acid metabolite, 2-{[7-(trifluoromethyl)-4-quinolinyl]amino}benzoic acid (FQB), and their respective internal standards, the methyl ester of FQB (EFQB) and 2-{[8-(trifluoromethyl)-4-quinolinyl]amino}benzoic acid (AFQB) were synthesized in the Department of Chemistry of Lers-Synthélabo. Their structures are presented in Fig. 1.

The analysis of antrafenine was carried out on a Micromeritics 7000B liquid chromatograph equipped with a Micromeritics 785 UV visible spectrophotometer and a slightly modified Micromeritics 725 automatic injector with a 500-μl injection loop. After the last analysis the liquid chromatograph was switched off by the automatic injector, the detector and the recorder were switched off by a timer.

The mobile phase, acetonitrile—0.1 *M* sodium acetate (72.5:27.5, v/v) was adjusted to a flow-rate of between 2.00 and 1.25 ml/min (depending on the age of the column) through a 15 cm × 4.6 mm I.D. stainless-steel column, packed according to the technique described by Broquaire [4] with Spherisorb 5-μm ODS, batch 17/3 (Soparès, Gentilly, France). The detector wavelength was set at 353 nm, corresponding to the maximum UV absorption of both antrafenine and its internal standard (Fig. 2). Analysis of the acid metabolite (FQB) was performed on a Perkin-Elmer 3920 B gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD).

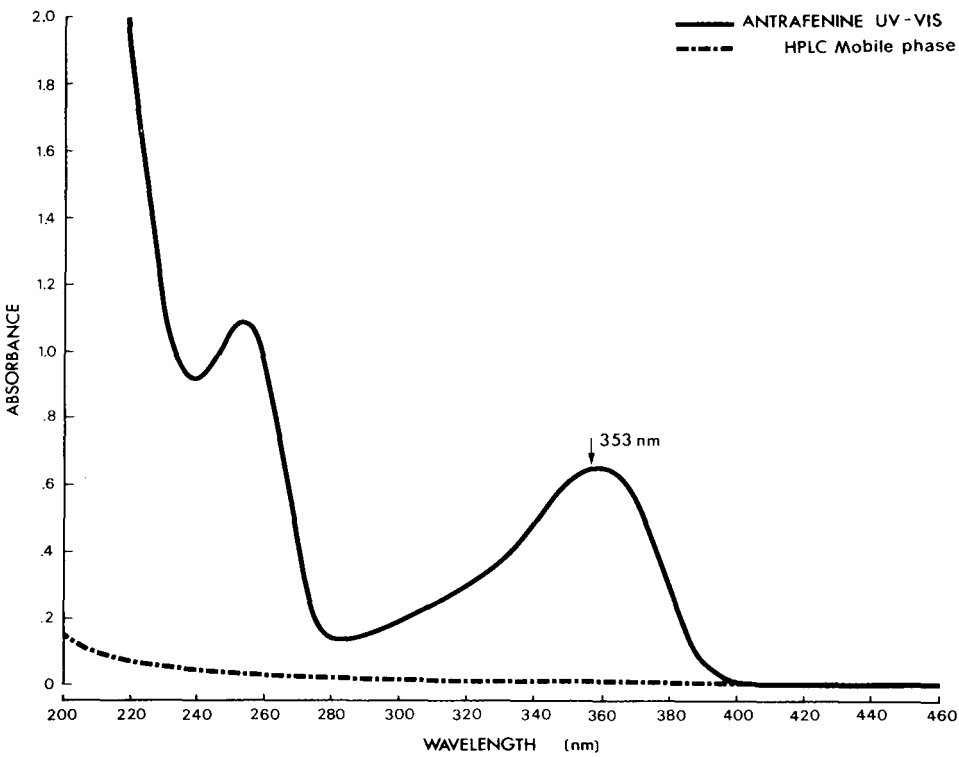

Fig. 2. Absorbance of antrafenine relative to that of HPLC mobile phase at different wavelengths.

A 1 m × 4 mm I.D. glass column was packed with 3% OV-225 coated on Gas-Chrom Q (100—120 mesh) and conditioned for 24 h at 245° C with an argon—methane (95:5) flow-rate of 65 ml/min. The operating conditions were: column temperature, 240° C; injector and detector temperatures, 300° C; and argon—methane carrier gas flow-rate, 65 ml/min.

Extraction procedures

Antrafenine. Plasma, blood or urine (2 ml) was added to a conical tapered tube containing 25 μl of an acetonitrile solution of internal standard (5 ng/μl). A 1-ml aliquot of a solution of 0.02 *M* potassium chloride acidified to pH 2.0 with hydrochloric acid was then added, the tube was vigorously shaken on a Vortex mixer and the pH adjusted to 2.8—3.2 with 10% hydrochloric acid, if necessary. The final solution was extracted with diethyl ether (8 ml) on a rock'n roll shaker for 20 min. The two phases were then separated by centrifugation at 4° C (1000 *g* for 5 min). The aqueous phase was discarded, and the ether phase transferred to another tube and evaporated to dryness at 37° C under a gentle stream of nitrogen. The dry extract was then dissolved in 870 μl of an acetonitrile—0.1 *M* sodium acetate solution (1 : 1) and each tube was shaken twice on a Vortex mixer for 10 sec. An aliquot of 860 μl of this final solution was transferred into an injection vial. The vial was placed on the automatic injector and 500 μl were injected into the liquid chromatograph.

FQB metabolite. A 25-μl aliquot of the AFQB internal standard solution (10 ng/μl in 0.1 *N* sodium hydroxide) was transferred to a conical tapered tube and mixed with 1.0 ml of 0.1 *N* hydrochloric acid and 1 ml of plasma. For urine 1 ml of the acidified potassium chloride solution was added. The final pH was approximately 4. The mixture was extracted twice with 2.5 ml of diethyl ether on a rock'n roll shaker for 20 min and then centrifuged (1000 *g* for 10 min). The two ether extracts were combined in a second tube and evaporated to dryness in a water bath (37° C) under a gentle stream of nitrogen. The TMPAH solution (250 μl) was added to the drug extract, Vortex mixed for 15 sec and 0.5—1.0 μl were injected into the chromatograph.

RESULTS

Antrafenine

Chromatograms from the analysis of plasma samples obtained after the oral administration of 450 mg of antrafenine are presented in Fig. 3a and b. The peaks corresponding to antrafenine and the internal standard were well resolved. Comparison with the blank plasma extracts showed that endogenous compounds present in plasma did not interfere with the analysis. A plasma pH of 2.8—3.2 was selected because under these conditions the extraction of endogenous interfering compounds was minimized (Fig. 3). The retention times were maintained at approximately 3.00 and 6.00 min for the internal standard and antrafenine, respectively, by decreasing the flow-rate from 2.00 to 1.25 ml/min depending on the age of the column. At 1.25 ml/min the reduced height equivalents to a theoretical plate (HETP) were 5.7 for the internal standard and 6.2 for antrafenine (mean diameter of the stationary phase was 5.0 μm, determined with a Coulter Counter). Time of analysis was 7

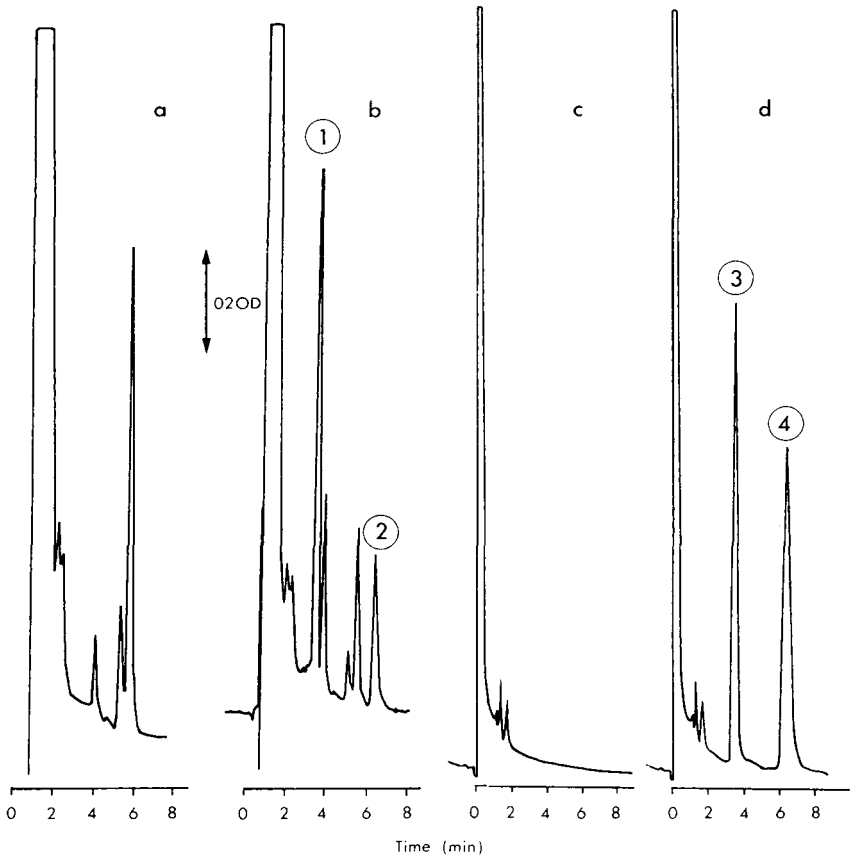

Fig. 3. Typical chromatograms after oral administration of antrafenine: "old column" HPLC of plasma extract, (a) blank); (b) containing antrafenine (2) and EFQB (1); GLC of plasma extract (c) blank, and (d) containing FQB (3) and AFQB (4). For conditions see text.

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD FOR THE MEASUREMENT OF ANTRAFENINE AT DIFFERENT CONCENTRATIONS

Spiked concentration (ng/ml)	No. of observations	Found concentration (ng/ml)	Standard deviation (ng/ml)	C.V. (%)
5	4	6.2	1.5	24
10	6	10.1	1.6	16
25	9	24.7	2.5	10
50	8	48.8	3.9	8
100	11	100.1	7.5	7.5
250	8	250.3	7.5	3

min between two injections. No strongly retained peaks appeared after this time.

The procedure was quantified using the peak ratio method. The calibration curve was linear between 5 and 1000 ng/ml (higher concentrations were not investigated). The limit of sensitivity was 5 ng/ml with a relative standard deviation of 24%. The reproducibility and the accuracy of the method were established by the injection of several samples at known concentrations. The number of analyses, the mean values, the standard deviation and the coefficient of variation (C.V.) are listed in Table I: the C.V. varied between 24 and 3% for concentrations of antrafenine between 5 and 250 ng/ml. Day-to-day variations of the calibration curve slope were less than ± 6%.

FQB metabolite

A typical GLC analysis of FQB and its internal standard (AFQB) in plasma is shown in Fig. 3c and d. Under the GLC conditions used, the retention times of FQB and AFQB were 3.45 and 6.25 min, respectively. The HETP was 2.25 mm for both compounds and the symmetry factors were 0.94 (FQB) and 0.75 (AFQB). The injector temperature was found to be a critical parameter because the dimethylation was quantitative only at temperatures above 300°C [3].

The response of the electron-capture detector was linear over the range 10—500 ng/ml for the biological specimens (blood, plasma, urine) analysed.

TABLE II

REPRODUCIBILITY OF THE GLC METHOD FOR THE MEASUREMENT OF FQB AT DIFFERENT CONCENTRATIONS

No. of observations = 6.

Spiked concentration (ng/ml)	Found concentration (ng/ml)	Standard deviation (ng/ml)	C.V. (%)
10	10.0	0.92	9.2
50	48.9	2.6	5.3
250	252.0	7.1	2.8
500	499.0	15.5	3.1

For higher concentrations of FQB the sample was diluted with TMPAH and reinjected. The C.V. was between 3.1 and 9.2% over the concentration range considered (Table II).

A typical plasma drug concentration—time curve of antrafenine and its acid metabolite in an adult subject following a single oral dose of 900 mg of antrafenine is shown in Fig. 4.

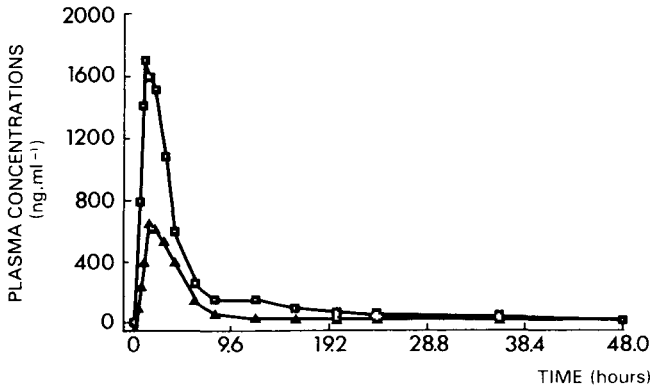

Fig. 4. Pharmacokinetic profile of antrafenine (▲) and its acid metabolite (■) in a healthy volunteer following single oral dosage of 900 mg.

DISCUSSION

Under the analytical conditions used for the HPLC analysis of antrafenine, the retention times were variable, depending on the age of the column. The flow-rate necessary to maintain constant retention times decreased from 2.0 ml/min for a new column to 1.25 ml/min for an old column. At this point an equilibrium appeared and the retention time did not decline further. A new column needed the injection of about 200 samples before reaching equilibration, and at that point, after more than 300 analyses, there was no loss in the efficiency of the column. The use of a large injection volume of non-eluting solvent (500 μl) did not alter the chromatographic separation and the reduced efficiencies were superior to those obtained with a classical injection valve [5]. The non-eluting solvent [acetonitrile—sodium acetate (1:1, v/v)] maintained antrafenine and its internal standard in a sharp band on the top of the column during all the injection time. Thus, there was no band-broadening due to the injected volume [6].

In a previous method [7] using manual injection, the sensitivity was 10 ng/ml of plasma, while with the automatic injector, the sensitivity was increased down to 5 ng/ml, using 2 ml of plasma, after injection of half of the total extract. The accuracy of the automatic injection method was twice that of the manual injection method. The improvement in accuracy of this method, considering that the same equipment was used in both cases, can be explained by a better dissolution in the large sample volume (870 μl) used for the automatic injection method than in a very small injection volume (25—50 μl) used in the manual technique.

Furthermore, in the present method, the injection of a large volume of solvent, different from that of the mobile phase, did not require a "saturation period" which was observed for other experiments with a large injection volume [5].

FQB and the isomer used as internal standard were chromatographed as their dimethyl derivatives.

The on-column methylation performed with TMPAH gave not only the methyl ester but also the methylamino derivative of FQB and AFQB, which was confirmed by GLC—mass spectrometry [3]. Derivatization was necessary to increase the volatility of FQB and AFQB, in order to make possible their analysis by GLC. The best results in terms of peak symmetry, resolution and sensitivity were obtained using a GLC column packed with 3% OV-225. The presence of a CF_3 group in the molecule of FQB and AFQB made the analysis possible using an electron-capture detector. The minimum sensitivity of this analysis was 10 ng/ml of FQB with a C.V. of ± 10% when 1 ml of plasma was analysed. The response of the electron-capture detector was linear for concentrations of FQB from 10—500 ng/ml of plasma. However, with higher concentrations of FQB (after oral doses of 300—600 mg) the analysis was performed by decreasing the volume of sample injected into the gas chromatograph (0.2—0.5 μl). In this case, the response of the electron-capture detector remained linear up to 2000 ng/ml.

In conclusion, the methods described, using both HPLC and GLC procedures, are suitable for the specific analysis of both antrafenine and its acid metabolite, FQB, at therapeutic concentrations in plasma and urine.

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DETERMINATION OF THE ANTI-TUMOR AGENT, 10-CHLORO-5-(2-DIMETHYLAMINOETHYL)-7H-INDOLO[2,3-C]QUINOLIN-6(5H)-ONE IN BLOOD OR PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific high-performance liquid chromatographic assay was developed for the determination of 10-chloro-5-(2-dimethylaminoethyl)-7H-indolo[2,3-C]quinolin-6(5H)-one [I] in blood or plasma with an overall recovery of 100.3 ± 9.1% and a limit of quantitation of 1.0 ng per ml of blood or plasma. The assay was used to determine blood concentrations of the drug in the rat following oral administration by intubation of a 1.17-mg dose of [I] · HCl.

INTRODUCTION

The compound 10-chloro-5-(2-dimethylaminoethyl)-7H-indolo[2,3-C]-quinolin-6(5H)-one, [I] (Fig. 1), is a member of a series of indoloquinolinones synthesized by Walser and co-workers [1, 2] and is of interest as an anti-tumor agent [3].

A sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of [I] in blood or plasma. The assay was used for the determination of blood concentrations of the drug in the rat following oral administration by intubation of a 1.17-mg dose of [I] · HCl.

The method quantitates compound [I] by fluorescence following normal-phase HPLC separation. The analog, 10-methoxy-5-(dimethylaminoethyl)-7H-indolo[2,3-C]quinolin-6(5H)-one, [II], was used as the internal standard in the assay because of its favorable retention time on HPLC analysis, and comparable luminescence properties.

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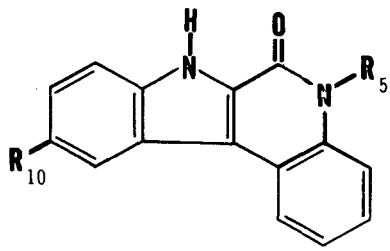

Compound	R_{10}	R_5	Retention time (min)	k'
I	Cl-	$-CH_2-CH_2-N(CH_3)_2$	4.4	2.4
II	CH_3O-	$-CH_2-CH_2-N(CH_3)_2$	5.9	3.5
III	Cl-	$-CH_2-CH_2-NH(CH_3)$	3.9	2.0
IV	Cl-	$-CH_2-CH_2-NH_2$	14	9.8
V	Cl-	$-CH_2-CH_2-OH$	14.5	10.2
VI	Cl-	-H	2.7	1.1

Fig. 1. Chemical structures, retention time and capacity factors (k') of some 10- and 5-substituted 7H-indolo[2,3-C]quinolin-6(5H)-ones.

EXPERIMENTAL

Column

The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing Partisil PXS 10/25 silica gel, 10 μm (Serial No. 1B1709, Whatman, Clifton, NJ, U.S.A.).

Instrumental parameters

A Waters Model 6000A high-pressure liquid chromatography pump, equipped with a Model U6K injection system and a pre-column filter (2-μm) (Waters Assoc., Milford, MA, U.S.A.), was used for chromatography. A Schoeffel Model FS-970 fluorescence detector operated at 258 nm for excitation and emission at wavelengths greater than 340 nm (Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.) was used for fluorimetric detection.

The isocratic mobile phase consisted of hexane—tetrahydrofuran—methanol—concentrated ammonium hydroxide (75 : 15 : 9.75 : 0.25) pumped at a constant flow-rate of 2.0 ml/min. Under these conditions, the retention time of

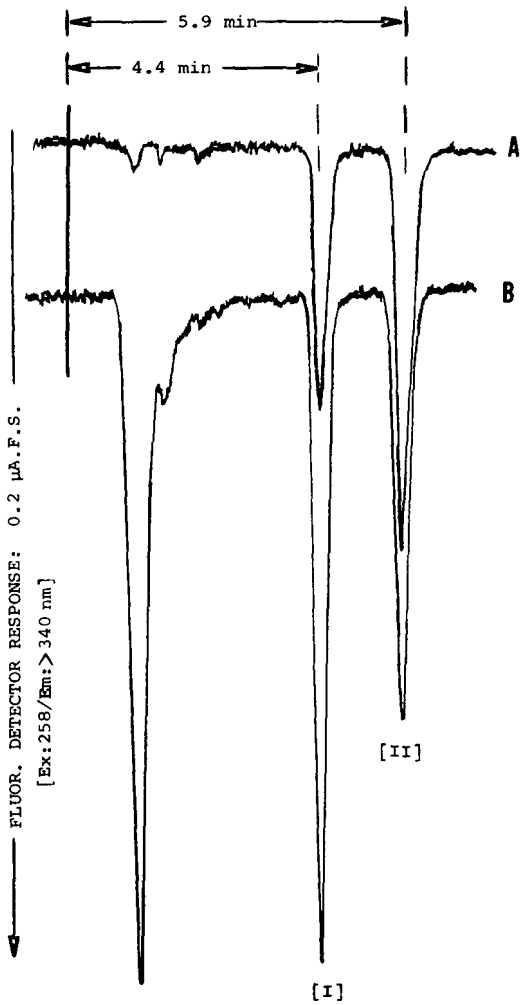

Fig. 2. Chromatograms of (A) authentic standard of 25 ng [I] and 10 ng [II] injected and (B) diethyl ether extract of 0.1 ml rat blood, 30 min post oral dose, containing added authentic [II].

compound [I] was 4.4 min while that of compound [II] was 5.9 min (Fig. 2), with capacity factors (k') of 2.4 and 3.5, respectively. The chart speed of the Hewlett-Packard dual-channel recorder (Model 7132A with option 108) was 0.5 in./min.

Spectrophotometric/fluorimetric instrumentation

Ultraviolet absorbance spectra were recorded using a double-beam ratio-recording spectrophotometer (Coleman Model EPS-3T Hitachi Spectrometer, Coleman Instruments, Maywood, IL, U.S.A.). Corrected luminescence excitation and emission spectra (10 nm bandpass) were recorded using a spectrofluorimeter equipped for direct recording of corrected excitation and corrected

emission spectra (Farrand Mark I, serial No. 947, Farrand Optical Co., Valhalla, NY, U.S.A.). Quantitative analytical fluorimetric data were generated in the uncorrected mode. Cryogenic (77°K) luminescence data were generated using commercially available accessories and equipment, made "in house", which has been previously described [4]. (An analogous cryogenic unit is now available from Farrand Optical Co.)

Reagents

All inorganic reagents were analytical-reagent grade (A.C.S.). All aqueous solutions were prepared in distilled, carbon-filtered, deionized water filtered through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.).

Organic solvents, suitable for spectrophotometry and liquid chromatography were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), with the exception of ethanol (200 proof, absolute) purchased from Pharmco (Publicker Industries, Linfield, PA, U.S.A.) and diethyl ether (anhydrous, absolute, reagent grade) purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Diethyl ether used for extraction of biological samples was used from a freshly opened can before each analysis and was shaken with granular zinc (about 1 g per 450 ml of ether) to reduce the peroxides, which might otherwise decompose low concentrations of [I].

Analytical standard solutions

Stock solutions of compound [I], ($C_{19}H_{18}ClN_3O$, mol. wt. 339.83 (base), m.p. 292—298°C) and compound [II] ($C_{20}H_{21}N_3O_2$, mol. wt. 335.41 (base), m.p. 285—287°C, used as the internal standard) were prepared containing 100 μg/ml of hexane—tetrahydrofuran—methanol (75:15:10). The stock solutions of [I] and [II] were used to prepare mixed standard solutions (Table I) by suitable dilutions in the mobile phase hexane—tetrahydrofuran—methanol—concentrated ammonium hydroxide (75:15:9.75:0.25). Aliquots (250 μl) of these mixed standard solutions were added to control (drug-free) blood or plasma as the processed standards to establish a calibration curve for the determination of the concentrations in the unknowns and for the determination of percent recovery.

TABLE I

STANDARD SOLUTIONS TO BE USED FOR HPLC ANALYSIS

Standard solution	Compound I (ng/250 μl)	Compound II (internal standard) (ng/250 μl)
1	250	10
2	100	10
3	25	10
4	5	10
5	0	10

Analysis of blood or plasma

A 250-μl aliquot of standard solution No. 5 (Table I) was transferred into a 15-ml glass-stoppered centrifuge tube as the internal standard [II] for each unknown. The organic solvent was evaporated at 40°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean dry nitrogen. Then a suitable aliquot (0.1—1.0 ml) of the blood or plasma sample was added and mixed well.

A separate set of standards was prepared by transferring 250-μl aliquots of mixed standard solutions 1, 2, 3, or 4 (Table I) into separate 15-ml centrifuge tubes, evaporating the organic solvent, and adding a suitable aliquot of control blood plasma and mixing well.

The samples were mixed vigorously and 1 ml of 1 *M* ammonium hydroxide and 10 ml of fresh diethyl ether were added. Each tube was stoppered (PTFE No. 13 stopper), and shaken for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes/min. The samples were centrifuged at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, Rotor No. 253; Damon/IEC, Needham, MA, U.S.A.) at 5°C and a 9.0-ml aliquot of the supernatant was transferred into another 15-ml conical centrifuge tube.

The ether extract was evaporated to dryness at 40°C in the N-EVAP evaporator under a stream of clean, dry nitrogen. The residue was dissolved in 250 μl of the mobile phase, 25 μl of which were injected for HPLC analysis using normal-phase (adsorption) chromatography. The Schoeffel fluorescence detector was set between 1.0 and 0.02 μA depending on the sensitivity desired. Typical chromatograms are shown in Fig. 2.

The concentration of [I] in each unknown was determined by interpolation from the calibration curve of the standards processed along with the unknowns, using the peak height ratio (ratio of peak height of compound [I] to the peak height of the internal standard [II] versus concentration) technique.

RESULTS AND DISCUSSION

Compound [I] and its analogs (Fig. 1) are conjugated heteroatomic molecules which possess strong UV absorption and intense luminescence emission characteristics. The spectral characteristics of [I] were determined in selected solvents, including the mobile phase used for HPLC analysis (Table II). The absorption, corrected excitation and luminescence emission spectra of [I] in the mobile phase used for HPLC analysis are shown in Fig. 3. The major UV absorption band of [I] occurs at 250—260 nm for all the solvents listed, the absorptivity (258—260 nm) ranges from 109 to 116 l/g · cm. The corrected fluorescence emission spectra determined at ambient temperature (25°C) show a hypsochromic "blue" shift from a broad, structureless emission (λ_{max} = 465 nm) with a high quantum efficiency at acidic pH; Φ_F = 0.43 in methanol—2 *N* HCl (1:1), to a sharper more structured emission spectrum (λ_{max} = 365, 380 nm) with lower quantum efficiency, Φ_F = 0.075 in the mobile phase, at basic pH. This suggests that at mildly acidic pH the compound exists as an ionized species in the excited state, but not in the ground state, since the UV absorption spectra do not exhibit wavelength shifts with pH. Optimal HPLC separation of [I] and [II] from endogenous interferences could only be achieved how-

TABLE II

UV ABSORPTION, 25°C FLUORESCENCE, AND 77°K LUMINESCENCE CHARACTERISTICS OF COMPOUND I IN SELECTED SOLVENTS

Solvent	UV Absorption		25°C Fluorescence		77°K Total luminescence		77°K Phosphorescence		
	Maximum λ_{ex} (nm)	Absorptivity (l/g · cm)	Maximum λ_{em} (nm)	Quantum efficiency (Φ_F)	Maximum λ_{em} (nm)	Quantum efficiency (Φ_L)*	Maximum λ_{em} (nm)	Approx. efficiency (Φ_P)**	Phosphorescence lifetime (sec)
Methanol—2 *N* HCl (1:1)	220 235 253 258 274 300 313 342 358	116	465	0.43	364, 380, 400, 493, 525	≈0.13	493, 525	≈0.01	1.4
1% Conc. H_2SO_4 in ethanol	253*** 260	115	380, 450	0.28	360, 377, 395, 492, 522	≈0.95	492, 522	≈0.03	1.4

1% 0.1 *N* potassium phosphate buffer (pH 7) in ethanol	253*** 260	109	382	0.23	N.M.§	N.M.	N.M.	N.M.	N.M.
1% Conc. NH_4OH in ethanol	252*** 259	116	365, 380	0.077	360, 375, 395, 480, 510	≈1.0	495, 520	≈0.03	1.3
Hexane—tetrahydrofuran—methanol—conc. NH_4OH (75:15:10:0.25)§§	225 240 253 260 277 300 313 340 357	111	364, 380	0.075	360, 375, 395, 480, 510	≈1.05	480, 510	≈0.055	1.25

* 77°K total luminescence includes both cryogenic fluorescence and phosphorescence. The fluorescence component is in the 350—450 nm spectral region while the phosphorescence component is in the 450—550 nm region. The light scatter from cracked solvent glasses severely limits the accuracy of Φ_L (total luminescence efficiency at 77°K).

** Φ_P is calculated from uncorrected spectra and must be considered approximate only.

*** Only the two most intense maxima are recorded here, the rest of the UV absorption spectrum is closely similar to that recorded for the mobile phase.

§ N.M. = not measured. Upon freezing this solvent scatters exciting light to the extent that the luminescence is masked, leading to erroneous measurements.

§§ Mobile phase for HPLC analysis; forms a snowy matrix upon freezing, thus Φ_L and Φ_P are highly approximate.

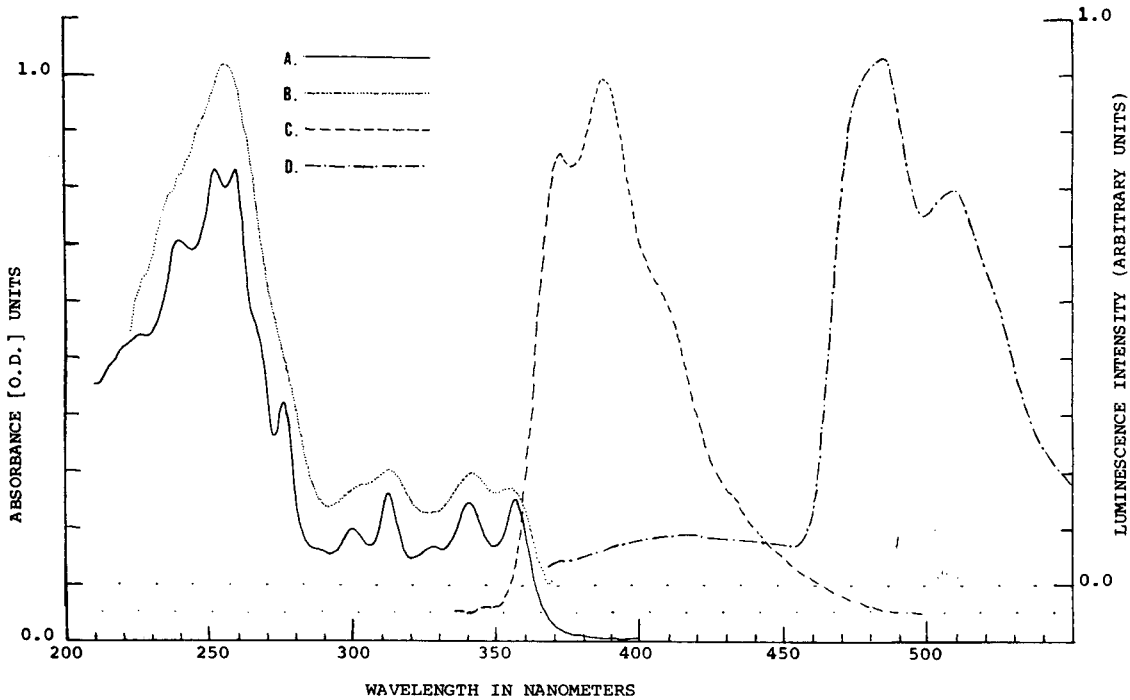

Fig. 3. (A) UV absorption spectrum (7.5 μg/ml); (B) corrected fluorescence excitation spectrum (0.25 μg/ml, λ_{em} = 380 nm, 10 × attenuation); (C) corrected fluorescence emission spectrum (0.25 μg/ml, λ_{ex} = 258 nm, 10 × attenuation); and (D) uncorrected phosphorescence emission spectrum (0.25 μg/ml, λ_{ex} = 258 nm, 3 × attenuation) of compound [I] in the mobile phase used for normal-phase HPLC analysis.

ever at basic pH, at the expense of higher sensitivity. Although the UV absorbance of [I] at 260 nm is sufficiently intense for quantitation in biological fluids using a tubeable UV detector, fluorescence detection was preferred due to higher sensitivity, specificity, and better overall background signal from the sample extract.

The cryogenic (77°K) luminescence characteristics demonstrate similar emission maxima regardless of pH and contain both fluorescence (singlet—singlet emission) in the 350—450 nm region and phosphorescence (triplet—singlet emission) in the 450—550 nm region. Total luminescence quantum efficiency, Φ_L, ranged from 0.13 at acidic pH to about 1.0 at basic pH. The phosphorescence quantum efficiency, Φ_P increased five-fold from acidic pH to basic pH, based on estimations from uncorrected spectra obtained in the phosphorescence mode. Phosphorescence lifetimes ranged from 1.40 to 1.25 sec over the pH range studied. The mobile phase forms a "snowy" matrix at 77°K, impairing the accuracy of the determination of Φ_L and Φ_P due to scatter effects.

The luminescence characteristics of the compound were used initially, to develop a thin-layer chromatographic (TLC)—spectrofluorimetric assay based on the intense fluorescence emission of [I] in acidic alcohol solutions. The analysis of [I] in blood or urine involved buffering the sample with 1 *M*

K_2HPO_4 (pH 8.8—9.1), double-extraction into diethyl ether, concentration of the combined ether extract, transfer of the ether concentrate containing [I] onto a 20 × 20 cm, E. Merck (Darmstadt, G.F.R.) pre-coated 60-μm silica gel F-254 TLC plate and ascending chromatography in benzene—methanol (1:1) as the developing solvent (Fig. 4). Compound [I] (R_F = 0.50) was then eluted from the silica gel with 5 ml of methanol, the residue of which was dissolved in 5 ml of methanol—2 *N* HCl (1:1) and quantitated in a 1-cm quartz cuvette by spectrofluorimetry at 460 nm, with excitation at 350 nm. The overall recovery of [I] by this procedure was 65 ± 7%, with a sensitivity limit of 70 ng/ml of sample.

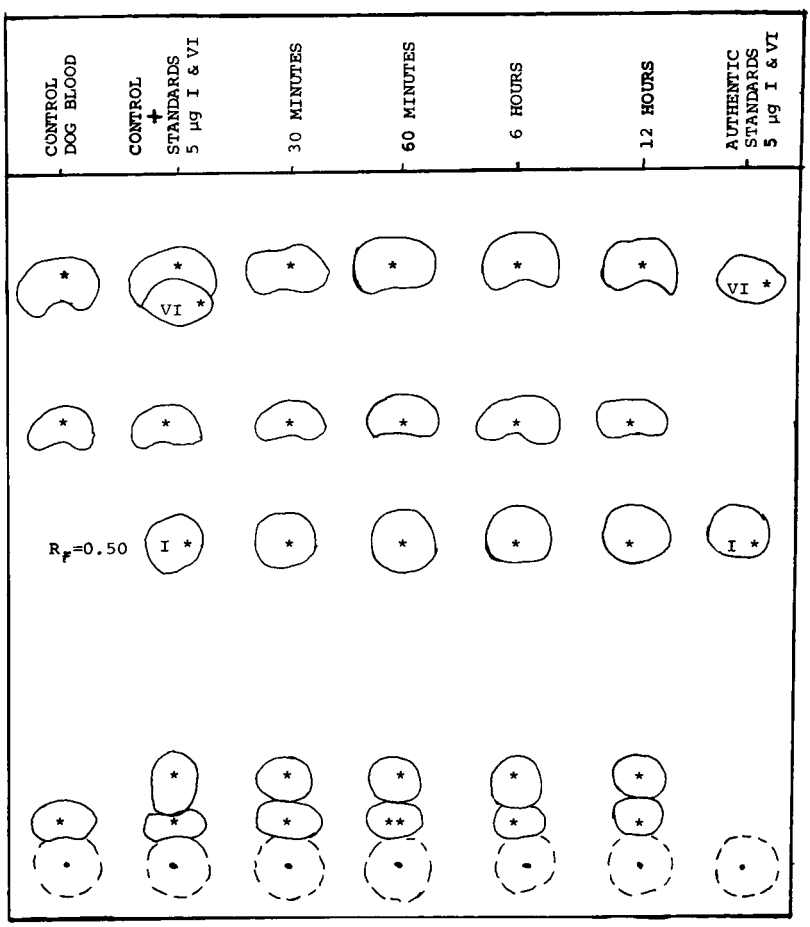

Fig. 4. TLC of ether extracts of dog blood after intravenous administration of 10 mg [I]/kg. Solvent system benzene—methanol (50:50). * = Fluorescent material.

The TLC—spectrofluorimetric assay was used in the early phases of pre-clinical development of the drug. The assay was tedious, time-consuming and was replaced by the HPLC assay described. The HPLC assay is a simpler more straightforward three-step operation which involves selective extraction, sample concentration, and direct analysis by HPLC with quantitation by spectrofluori-

metric detection. It provides better sensitivity, precision and higher sample throughput than the TLC procedure, hence it is the method of choice.

Recovery, precision and sensitivity limits of the HPLC assay

The mean recovery of compound [I] from human plasma over the concentration range 1.0—250 ng/ml is 100.3 ± 9.1% (S.D.) (Table III). The inter-assay precision over this concentration range showed a mean relative standard deviation of about ± 7.4%. The sensitivity limit of quantitation is 1.0 ng of [I] per ml of blood or plasma using a 1-ml sample per assay.

TABLE III

RECOVERY AND PRECISION OF ASSAY FOR COMPOUND [I] IN HUMAN PLASMA

Compound [I] added (ng/ml)	Replicates (n)	Average amount of [I] recovered* (ng/ml)	Mean recovery** (%)	Relative standard deviation** (± %)
1.0***	3	1.047	104.7	8.6
5.0	4	4.88	97.6	7.9
25	5	23.08	92.3	6.0
100	5	98.9	98.9	6.4
250	5	272.5	109.0	8.0

*Corrected for 9/10 ether aliquot in extraction procedure; calculated from a least squares regression analysis of HPLC response to external standards of [I], based on a logarithmic curve: $Y = aX^b = 0.0688\ X^{0.9761}$ with a correlation coefficient of 0.9983. Average percent deviation of the curve fit is ± 8.5%.

**Overall average recovery = 100.3% with an overall relative standard deviation of ± 9.1% (n = 22).

*** Limit of quantitation.

Analysis of biological specimens

The initial TLC—spectrofluorimetric assay was used to determine compound [I] in a pilot study in a dog following the intravenous administration of a 10 mg/kg dose (total dose = 80 mg [I] administered as the free base). Blood concentrations of [I] were measured over 48 h (Table IV), with an apparent half-life of elimination [$t_{1/2\beta}$] of about 5.4 h. The blood samples were also analyzed for compound [VI] as a potential metabolite (Figs. 1 and 4), however, none was found (sensitivity limit ≈ 0.07 μg/ml). The assay was specific by virtue of the TLC separation step.

The HPLC assay was subsequently used to determine compound [I] in a pilot study in one rat (400—450 g) following the oral administration by intubation of 1.5 ml of an ethanol—water (50:50) solution containing 1.17 mg of [I] · HCl. Blood concentrations of [I] were observed over 1.5 h, sampled via caudal venipuncture (Table V). None of the analogs III, IV, V, or VI considered to be potential metabolites (all of which were chromatographically resolved) (Fig. 1) were detected in these samples.

The sensitivity and specificity of the HPLC assay should be sufficient for future clinical pharmacokinetic studies.

TABLE IV

BLOOD CONCENTRATIONS OF COMPOUND [I] IN THE DOG FOLLOWING A SINGLE INTRAVENOUS ADMINISTRATION OF A 10 mg [I]/kg DOSE (TOTAL = 80 mg)

Time	Concentration* (μg/ml)
2.5 min	1.8
5.0 min	2.5
10 min	1.7
20 min	2.1
30 min	1.8
45 min	1.3
1.0 h	1.0
1.5 h	0.75
2 h	1.2
3 h	0.85
4 h	0.90
6 h	0.55
7 h	0.40
11 h	0.30
24 h	0.07
48 h	–**
72 h	–

* Determined by TLC—fluorimetric procedure.
** – = below the limit of quantitation (0.07 μg/ml of blood).

TABLE V

BLOOD CONCENTRATION OF COMPOUND [I] IN THE RAT FOLLOWING A SINGLE ORAL ADMINISTRATION OF 1.17 mg [I] · HCl BY INTUBATION

Time (min)	Concentration* (μg/ml)
15	2.3
30	0.62
45	0.68
60	0.42
75	0.83
90	0.25

* Determined by HPLC analysis with fluorimetric detection.

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CHROMBIO. 769

DETERMINATION OF THE MAJOR URINARY METABOLITE OF FLURAZEPAM IN MAN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of N-1-hydroxyethylflurazepam, the major urinary metabolite of flurazepam, in human urine is described. Urine specimens were incubated enzymatically to deconjugate N-1-hydroxyethylflurazepam glucuronide (metabolite) and were then extracted at pH 9.0 to extract the metabolite. The extracts were chromatographed on a microparticulate silica gel column using automatic sample injection, isocratic elution at ambient temperature and UV monitoring at 254 nm. The internal standard was 7-chloro-5-(2'-chlorophenyl)-1,3-dihydro-1-2-dimethylaminoethyl-2H-1,4-benzodiazepine-2-one. The recovery from urine, in the 0.5—25.0 μg/ml range, was 96.5 ± 11.5% (S.D.), and the sensitivity limit was 0.5 μg/ml. The method was found to be specific for N-1-hydroxyethylflurazepam in the presence of intact flurazepam and other possible urinary metabolites of flurazepam. The method was successfully applied to urine specimens collected from human subjects following the administration of 30-mg single oral doses of flurazepam dihydrochloride.

INTRODUCTION

Flurazepam dihydrochloride, [I] · 2 HCl, a hypnotic of the 1,4-benzodiazepine class marketed as Dalmane®, undergoes extensive biotransformation in man [1—3] to form the mono-desethyl [I-A], the didesethyl [I-B], the N-1-hydroxyethyl [II], the N-desalkyl [III] and the N-desalkyl-3-hydroxy [IV] metabolites (Table I). The major urinary metabolite, [II], present largely as the glucuronide conjugate, accounted for 30—55% of an orally administered dose [4].

Methods for the quantitation of [I] and its metabolites in blood have included luminescence [4], electron-capture—gas—liquid chromatography [5—9],

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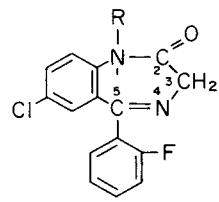
TABLE I

CHEMICAL STRUCTURES AND RELATIVE RETENTION TIMES OF FLURAZEPAM AND ITS MAJOR METABOLITES



	R		Relative retention time
Flurazepam	$-(CH_2)_2-N-(C_2H_5)_2$	[I]	0.88
Monodesethylflurazepam	$-(CH_2)_2-NH-C_2H_5$	[I-A]	0.55
Didesethylflurazepam	$-(CH_2)_2-NH_2$	[I-B]	2.43
N-1-Hydroxyethylflurazepam	$-CH_2-CH_2OH$	[II]	1.00
N-1-Desalkylflurazepam	-H	[III]	0.89
N-1-desalkyl-3-hydroxyflurazepam	-H	[IV], 3 > CHOH	3.75
Internal standard for HPLC	$-(CH_2)_2-N-(CH_3)_2$	[V], 2'-Cl	1.56

spectrofluorodensitometry [10], radioimmunoassay [II] and gas chromatography—mass spectrometry [12]. The pharmacokinetics of [I] and its metabolites [13] led to the consideration of utilizing the urinary excretion of the major metabolite, [II]-glucuronide, as a means of assessing bioavailability of [I] in man, since [II] accounts for more than 40% of a given dose over a 24-h excretion period [3,4]. Although bioavailability of dosage forms is commonly evaluated by measurement of unchanged drug in blood after the administration of a single dose of drug, utilization of urinary excretion data of unchanged drug or a major metabolite is accepted as a viable alternative [14].

Previously reported methodology utilizing enzymatic deconjugation, selective extraction and either spectrofluorometry [4] or differential pulse polarography [5] suggested the practicality of using the rate of excretion of [II] as a means of comparing bioequivalence of formulations of [I] · 2 HCl in man. In order to establish urinary excretion profiles following a single dose of [I] · 2 HCl in man, it is desirable to collect a series of specimens, over short time intervals, namely, 1—2-h intervals during the first 8 h, followed by 4-, 12-, and 24-h intervals up to 72 h post dose.

This report describes a more direct and rapid method than those previously reported [4, 5] for the quantitation of [II] in urine. The sample preparation is similar to a previously reported method [5], but is simplified by use of high-performance liquid chromatography (HPLC) for the separation of [II] from the other possible urinary metabolites of [I], thus eliminating the need for selective solvent extraction prior to enzymatic sample treatment. The method presented here involves enzymatic hydrolysis followed by solvent extraction at pH 9.0, followed by HPLC of the sample using a microparticulate silica gel column, with isocratic elution and UV detection at 254 nm. The limit of sensitivity of

the assay was 0.5 μg/ml of urine, and effectively resolves [II] in the presence of [I] and its metabolites, [I-A], [I-B], [III], and [IV]. In addition, the method was readily automated by using an automatic sample injector and includes use of 7-chloro-5-(2′-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one, [V], (Table I), as the internal standard for HPLC analysis.

EXPERIMENTAL

Reagents

Reagent grade chemicals were used to prepare: phosphate buffer, 1 *M*, pH 5.4 (prepared by mixing 820 ml of 1 *M* potassium dihydrogen orthophosphate with 180 ml of 1 *M* dipotassium hydrogen orthophosphate and adjusting the mixture to pH 5.4); sodium hydroxide, 6 *N*; hydrochloric acid, 0.25 *N*. Other reagents included: ammonium hydroxide, (J.T. Baker, Phillipsburg, NJ, U.S.A.); diethyl ether, anhydrous (Mallinckrodt, St. Louis, MO, U.S.A.); methylene chloride and methyl alcohol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

The HPLC mobile phase was composed of: methylene chloride and a mixture of methanol—water—ammonium hydroxide (concentrated) (150:9:1, v/v) in a 500:25 (v/v) ratio, prepared fresh for each chromatographic run, and vacuum degassed for approximately 5 min with ultrasonic vibrating prior to use.

Standards. N-1-Hydroxyethylflurazepam, [II], [7-chloro-1-(2-hydroxyethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one], mol. wt. 332.76. Prepare a stock solution (A) by weighing 10.0 mg of [II] into a 10-ml volumetric flask and dissolving it in methanol to yield a 1 mg/ml solution. Prepare working solutions (B) and (C) to contain 100 μg and 10 μg [II] per ml respectively, in 10 ml of mobile phase by sequential dilution of stock solution (A).

Internal standard, [V]. 7-Chloro-5-(2′-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one, mol. wt. 376.29. Prepare a stock solution (A′) containing 100 μg [V] per ml, by weighing 10.0 mg into a 100-ml volumetric flask and dissolving in the mobile phase. Prepare a working solution (B′) containing 5 μg/ml by transferring a 5-ml aliquot of (A′) into a 100-ml volumetric flask and diluting with mobile phase. This solution is used to reconstitute the extracted urine samples prior to injection into the HPLC.

Instrumentation

The liquid chromatographic system used consisted of the following components: a constant-flow high-pressure solvent delivery pump Model 6000A, and a fixed-wavelength dual-channel UV detector (254 nm) Model 440 (Waters Assoc., Milford, MA, U.S.A.); automatic sample injector with a 50-μl fixed-volume loop Model 725 Auto Injector (Micromeritics, Norcross, GA, U.S.A.); a microparticulate (10 μm) silica gel column, 25 cm × 4.6 mm I.D. Partisil PXS (Whatman, Clifton, NJ, U.S.A.) and a 10-mV dual-channel recorder Model 7130A (Hewlett-Packard, Avondale, PA, U.S.A.).

The instrumental parameters used to obtain the separation were: flow-rate, 1.5 ml/min; operating pressure, 45 bar; ambient column temperature; detector

sensitivities, 0.05 and 0.5 a.u.f.s.; recorder chart speed, 0.5 in./min.

Under these conditions of analysis, 150 ng of [II] injected gave nearly full-scale response and 250 ng of [V] injected gave 60% of full-scale response at 0.05 a.u.f.s. The retention times of [II] and [V] were 4.5 and 7.0 min, respectively (Fig. 1).

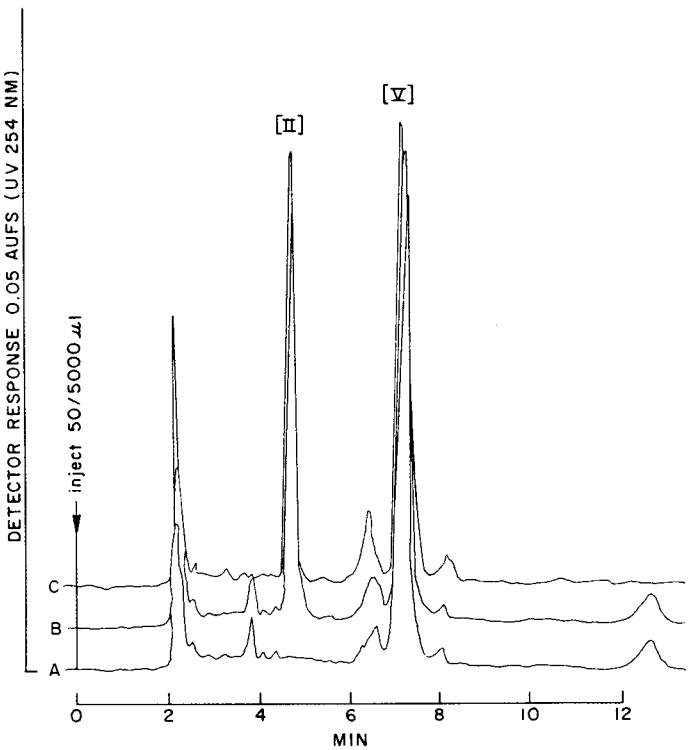


Fig. 1. Chromatograms of urine extracts of urine specimens post-glusulase incubation: (A) control urine spiked with internal standard [V]; (B) control urine spiked with 10 µg [II] per ml; (C) urine specimen from a subject following oral administration of 30 mg [I] · 2 HCl, 2—3 h collection, 1 ml urine assayed.

Preparation of samples for automatic injection into the HPLC instrument

Aliquots of 1.0 ml of urine were transferred into 50-ml glass centrifuge tubes using a pipetting device Pipetman, Model P1000D (Gilson, Middleton, WI, U.S.A.). The pH values of the samples were measured with a Model 125 digital pH meter, using a micro combination electrode (Corning Glass Works, Corning, NY, U.S.A.) and adjusted to pH 5.4 by dropwise addition of 0.25 *N* hydrochloric acid. Then 2 ml of 1 *M* phosphate buffer, pH 5.4, were added and the sample mixed well. Enzyme preparation: 0.10 ml Glusulase® (Endo Labs., Garden City, NY, U.S.A.) was added to each sample using a 1-ml glass hypodermic syringe, fitted with a stainless-steel needle. The samples were then placed in a rack, loosely stoppered with cotton and incubated overnight (about 16 h) at 37°C in a mechanical incubation shaker (Dubnoff, Precision Scientific, Chicago, IL, U.S.A.).

Following incubation, the samples were removed from the water bath, allowed to equilibrate to room temperature, and adjusted to pH 9.0 by the addition of 6 *N* sodium hydroxide (usually only 3—4 drops required), mixed well and checked on a pH meter. The samples were then extracted twice with 12-ml portions of anhydrous diethyl ether (sealing these tubes using PTFE stoppers) by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.), and then centrifuging for 10 min at 2500 rpm (1000 *g*) at 5°C in a refrigerated centrifuge (Model PR 6000, Rotor No. 253, IEC/Damon, Needham Heights, MA, U.S.A.). Following each centrifugation the ether extracts were transferred to 15-ml glass centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen in a water bath at 40°C.

The residues were then reconstituted in solution (B′) containing 5 μg/ml of internal standard [V] in the mobile phase. The volume of solution used varied from 1.5 to 5.0 ml depending on the concentration of [II] in the unknowns. Aliquots (about 500 μl) of the samples were then transferred to vials (Micromeritics; capacity about 750 μl) and capped for automatic injection using a 50-μl loop. Each sample vial in the autoinjector carousel was separated by a wash vial containing mobile phase, programmed not to be injected.

Typical chromatograms following automatic sample injection are shown in Fig. 1 for (A) control urine extracts, (B) control plus added standards, and (C) an extract from a subject following the administration of 30 mg [I] · 2 HCl.

Quantitation of [II] in the unknown samples

Along with each set of unknowns to be determined, a set of standards of [II] added to control (drug-free, predosing) urine was assayed. Concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 25.0 μg [II] per ml of urine (prepared by evaporation of the appropriate aliquot of standard solution (B) or (C) were processed along with the unknowns. These calibration standards were used to establish a least squares linear regression curve, using the peak height ratio of [II]/[V] for each concentration of standard, for the measurement of the unknowns. The equation of a typical calibration curve from 0.5—25.0 μg [II] per ml was $y = 2.842\,x + 0.004$ and the coefficient of correlation was 0.9999.

RESULTS

Recovery, reproducibility, linearity and sensitivity limits

The recovery of [II] was found to be 96.5 ± 11.5% (S.D.) over the concentration range 0.5—25.0 μg [II] per ml urine. The sensitivity limit ranged from 0.5 to 1.0 μg [II] per ml depending on the endogenous material in control urine. The precision of the autoinjector was 1.9%.

Specificity of the method

The other minor metabolites of [I] (Table I) were chromatographed as pure standards to determine their relative retention times compared to [II]. Interferences from endogenous compounds in the urine extracts were minimized or eliminated by injecting only 1—3% of the reconstituted extracts (50 μl from 1.5—5.0 ml of reconstituted extract).

Substances other than [I] and its metabolites, and the internal standard [V],

were not determined in this system for potential interference, since no other drugs were co-administered in these controlled human studies. However, other benzodiazepines and/or their metabolites could potentially interfere with the specificity of this method since they are also amenable to HPLC analysis [15].

Application of the method for bioavailability assessment

The urinary excretion profiles of [II] were determined in two normal volunteers following the administration of single 30-mg oral doses of [I] · 2 HCl. Urine specimens were collected over the following time intervals: −12—0 h (pre-dosing control), 0—1, 1—2, 2—3, 3—4, 4—6, 6—8, 8—12, 12—24, 24—48,

TABLE II

URINARY EXCRETION DATA OF THE N-1-HYDROXYETHYLFLURAZEPAM LEVELS IN TWO SUBJECTS WHO RECEIVED 30-MG ORAL DOSES OF FLURAZEPAM DIHYDROCHLORIDE*

Subject no. 1: age, 20 years; sex, male; weight, 59.47 kg; dose, 0.50 mg [I] · 2 HCl per kg.
Subject no. 2: age, 51 years; sex, male; weight, 61.29 kg; dose, 0.49 mg [I] · 2 HCl per kg.

Time interval (h)	Concentration** (μg/ml)	mg per time interval**	mg per h**	Percentage of dose excreted***
Subject no. 1				
−12—0	—	—	—	—
0—1	67.65	1.759	1.759	8.12
1—2	38.39	2.284	1.142	10.55
2—3				
3—4	11.24	0.354	0.354	1.63
4—6	6.49	0.454	0.227	2.10
6—8	2.34	0.367	0.184	1.69
8—12	0.64	0.685	0.171	3.16
12—24	0.85	0.481	0.040	2.22
24—48	0.10	0.261	0.011	1.21
48—72	0.05	0.121	0.005	0.56
0—72	—	6.766	—	31.24
Subject no. 2				
−12—0	—	—	—	—
0—1	13.94	0.523	0.523	2.42
1—2	26.21	1.979	1.979	9.14
2—3	5.47	1.001	1.001	4.62
3—4	1.62	0.365	0.365	1.69
4—6	4.14	0.513	0.257	2.37
6—8	1.38	0.221	0.111	1.02
8—12	0.58	0.293	0.073	1.35
12—24	0.62	0.397	0.033	1.83
24—48	0.25	0.465	0.019	2.15
48—72	0.17	0.196	0.008	0.19
0—72	—	5.953	—	27.50

* 30 mg flurazepam dihydrochloride administered equivalent to 25.25 mg flurazepam free base.
** All expressed in terms of N-1-hydroxyethylflurazepam.
*** Expressed in terms of flurazepam free base.

and 48—72 h post dose. The total urine volume voided in each interval was recorded, and 50-ml aliquots were stored at −17°C for analysis.

The urinary concentrations of [II] determined in specimens collected from the two subjects are shown in Table II. The urinary excretion rate data (mg per h) for [II] are shown in Table II and plotted in Fig. 2. The percentages of dose excreted at each interval and cumulatively, expressed in terms of [I] free base are also shown in Table II. These excretion profiles were shown to be reproducible within subjects and consistent between subjects.

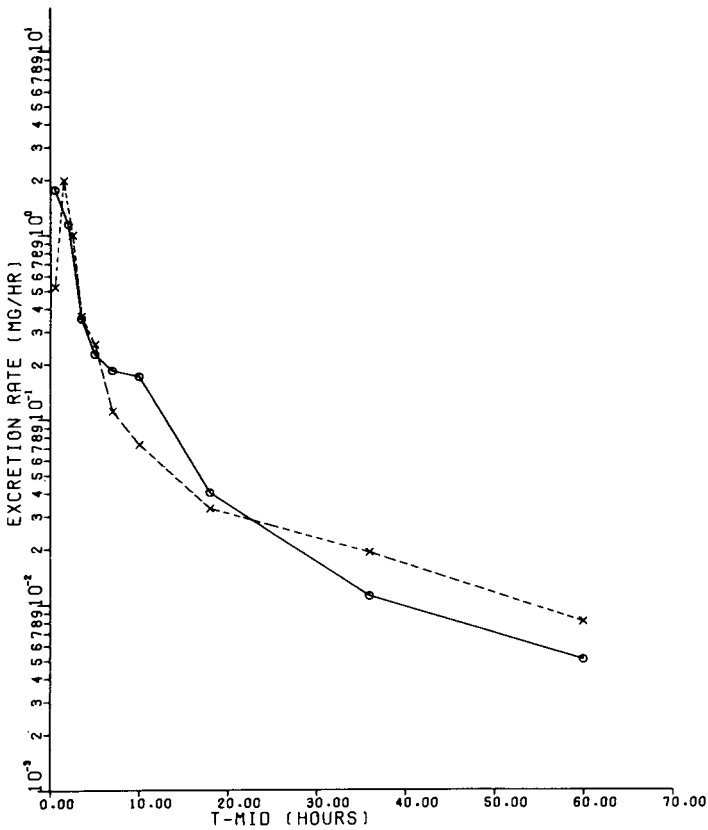


Fig. 2. Urinary excretion rate—time profiles of N-1-hydroxyethylflurazepam following oral administration of 30-mg doses of flurazepam dihydrochloride capsules to two human volunteers. o——o, Subject 1; x — — — x, Subject 2.

DISCUSSION

The UV absorbances of [I] and its metabolites, [I-A], [I-B], [II], [III] and [IV] in the HPLC mobile phase at 254 nm were sufficient to detect as little as 10 ng of pure standards injected at a detector sensitivity of 0.01 a.u.f.s. It has been reported that following single oral 90-mg doses of [I] · 2 HCl in man, about 0.5% of the dose could be accounted for in a 24-h excretion period as unconjugated [I], [II], [III], and [IV] while significant amounts of uncon-

jugated [I-A] and [I-B] (0.49—2.98% and 3.63—13.1% of the dose, respectively) and conjugated [II] (29.7—32.1% of the dose) were found [4].

Attempts to quantitate [I-A] and [I-B] by the HPLC method described were unsuccessful due to the presence of endogenous interfering substances. Modification of the method to resolve this problem was not attempted since no interferences were encountered for the analysis and quantitation of [II], the compound of interest.

A suitable HPLC procedure was developed for the quantitation of [II] in human urine following the administration of single 30-mg oral doses of [I] · 2 HCl and was used to determine the rate of excretion of conjugated [II] as a facile non-invasive means of determining the bioavailability of [I] · 2 HCl. The feasibility of this approach is demonstrated by the data shown in Table II and graphically in Fig. 2 for two subjects. The data are in agreement with previously reported results for percent of dose recovered as [II] in 24 h [3, 4]. The importance of urine sample collections over short excretion intervals to assess both rate and extent of bioavailability was shown in the data tabulation (Table II). If too few collection intervals are obtained, however, the only evaluation that could be made would be the extent of recovery of dose.

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CHROMBIO. 779

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF GENTAMICIN IN BIOLOGICAL FLUIDS

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SUMMARY

A selective and sensitive method for the determination of gentamicin in plasma and urine by high-performance liquid chromatography has been developed. Following deproteinization, the gentamicin is reacted with fluorescamine to produce a fluorescent derivative. This reaction mixture is directly chromatographed on a cation-exchange column using as mobile phase acetonitrile—phosphoric acid (7:3). The gentamicin components elute as a single peak. Using 0.1 ml of plasma, quantitation of gentamicin concentrations as low as 1 mg/l are possible. Possible interference from other aminoglycosides and antibiotics is discussed.

INTRODUCTION

A variety of techniques are available for the quantitative determination of gentamicin and other aminoglycosides in biological fluids. Until recently sensitive high-performance liquid chromatographic (HPLC) methods were not available since gentamicin does not possess an absorbing chromophore and, therefore, does not lend itself to UV detection. This necessitates derivatization of gentamicin to allow detection with the required sensitivity. Aminoglycosides have been derivatized with *o*-phthalaldehyde (OPA) [1—3], dansyl chloride [4] and 1-fluoro-2,4-dinitrobenzene (FDNB) [5], all of which are suitable for HPLC analysis using either fluorescent or UV detection. Those assays using OPA utilize a normal column chromatographic technique for sample preparation. Thereafter, HPLC results in multiple peaks which correspond to the various components [1—3]. Peng et al. [4] report derivatization of gentamicin with dansyl chloride. Their derivatization procedure re-

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quires heat and is time-consuming. Also, in our laboratory, duplication of their reaction and chromatographic conditions resulted in variable chromatograms with more than their reported two peaks. The derivatization procedure using FDNB is the most time-consuming, requiring incubation of gentamicin with FDNB at 80°C for 45 min [5].

Quantitation of the individual components may be important from a research aspect. However, in the clinical situation quantitation of the individual components or combinations thereof, is of little value.

Fluorescamine is a third derivatizing agent which produces fluorescent products [6—8]. Fluorescamine has advantages over the other two fluorescent derivatizing agents, in that (1) the nucleophilic reaction with an amine proceeds at a much faster rate, being complete within seconds [6] and (2) excess fluorescamine is hydrolyzed to non-fluorescent products [6].

We report the development of an HPLC assay which utilizes fluorescamine as the derivatizing agent. On chromatography the derivatized gentamicin yields only a single product, which we feel is an advantage. The assay has been used to analyse serum and urine samples obtained following a single intravenous dose of gentamicin to rabbits.

MATERIALS AND METHODS

Reagents

The components of gentamicin sulphate and the mixture (referred to as gentamicin) were donated by Schering (Bloomfield, NJ, U.S.A.). The labelled potencies are 620, 641, 788 and 551 μg/mg of powder for C_1 C_{1a} C_2 and gentamicin respectively. Fluorescamine [4′-phenylspiro(furan-2(3H),1′-phthalal)-3,3′-dione] was obtained from Sigma (St. Louis, MO, U.S.A.). All organic solvents were glass distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Other chemicals were reagent grade.

Chromatographic system

The chromatographic system consisted of a solvent delivery pump (Altex Model 100A), connected to a sample loop injector (Altex Model 905-42) fitted with a 175-μl sample loop. A chromatographic fluorescence detector (Schoeffel FS 970) monitored the effluent. The excitation wavelength was set at 275 nm and a KV 418 emission cut-off filter was used to select the fluorescence for detection. The mobile phase consisted of acetonitrile—phosphoric acid (5 g/l) (7:3). This was pumped through a cation-exchange column (Partisil SCX; 250 × 3.6 mm, particle size 10 μm) at 2 ml/min. The chromatograms were recorded on a 10-mV potentiometric chart recorder (Hewlett-Parkard Model 7101B-06-07) at a chart speed of 0.2 in./min.

Analytical variables affecting the gentamicin—fluorescamine reaction in water

Initially, the reaction between gentamicin, dissolved in water and fluorescamine, dissolved in acetone, was investigated with respect to the following variables: (1) pH of the reaction mixture; (2) buffer strength of the reaction mixture; (3) amount of fluorescamine required. Optimum conditions were evaluated on the basis of peak height using the aforementioned chromatographic conditions.

Sample preparation

Plasma. In a clean dry 15-ml test tube, 100 µl of a plasma sample and 800 µl of 0.05 mol/l potassium phosphate (pH 4.5) were mixed with 50 µl of 1.0 mol/l sodium hydroxide. After briefly vortexing, 2.5 ml of acetonitrile are added. The tube was again vortexed and then centrifuged at RFC = 2500 *g* for 2 min. After centrifugation the supernatant was decanted into a second tube containing 2.0 ml of methylene chloride. This tube was vortexed for 30 sec and then centrifuged for 2 min. Following centrifugation, 500 µl of the upper aqueous layer were pipetted into a third tube with 40 µl of 0.1 mol/l hydrochloric acid. The tube was then vortexed and during mixing, 200 µl of acetone containing 240 µg of fluorescamine were added. Fifty microlitres of the resultant clear yellow solution were chromatographed. Standards of 1, 2, 5, 10, 15, 30 and 40 mg/l were prepared and treated in an identical fashion to that described above.

Gentamicin in human plasma can be quantitated by the above method. However, prior extraction with methylene chloride is not necessary.

Urine. Into a clean dry 15-ml test tube, 50 µl of a urine sample were mixed with 500 µl of 0.05 mol/l phosphate buffer (pH 7.35). During mixing, 200 µl of acetone containing 240 µg of fluorescamine were added and 50 µl of the resulting clear yellow solution were injected into the liquid chromatographic system.

Rabbit study

Rabbits were weighed and placed in restraining cages. Angiocaths (Deseret; Sandy, UT, U.S.A.) were placed in the marginal veins of each ear. Under mesmerization, a Foley catheter (No. 8 Fr. Paediatric Foley Catheter; Acmi, Sullivan, IN, U.S.A.) was inserted into the bladder via the urethra. The dose of gentamicin (Cidomycin, 3.5 mg/kg; Roussel, Montreal, Canada) was administered via one of the ear veins, thereafter plasma and urine samples were taken at selected times over a 6-h period.

RESULTS

Reaction conditions in aqueous solution

The reaction between gentamicin (in aqueous solution) and fluorescamine (dissolved in acetone), was investigated with respect to pH, buffer strength and the amount of fluorescamine required. From these investigations, it appeared that gentamicin reacts optimally with fluorescamine between a pH of 7.2 and 8.0 when the molar strength of the phosphate buffer (pH 7.35) exceeds 0.03 *M*.

The amount of fluorescamine required to give greatest peak height and linear standard curves was determined to be about 10 *M* fold. This 10 *M* fold excess is required (to maintain linearity in a standard curve), regardless of pH, or amount of gentamicin. Once the molar ratio of fluorescamine to gentamicin falls below 10 the standard curve begins to deviate from linearity.

Using these optimum conditions, equal concentrations of each of the three major components of gentamicin, C_1 C_{1a} and C_2 were derivatized and chromatographed individually and together. When chromatographed individually, the peak heights and retention times were identical. When chromatographed together the components eluted as a single peak.

Recovery from plasma

The analysis of gentamicin utilizing the above-mentioned optimum reaction conditions and only an initial protein precipitation step, using acetone, proved to be sufficient for human plasma. However, attempts to reproduce these results with gentamicin in rabbit plasma yielded less than adequate recovery. This problem was circumvented by utilizing the extraction mentioned under Methods.

Since it was expected that amines in plasma would react with fluorescamine, the 10-fold molar ratio was re-investigated for a 100-μl sample of rabbit plasma containing 40 mg/l of gentamicin sulphate. The results, plotted in Fig. 1, demonstrate that at least an 80—100 fold molar excess is required to give maximum peak height and, therefore, linear standard curves in the concentration range from 0—40 mg/l of gentamicin sulphate.

Evaluation of protein precipitants (acetone and acetonitrile) and back-extracting solvents (diethyl ether, ethyl acetate, hexane, and methylene chloride) led to the choice of acetonitrile—methylene chloride as the best protein precipitant/back-extracting solvent combination. In addition to extracting the greatest amount of interfering substance, this combination of solvents leaves the aqueous layer (containing gentamicin) above the organic layer, making it easier to remove.

Interference from other drugs was tested by subjecting them to the procedures developed for gentamicin and chromatographing the products. Of the drugs tested (quinine, quinidine, amiloride, tobramycin, amikacin, netilmicin, ampicillin, cloxacillin, ticarcillin and cefazolin) only tobramycin and netilmicin produced a peak sufficiently close to the gentamicin peak to interfere with its quantitation. Retention times for gentamicin, tobramycin and netilmicin were 2.25, 2.42 and 2.25 min respectively. The recovery of gentamicin from a 100-μl plasma sample relative to water is about 93%.

Comparison of a series of standard curves for gentamicin in rabbit plasma,

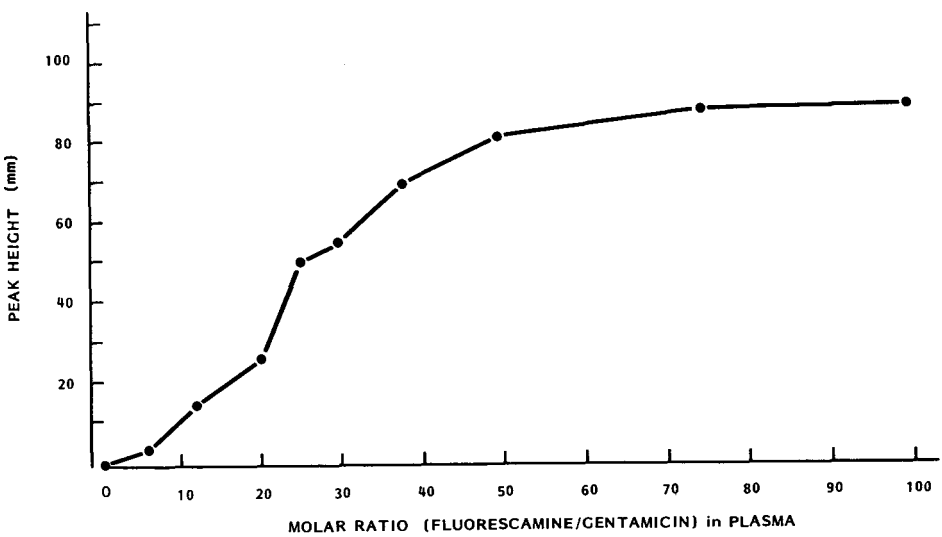

Fig. 1. Investigation into the optimum molar ratio of fluorescamine/gentamicin required with a 100-μl rabbit plasma sample.

produced on different days, demonstrates the reproducibility of the overall assay procedure (Table I). The intra-assay coefficient of variation, which never exceeded 3.5%, and the coefficients of determination (r^2) indicate that gentamicin concentrations in plasma produce linear and reproducible standard curves in the range from 0 to 40 mg/l. Replicates of unknowns, analysed on different days, demonstrated that the inter-assay coefficient of variation is less than 2%. Blank plasma showed no peaks which would interfere with the peak height quantitation of gentamicin (Fig. 2). The limit of sensitivity of this assay is about 1.0 mg/l of gentamicin sulphate. This corresponds to about 5 ng injected.

Standard curves in urine, according to the method described previously, were linear in the range from 0 to 71 mg/l. This, and the reproducibility of the overall assay technique is demonstrated by the low coefficients of variation and r^2 values observed in standard curves (Table II). Blank rabbit urine showed no peaks which would interfere with the peak height quantitation of gentamicin (Fig. 3). The limit of sensitivity of this procedure is about 1.0 mg/l.

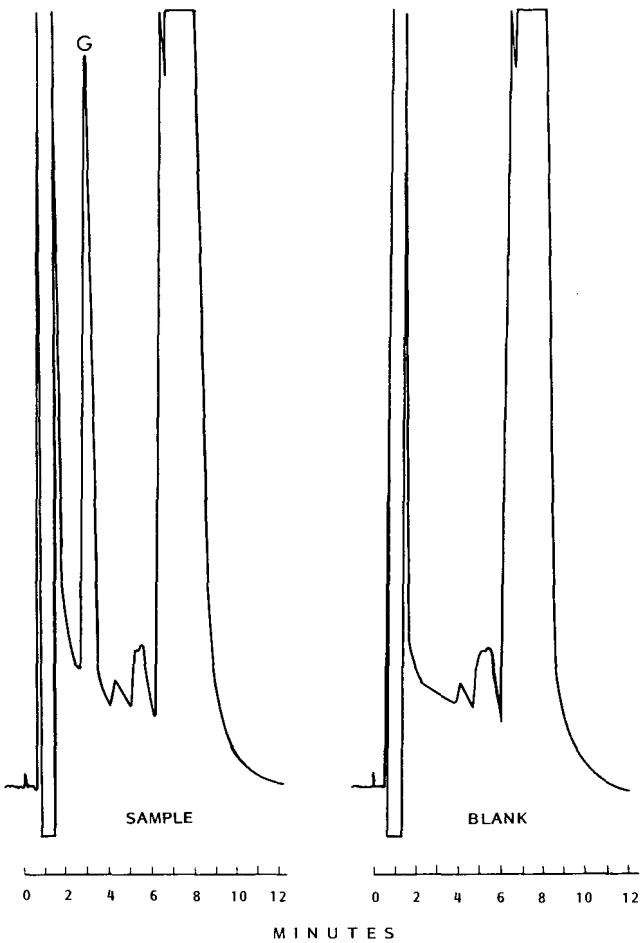

Fig. 2. Typical chromatograms of a blank rabbit plasma sample and one containing 19.1 μg/ml of gentamicin (G).

TABLE I

COMPARISON OF STANDARD CURVES FOR GENTAMICIN SULPHATE IN RABBIT PLASMA

Curve No.	Curve regression parameters			Coefficient of variation (%) at various gentamicin concentrations*		
	r^2	Slope	Intercept	Concentration (μg/ml)		
				5.93	11.68	19.10
1	0.9987	3.25	−0.21	2.56	0.80	0.65
2	0.9994	4.26	−0.79	2.47	2.89	1.09
3	0.9999	2.59	0.17	2.24	1.94	1.43
4	0.9982	2.49	0.73	0.00	3.45	2.11
5	0.9996	3.81	0.25	3.27	1.12	2.69
6	0.9977	3.17	−0.65	3.02	1.02	0.00
7	0.9996	2.86	0.24	0.00	0.86	2.80

*Coefficient of variation based on at least four replicates.

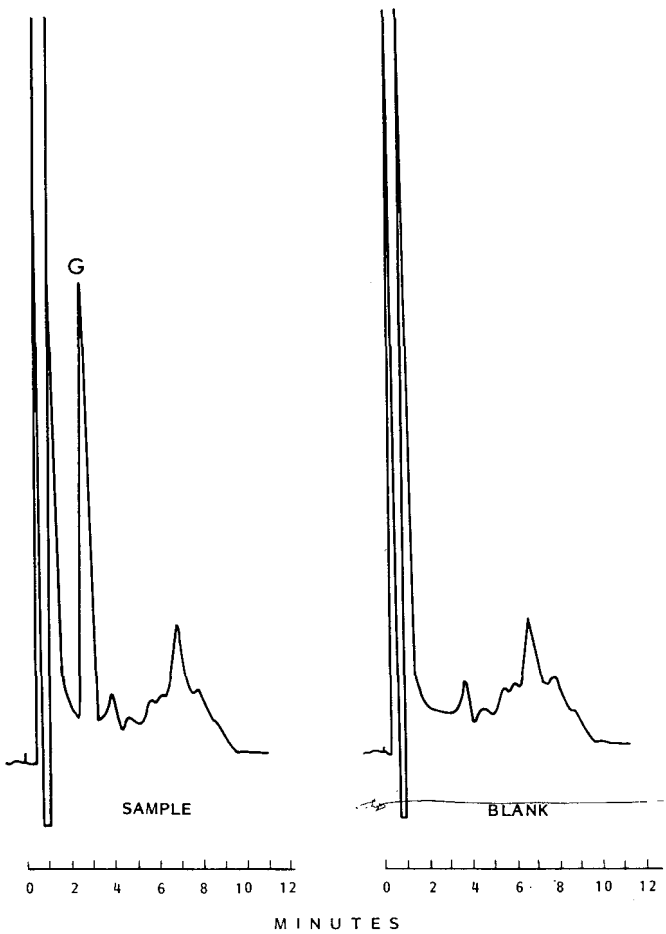

Fig. 3. Typical chromatograms of a blank rabbit urine sample and one containing 52.3 μg/ml of gentamicin (G).

This assay has been used extensively to monitor gentamicin concentrations in plasma and urine in rabbits. These rabbits were given approximately 3.5 mg/kg of gentamicin as an intravenous bolus. Gentamicin shows a biexponential decline with a mean half-life ($t_{1/2\beta}$) of about 54 min (21 experiments) (Fig. 4). This agrees closely with the half-life reported by Peng et al. [4] using their dansyl chloride derivatization assay.

TABLE II

COMPARISON OF STANDARD CURVES FOR GENTAMICIN SULPHATE IN RABBIT URINE

Curve No.	Curve regression parameters			Coefficient of variation (%) at various gentamicin concentrations*			
	r^2	Slope	Intercept	Concentration (μg/ml)			
				17.00	36.46	52.31	71.00
1	0.9997	2.09	−0.64	0.00	1.88	3.28	0.39
2	0.9963	2.13	1.60	0.92	0.00	0.00	0.54
3	0.9995	2.33	0.55	0.00	2.38	0.47	0.94
4	0.9989	1.90	−0.10	2.32	1.00	0.70	1.06

*Coefficient of variation based on at least four replicates.

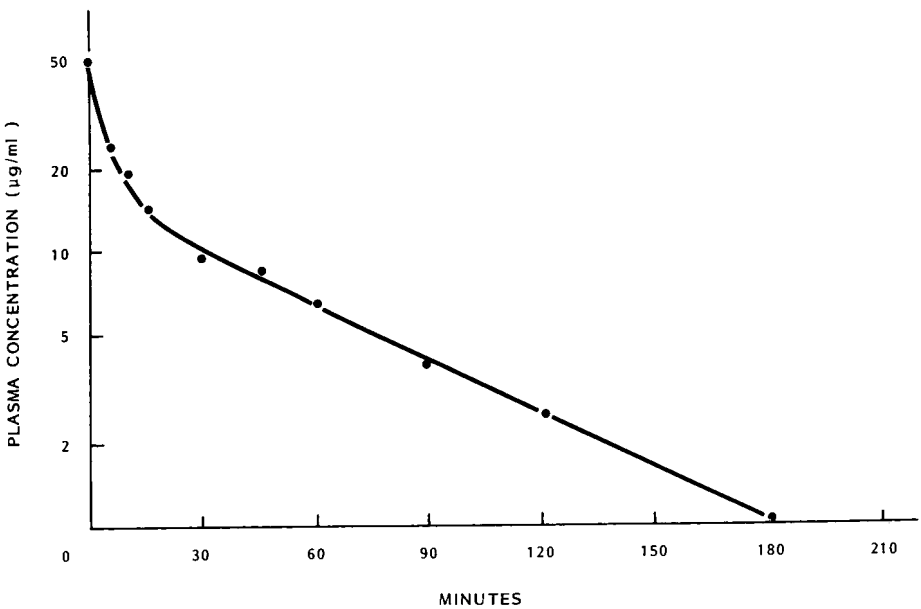

Fig. 4. Plasma concentration—time profile in a 4-kg rabbit following a 3.5 mg/kg dose of gentamicin given as a bolus over 30 sec.

DISCUSSION

We have described a HPLC procedure for the determination of gentamicin in rabbit and human plasmas, and in rabbit urine. In each case the quantitation of gentamicin was achieved after derivatization with fluorescamine.

After the establishment of optimum conditions (when working with human plasma), a protein precipitation step was required before buffer addition and derivatization. However, with rabbit plasma derivatization immediately following protein precipitation resulted in extremely low recovery of gentamicin. The reason for this effect is unknown but it is not unlike the situation reported by Peng et al. [4]. Dilution of the plasma with phosphate buffer alkalinised with sodium hydroxide circumvented this problem. It also produced a very dilute supernatant with respect to gentamicin. Back-extraction of this supernatant not only removed some of the acetonitrile, but also basic compounds (possibly amines) which could interfere with the derivatization process. Since gentamicin is very soluble in water and insoluble in organic solvents, no gentamicin was lost during this procedure.

The back-extraction procedure did not remove all of the endogenous amines from the plasma as evidenced by the large peak which elutes from the column between 6 and 8 min. This large peak remained regardless of the extracting solvent used. Consequently, the elution time for each sample was significantly increased.

The arrival at our chromatographic system was quite pragmatic. The reaction of fluorescamine with an amine produces a carboxyl group on the fluorescamine moiety. Initially, assay development was centred around this carboxyl group using an anion-exchange column and a mobile phase of pH 6—7. However, the appearance of broad, asymmetrical peaks suggested the presence of unreacted amino groups. Therefore, we selected to use a cation-exchange column with a solvent of low pH to suppress ionization of the carboxyl group and to effect separation on the basis of protonated amino groups. Thus, we developed a chromatographic system which produced sharp symmetrical peaks due to gentamicin from human and rabbit plasmas, and rabbit urine.

HPLC methods for gentamicin have the advantages of rapid turnover time, specificity and sensitivity. Furthermore, we feel that this assay has a certain advantage over other presently available HPLC methods, in that the gentamicin components elute as a single peak. This should be particularly useful in a clinical setting. Also, this method can be used directly to assay other aminoglycosides. However, slight modifications (in both reaction and HPLC conditions) may be necessary to optimise quantitation of these compounds.

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SIMULTANEOUS DETERMINATION OF TRIMETHOPRIM, SULPHAMETHOXAZOLE AND N^4-ACETYLSULPHAMETHOXAZOLE IN SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The simultaneous determination of trimethoprim, sulphamethoxazole and N^4-acetylsulphamethoxazole in serum and urine by high-performance liquid chromatography using sulphafurazole as internal standard is described. The separation was achieved on a reversed-phase column employing acetic acid—methanol as the mobile phase with spectrophotometric detection at 230 nm. Precise simultaneous quantitative analysis of the relative components has been achieved at levels of 0.1 μg/ml for trimethoprim and 1.0 μg/ml for both sulphamethoxazole and its N^4-acetyl metabolite using 1 ml of serum or urine.

INTRODUCTION

Co-trimoxazole, a combination of trimethoprim (TMP) and sulphamethoxazole (SMZ) in a 1:5 ratio is a highly effective broad spectrum chemotherapeutic formulation [1].

Several high-performance liquid chromatographic (HPLC) assay methods for TMP, SMZ and other related sulphonamides in dosage forms or as pure drugs have been reported [2—8]. HPLC has also been used for the determination of sulphamethoxazole [9—11] and trimethoprim independently [10—15], whereas several different analytical procedures for the simultaneous determination of both drugs in pharmaceutical preparations [16, 17] and in biological fluids [18, 19] have also been described. Recently, however, HPLC methods have been reported describing the simultaneous determination of SMZ and N^4-acetyl-SMZ [20] and TMP, SMZ and N^4-acetyl-SMZ [21, 22] in biological fluids.

The HPLC method of Vree et al. [21] gives no account of the precision of the assay and lists the lower limit of sensitivity for TMP in serum and urine as

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0.75 μg/ml, whereas the method of Ferry et al. [22] does not completely resolve all the relevant components. In addition, the former method involves the use of 225 nm as the detection wavelength which results in the sulphonamide peaks being too large in relation to the TMP peak, making simultaneous determinations in serum difficult. The latter method utilizes a wavelength of 270 nm resulting in a reduction in the limits of sensitivity.

The HPLC method described here has a detection limit of 0.1 μg/ml TMP and, in addition, involves the use of an internal standard, the advantages of which have been well established [23, 24]. Using this procedure, the concentrations in serum and urine of TMP, SMZ and N^4-acetyl-SMZ, covering the entire concentration range normally encountered during therapy, can be simultaneously determined. Furthermore, this method can be used to quantitatively determine the relevant drugs and major sulphonamide metabolite in serum and urine after a single dose of co-trimoxazole as would be the case during a bioavailability study.

MATERIALS AND METHODS

Apparatus

A Perkin-Elmer Model 601 liquid chromatograph was used, equipped with a Perkin-Elmer LC-55 variable-wavelength detector and a strip chart recorder. A reversed-phase column (μBondapak C_{18}, particle size 10 μm, 30 cm × 3.9 mm, from Waters Assoc., Milford, MA, U.S.A.) housed in a temperature controlled oven was used and samples were injected using a Rheodyne Model 70-10 sample injection valve equipped with a 20-μl loop.

Reagents

TMP and SMZ were supplied by Wellcome (Kempton Park, South Africa), and the sulphafurazole (SFZ), which was used as the internal standard, by Maybaker (Port Elizabeth, South Africa). The N^4-acetyl-SMZ was prepared by acetylation of SMZ as described by Sharma et al. [9] (theoretical: %C, 48.81; %H, 4.44; %N, 14.23; found: %C, 48.75; %H, 4.50; %N, 14.25). All solvents and other reagents were purchased from E. Merck (Darmstadt, G.F.R.) and were of analytical grade. The water used was de-ionized and then glass distilled.

Mobile phase

The mobile phase was methanol—acetic acid, and was prepared by mixing methanol (200 ml) with 1% v/v acetic acid (800 ml), the latter being prepared from glacial acetic acid and water. The solvent mixture (pH 2.9) was simultaneously filtered and degassed through a 0.45-μm HA filter (Millipore, Bedford, MA, U.S.A.). This mobile phase was used for the analysis of both serum and urine samples.

Chromatography

For both serum and urine samples the flow-rate of the mobile phase was set at 1.5 ml/min at a pressure of 100 bar and the wavelength of detection was 230 nm. The oven temperature was set at 30°C for the analysis of serum extracts whereas urine samples were analysed at ambient temperature (20—24°C). Peak

height ratios were used for quantitation based upon calibration curves established on the same day. The calibration curves were prepared from the results of assays on serum and urine spiked with known quantities of TMP, SMZ, N^4-acetyl-SMZ and the internal standard, SFZ.

Extraction

Serum samples. Acetonitrile (3 ml) containing SFZ (6 μg/ml) as internal standard was added to serum (1 ml). This was mixed in a tube (Vacutainer, Becton-Dickinson, Parsippany, NJ, U.S.A.) using a vortex mixer for 0.5 min. The mixture was allowed to stand for 15 min to ensure complete protein precipitation. Ethyl acetate (3 ml) was then added and mixed on a vortex mixer set at a low speed for 1 min. The sample was allowed to stand for 30 min and then mixed again. Sodium chloride (0.4 g) was added to the mixture in order to saturate the aqueous layer. The tube was vortexed briefly and allowed to stand for a further 30 min. Finally the tube was mixed again and then centrifuged at 1700 *g* for 15 min. Five millilitres of the clear upper layer were then transferred to a finely tapered centrifuge tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The sides of the tube were carefully washed down with methanol during drying. The dry extract was reconstituted in 50 μl of mobile phase, mixed, placed in an ultrasonic bath for 1 min and allowed to stand for 15 min. After centrifugation at 1700 *g* for 15 min, 1—10 μl of the clear supernatant were injected onto the column. Relevant chromatograms are depicted in Fig. 1.

Urine samples. The sample (1 ml) was prepared by adding Sorensen's phosphate buffer (1 ml, pH 7.2) [25]. The mixture was vortexed briefly and the pH

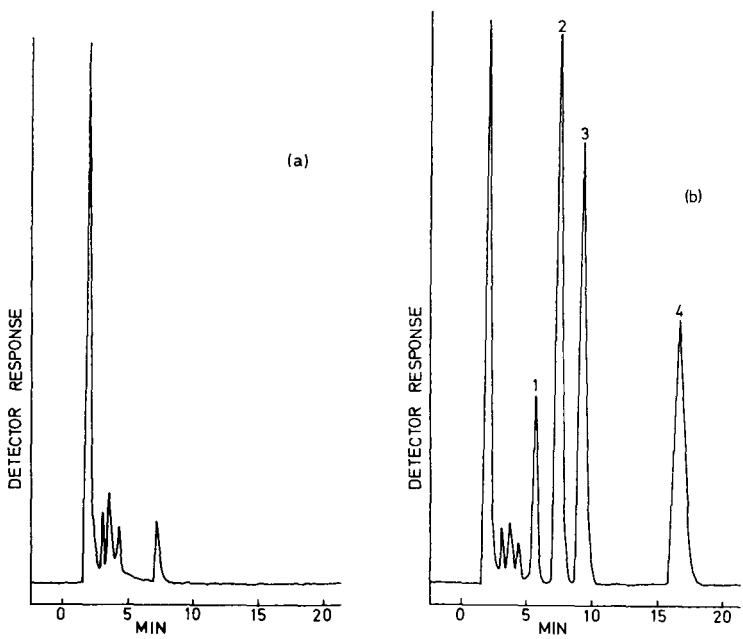

Fig. 1. (a) Chromatogram of blank serum extract. (b) Chromatogram of an extract of serum containing TMP (1), SMZ (2), SFZ (3) and N^4-acetyl-SMZ (4).

readjusted to 7.2 by the addition of a suitable quantity of 0.1 *M* sodium hydroxide. Acetonitrile (2 ml) containing SFZ (75 μg/ml) was added as internal standard. This was mixed for 1 min on a vortex mixer and allowed to stand for 30 min. Sodium chloride (1 g) was added and the mixture was agitated again. It was left to stand for 30 min and then remixed and centrifuged at 1700 *g* for 15 min. The upper acetonitrile layer (1 ml) was removed, placed in a tapered centrifuge tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The sides of the tube were carefully washed down with methanol during drying. The dry extract was reconstituted in 200 μl of mobile phase, mixed thoroughly and then allowed to stand for 30 min. Finally the solution was centrifuged at 1700 *g* for 15 min and 1—10 μl injected onto the column. Relevant chromatograms are depicted in Fig. 2.

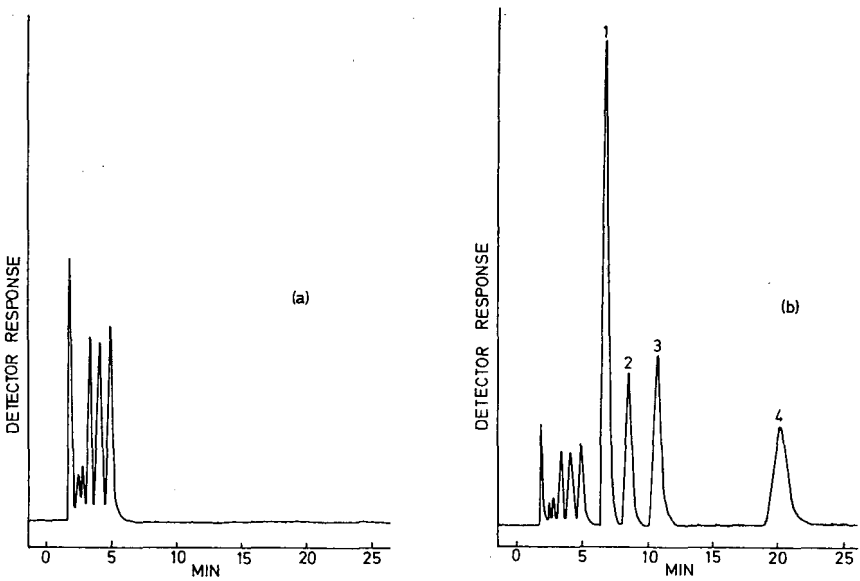

Fig. 2. (a) Chromatogram of blank urine extract. (b) Chromatogram of an extract of urine containing TMP (1), SMZ (2), SFZ (3) and N^4-acetyl-SMZ (4).

RESULTS

Extraction efficiency

Known amounts of each component were added to drug-free serum and urine. All samples were extracted as previously described except that the internal standard was added as a final step to each extract. Three different concentrations of each component in both serum and urine were assayed in triplicate. The results were compared with those obtained from the injection of equivalent concentrations of pure drugs in mobile phase solution. Mean values obtained were 101.0%, 76.3% and 72.3% recovery for TMP, SMZ and N^4-acetyl-SMZ, respectively. The concentration ranges were 1—3 μg/ml for TMP, 6—60 μg/ml for SMZ and 3—30 μg/ml for N^4-acetyl-SMZ.

Precision

The within-run precision was established by spiking serum and urine with TMP, SMZ and N^4-acetyl-SMZ at two different concentrations corresponding, approximately, to the lower and upper limits likely to be encountered after the administration of a single oral dose of 160 mg TMP and 800 mg SMZ. The coefficients of variation for the analysis were determined to be 3.78% at a concentration of 0.6 μg/ml and 2.83% at a concentration of 1.6 μg/ml for TMP, 3.09% at a concentration of 12 μg/ml and 3.99% at a concentration of 35 μg/ml for SMZ and 2.60% at a concentration of 6 μg/ml and 3.52% at a concentration of 16 μg/ml for N^4-acetyl-SMZ. Six samples were assayed at each concentration of the three compounds.

Linearity

Calibration curves obtained by plotting the ratio of the peak height of each compound to that of the internal standard, SFZ, versus their respective concentrations using five different concentrations of serum and urine were linear over all the concentration ranges studied.

Interferences

Chromatograms of blank serum and urine extracts are shown in Figs. 1 and 2. Whereas no interfering peaks were evident in the urine control, a small peak corresponding to the elution time for caffeine was evident in the serum blank. This peak is not present if the volunteer refrains from consuming caffeine-containing beverages. Solutions of acetylsalicylic acid and salicylic acid were also tested for possible interference. Peaks for both compounds gave an identical retention time which did not interfere with any of the compounds of interest.

Sensitivity and detection limit

Under the conditions of this assay, the detection limits for TMP and the sulphonamides (SMZ and N^4-acetyl-SMZ) were established as 0.1 and 1.0 μg/ml respectively.

Patient serum and urine profiles

Application of the assay method was carried out by analyzing serum and urine from a volunteer. A single oral dose of 160 mg TMP and 800 mg SMZ (2 tablets Septran, Wellcome) was administered to the volunteer after an overnight fast. Blood samples were collected from a forearm vein at scheduled intervals and the serum separated by centrifugation and then frozen. Urine samples were collected until 48 h after administration of the dose and representative aliquots were frozen until analysis. The serum and urine concentration—time profiles of TMP, SMZ and N^4-acetyl-SMZ are listed in Table I. The cumulative amounts excreted in the urine over 48 h were TMP 98.46 mg, SMZ 98.20 mg and N^4-acetyl-SMZ 386.92 mg.

DISCUSSION

The method is well suited for routine application in the clinical laboratory because of the relatively simple extraction procedure which allows the simulta-

TABLE I

SERUM AND URINE CONCENTRATION–TIME PROFILES OF TMP, SMZ AND N^4-ACETYL-SMZ

All values are expressed in μg/ml.

Time (h)	TMP		SMZ		N^4-Acetyl-SMZ	
	Serum	Urine	Serum	Urine	Serum	Urine
1	1.57		54.68		2.11	
2	1.34	51.13	49.29	75.90	4.55	65.83
3	1.30		44.57		5.87	
4	1.22	42.54	47.82	50.22	7.43	118.23
6		81.47		53.68		167.11
8	0.78	89.56	32.60	73.81	7.36	246.56
12	0.55	62.37	25.14	97.57	6.43	310.56
19		65.82		19.75		230.71
22		41.72		20.55		251.29
24	0.18	10.42	11.66	13.75	3.45	64.09
25		3.02		6.19		20.12
29		24.67		27.62		169.58
31		11.18		16.81		62.52
32		2.86		4.61		16.01
33		5.39		9.35		32.52
36		12.94		8.37		78.05
47		9.19		5.57		92.23
48		1.93		3.95		36.01

neous determination of the three compounds. The assay provides adequate sensitivity and precision for monitoring therapeutic steady state concentrations as well as the subtherapeutic concentrations which are encountered following the administration of a single solid oral dose of co-trimoxazole for bioavailability assessment.

In view of the fact that the mean serum concentration ratio of SMZ to TMP is approximately 20:1 [26], it was necessary to have a detection system that is considerably more sensitive to TMP in order to obtain responses of a similar magnitude for all the relevant compounds. A detection wavelength of 230 nm proved to be optimal since at this wavelength the absorbance of TMP is about five times that of SMZ on a weight basis. This enabled the use of a single internal standard to quantitate both drugs and the major metabolite and also resulted in easy measurement of peak heights and a simultaneous analysis without the need to selectively attenuate certain peaks. Although the SMZ to TMP ratio in urine is approximately 1:1 [27], the same wavelength gave excellent results for the simultaneous analysis of all three components.

Reconstitution of the final dry extracts with mobile phase as opposed to methanol or acetonitrile markedly improved resolution and peak shapes. Band broadening occurred when the samples were reconstituted in either methanol or acetonitrile.

In summary, the HPLC method presented here is specific, highly sensitive, reliable, reproducible and extremely suitable for the simultaneous analysis of TMP, SMZ and N^4-acetyl-SMZ in both human serum and urine.

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CHROMBIO. 788

THE SIMULTANEOUS ANALYSIS OF CLOFIBRIC ACID AND PROBENECID AND THE DIRECT ANALYSIS OF CLOFIBRIC ACID GLUCURONIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and selective high-performance liquid chromatographic method for the simultaneous quantitative analysis of clofibric acid and probenecid in plasma and urine and for the direct analysis of clofibric acid glucuronide in plasma is described. Both methods involve direct injection of deproteinised body fluids. Concentrations of as low as 10 mg/l of clofibric acid and probenecid and 1.5 mg/l of clofibric acid glucuronide can be measured by the analysis. The coefficient of variance for these methods ranges from 1—7%.

INTRODUCTION

It has been recently reported in man that probenecid and naproxen co-administration produces an elevation in plasma naproxen concentration via a proposed mechanism involving the inhibition of glucuronide conjugation [1]. Like naproxen, clofibric acid, the active metabolite of clofibrate, is cleared predominantly as a glucuronide conjugate [2]. A study was instituted with clofibrate and probenecid to further examine the inhibition of glucuronide conjugation by probenecid, the results of which will be reported elsewhere [3]. To facilitate this study a method has been developed which enables the concentrations of clofibric acid and probenecid to be measured simultaneously in the same plasma or urine sample. In order to detect small concentrations of clofibric acid glucuronide (CAG) in plasma in the presence of high clofibric acid concentrations, a direct method has been developed for CAG which does not require its chemical or enzymatic hydrolysis to clofibric acid. This approach overcomes the problems of sensitivity and specificity inherent in indirect methods based on hydrolysis.

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EXPERIMENTAL

Materials

The sodium salt of clofibric acid, 2-(*p*-chlorophenoxy)-2-methylpropionic acid, was supplied by Imperial Chemical Industries (Melbourne, Australia). Probenecid, β-glucuronidase type VII from *E. coli*, phenolphthalein glucuronide and D-saccharic acid 1,4-lactone were purchased from Sigma (St. Louis, MO, U.S.A.). Flurbiprofen [2-fluoro-α-methyl(1,1′-biphenyl)-4-acetic acid] was supplied by the Boots Company (Sydney, Australia). The acetonitrile was chromatographic grade (Unichrome 210, Ajax Chemicals, Sydney, Australia). All other chemicals were of analytical grade.

Clofibric acid and probenecid analysis in plasma or urine

Plasma or urine, 0.1 ml, was added to a 1.5-ml capacity disposable centrifuge tube containing 0.250 ml of a 30 mg/l solution of flurbiprofen in acetonitrile as internal standard. The tubes were capped, shaken on a vortex mixer for 30 sec and centrifuged for 2 min at 10,000 *g*. A 0.02—0.05 ml aliquot of the supernatant was sampled with a 0.1-ml syringe and injected into the high-performance liquid chromatograph (Waters Model 6000A) via a Waters Model U6K injector (Waters Assoc., Milford, MA, U.S.A.). A 10-μm particle size reversed-phase column (Waters μBondapak C_{18}) was used for the analysis with a mobile phase of acetonitrile—glacial acetic acid—water (450 : 5 : 545) and a flow-rate of 2 ml/min. A variable-wavelength detector (Waters Model 450) operated at 235 nm was used to monitor the column effluent. The detector output was measured using a dual-pen recorder with 10 and 50 mV voltage spans. The detector was usually operated at 0.04 a.u.f.s. deflection.

Calibration curves were constructed by adding known amounts of both clofibric acid (as the sodium salt) and probenecid to plasma or urine to produce concentrations of 10, 20, 50, 100, 150 and 200 mg/l. These samples were analysed and peak height ratios of both clofibric acid and probenecid to the internal standard were plotted versus the concentration. The peak height ratios were divided by the concentration of clofibric acid or probenecid and multiplied by 100 to give a normalised peak height ratio for 100 mg/l. The mean normalised peak height ratio was used to determine the concentrations of clofibric acid or probenecid present in unknown samples. The coefficient of variation (C.V.) of the normalised peak height ratios provides estimates of the accuracy of the method over the range of the assay. The reproducibility of the method was also determined by analysing replicate samples containing 20 and 50 mg/l of clofibric acid and probenecid in plasma and urine. The specificity of the method was assessed by determining retention times of various drugs under the chromatographic conditions described.

Clofibric acid analysis after hydrolysis

β-Glucuronidase hydrolysis. One ml of plasma, or urine diluted with water, was added to an 8-ml capacity culture tube fitted with a PTFE-lined screw cap. To the tube was added 1 ml of 0.075 *M* phosphate buffer (pH 6.8) containing approximately 2000 Fishman units of β-glucuronidase activity. The tubes were mixed and incubated for 2 h at 37°C in a water bath. Preliminary experiments

had demonstrated that no significant increase in clofibric acid concentration occurred after 1 h under the conditions described. The concentrations of clofibric acid liberated by the enzyme were measured in a 0.1-ml sample, as described for the analysis in plasma and urine.

The method was calibrated and an estimate of its accuracy was obtained by adding known amounts of clofibric acid to plasma and urine to produce concentrations in the range of 20—200 mg/l. The calibration procedure used was that described for the analysis of clofibric acid and probenecid in plasma and urine.

Acid hydrolysis. Urine (1 ml) was hydrolysed in 3 *N* hydrochloric acid (final concentration) for 30 min at 100°C. On cooling, 0.5 ml of 10 *N* sodium hydroxide was added and 0.1 ml of the solution was analysed as described for clofibric acid and probenecid in urine or plasma. The method was calibrated over the range of 250—1500 mg/l by adding known amounts of clofibric acid to urine. The calibration procedure was as described for the analysis of clofibric acid and probenecid in plasma and urine.

Isolation and characterization of clofibric acid glucuronide

Urine samples were collected from a volunteer over 8-h periods, prior to (control) and after the ingestion of 1 g of clofibrate (CAG-containing urine). Samples were examined using the chromatographic conditions previously described except that the mobile phase was acetonitrile—0.01 *M* sodium citrate buffer, pH 3.0 (25:75). This chromatographic analysis was carried out for both control and CAG-containing urine, prior to and after treatment with β-glucuronidase, as described for CAG analysis by β-glucuronidase hydrolysis. Treatment with β-glucuronidase was also carried out in the presence of 30 mg/ml of D-saccharic acid 1,4-lactone.

Control and CAG-containing urine (100 ml) were adjusted to pH 2.0 with dilute sulphuric acid and extracted with 2 × 200 ml of ethyl acetate. The organic phase was dried with anhydrous calcium chloride, filtered and the ethyl acetate was removed at 45°C under reduced pressure. The residue was taken up in 5 ml of 0.01 *M* sodium citrate buffer (pH 3.0) and the solution was filtered. The ethyl acetate extracts were subjected to the same treatments and chromatographic analyses as the control and CAG-containing urine samples from which they were derived.

On the basis of the above treatments (see Results and discussion), a chromatographic peak was identified which corresponded to CAG. The column effluent corresponding to this peak was collected following a series of 0.3—0.5 ml injections of the CAG-containing extract. The acetonitrile content of this purified fraction was removed under reduced pressure at 76°C. The purified CAG fraction in citrate buffer was again subjected to the treatments and chromatographic analyses already described for urine and the ethyl acetate extract. In addition, the UV absorption spectrum of the CAG fraction was determined and compared with that of clofibric acid.

Direct analysis of clofibric acid glucuronide in plasma

Plasma containing CAG (0.1 ml) was added to a 1.5-ml capacity disposable centrifuge tube containing the internal standard, 0.2 ml of a 7 mg/l solution of

phenolphthalein glucuronide in 10% aqueous trichloroacetic acid. The samples were then treated as described for the analysis of clofibrate and probenecid in plasma or urine. The chromatographic conditions were those used in the isolation and characterisation of CAG. The method was calibrated by adding known amounts of chromatographically pure CAG to plasma (0.1 ml of CAG fraction in buffer to 10 ml of plasma) to produce concentrations of 1.5, 3.0, 7.5, 30, 60, 120 and 240 mg/l of CAG. The calibration procedures were those described for the analysis of clofibric acid and probenecid in plasma or urine.

RESULTS AND DISCUSSION

Clofibric acid and probenecid analysis in plasma or urine

The retention times of clofibric acid, probenecid and the internal standard were 4.6, 5.8, and 8.2 min, respectively, under the conditions described. A chromatogram of a plasma sample containing 20 mg/l of clofibric acid and probenecid is shown in Fig. 1a, while Fig. 1b shows the chromatogram of a control plasma sample. There was an absence of interfering peaks in control plasma and urine from five subjects examined. Samples containing salicylate, paracetamol, ibuprofen and naproxen also showed that these compounds do not interfere with the assay.

The reproducibility of the method over the range calibrated and at two fixed concentrations is shown in Table I. In the range of 10—200 mg/l the C.V. for

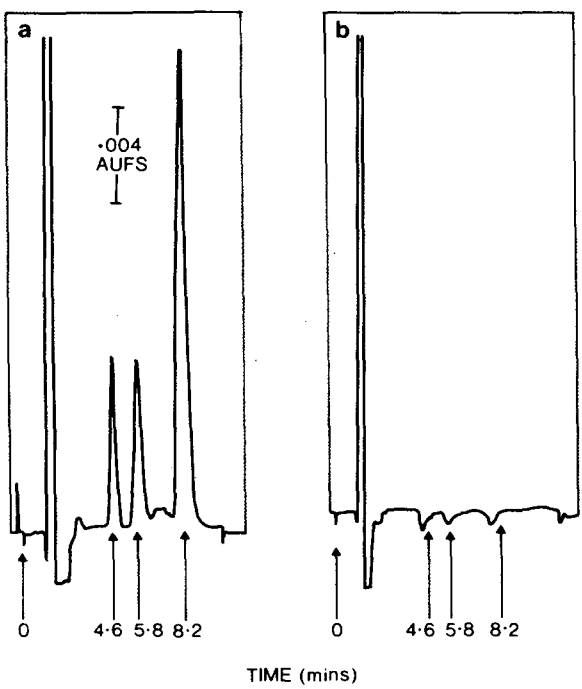

Fig. 1. (a) Chromatogram of plasma containing 20 mg/l of clofibric acid and probenecid with retention times of 4.6 and 5.8 min respectively; that of the internal standard, flurbiprofen (30 mg/l) is 8.2 min. (b) Chromatogram of blank plasma.

TABLE I
CALIBRATION DATA

	Concentration (mg/l)	Mean C.V. of normalised peak height ratio (%)	
		Clofibric acid	Probenecid
Plasma (N = 9)	10—200	3.8	4.7
Urine (N = 2)	10—200	1.9	2.1
Reproducibility			
Plasma (n = 4)	20	3.5	3.0
	150	2.8	2.9
Urine (n = 4)	20	0.9	6.1
	150	1.5	2.2

the normalised peak height ratio for either clofibric acid or probenecid is approximately 5% for plasma and 2% for urine. The reproducibility of replicates at either 20 or 150 mg/l is similar to that seen over the entire range of the method, the C.V. ranging from approximately 1 to 6%.

A number of high-performance liquid chromatographic methods have been reported in the literature for clofibrate [4—6] and probenecid [7, 8] in biological samples. Previously reported methods have used solvent extraction and evaporation or double extraction methods of sample preparation, whereas we use a more rapid and convenient approach of protein precipitation and direct injection of the resulting supernatant.

The reproducibility of the reported methods is similar to the present analysis. The sensitivity of our method is less than that reported by others. Common doses of clofibrate are 0.5 or 1 g twice daily which result in plasma clofibric acid concentrations of 65—100 mg/l [9]. Similarly, probenecid is commonly taken in doses of 0.5—1 g twice daily, resulting in plasma concentrations of 100—200 mg/l [9]. The present method which measures clofibric acid and probenecid concentrations down to 10 mg/l is more than sufficiently sensitive for multiple dosing studies and also satisfactory for single dose studies with the usual doses. The comparative lack of sensitivity of the present method is compensated for by the small sample volume required (0.1 ml) and the ease of sample preparation.

Clofibric acid glucuronide by hydrolysis

Chromatograms from plasma and urine samples treated with either β-glucuronidase or acid were essentially similar to those obtained from untreated samples, such as that shown in Fig. 1. No peaks arose from these treatments which interfered with those of clofibric acid or the internal standard. Reproducibility data for the method are shown in Table II. In the range of 20—200 mg/l, the C.V. of the normalised peak height ratio for the enzymatic method is less than 5%. The range (250—1500 mg/l) over which the method was established for the acid treatment, reflects the high concentrations of clofibric acid found in

TABLE II

CALIBRATION DATA FOR CLOFIBRIC ACID AFTER HYDROLYSIS

	Concentration (mg/l)	C.V. of normalised peak height ratio (%)
β-Glucuronidase hydrolysed plasma	20—200	4.8
β-Glucuronidase hydrolysed urine	20—200	2.1
Acid hydrolysed urine	250—1500	3.9

urine after acid hydrolysis rather than any limits of sensitivity. Over this range, the reproducibility of the acid hydrolysis method was comparable to that of the enzymatic method (Table II).

Purification of clofibric acid glucuronide

Chromatograms of CAG-containing urine showed a peak at 6.5 min which was not present in control urine and which was not due to clofibric acid which had a retention time of 20 min under these conditions. The peak at 6.5 min was not present in CAG urine which had been treated with β-glucuronidase, but was present in β-glucuronidase-treated urine which also contained D-saccharic acid 1,4-lactone, a specific inhibitor of the enzyme [10]. Treatment of CAG-containing urine with β-glucuronidase not only abolished to peak at 6.5 min but also produced a corresponding increase in the peak due to clofibric acid. A similar examination of the ethyl acetate extract of control and CAG-containing urine gave the same pattern of results as observed in urine.

Fig. 2a shows the chromatogram of the purified CAG fraction prior to treatment with β-glucuronidase. The absence of a peak at 20 min indicates that this fraction is uncontaminated with clofibric acid. Fig. 2b shows the chromatogram of the sample shown in Fig. 2a, after it has been treated with β-glucuronidase. All but approximately 5% of the peak at 6.5 min is removed by this treatment, and there is a corresponding increase in the clofibric acid peak at 20 min.

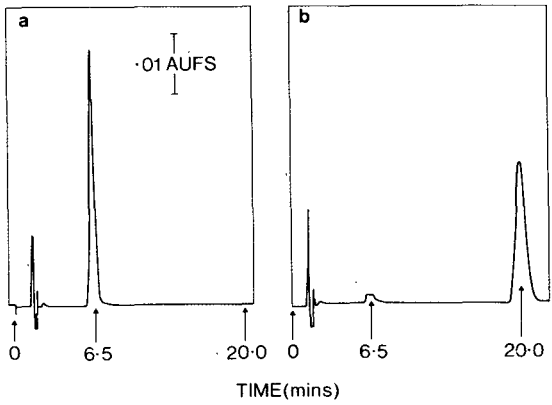

Fig. 2. (a) Chromatogram of purified extract of clofibric acid glucuronide which shows a single peak at 6.5 min. (b) Chromatogram of the same sample after β-glucuronidase hydrolysis; the peak due to clofibric acid glucuronide at 6.5 min has been replaced by a peak at 20 min due to clofibric acid.

No peaks other than that due to clofibric acid resulted from this treatment. The presence of D-saccharic acid 1,4-lactone during β-glucuronidase treatment prevented a reduction in the peak at 6.5 min and the appearance of a peak due to clofibric acid.

The UV absorbance spectrum of the peak at 6.5 min exhibited maxima at 227 nm and 277 nm, as did an authentic sample of clofibric acid. The ratio of the absorbance at 227 nm to that at 277 nm was 0.095 for the peak at 6.5 min and 0.088 for clofibric acid.

Assuming that conjugation of clofibric acid with glucuronic acid does not alter its UV absorbance characteristics, there was a stoichiometric relationship between the amount of CAG present in the purified extract and the amount of clofibric acid measured after enzymatic hydrolysis. All of the above data are consistent with the peak at 6.5 min of the purified fraction being due to CAG. Furthermore, this fraction is essentially pure by enzymatic, chromatographic and UV absorbance criteria.

Direct analysis of clofibric acid glucuronide in plasma

Under the chromatographic conditions described, the retention times of CAG and the internal standard (phenolphthalein glucuronide) were 10.5 and 8.1 min respectively (Fig. 3). Although some samples showed small peaks

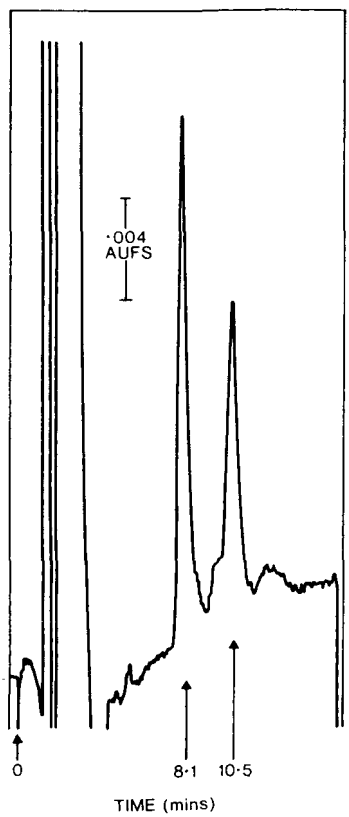

Fig. 3. Chromatogram of plasma containing the internal standard phenolphthalein glucuronide and 15 mg/l clofibric acid glucuronide (retention times 8.1 and 10.5 min, respectively).

which were not completely resolved from the CAG peak (Fig. 3), the presence of these peaks did not seriously reduce the accuracy of the method. The calibration curve for the direct analysis of CAG in plasma was linear. In the range of 1.5—250 mg/l, the C.V. of the normalised peak height ratio was 7.3% and the regression coefficient was 0.9988. A disadvantage of this approach is that under the chromatographic conditions required to separate CAG from interfering peaks in plasma, slowly eluting peaks occur, including that due to clofibric acid, which permit relatively few (10—15) samples to be assayed in a day.

The usual methods by which glucuronides are measured require an estimate of the aglycone to be made before and after enzymatic or chemical hydrolysis. The difference of these two measurements is then taken as an estimate of the glucuronide present. This approach has two major disadvantages. The method becomes less accurate as the ratio of aglycone to conjugate increases and such an approach might fail to detect small concentrations of CAG in plasma in the presence of high clofibric acid concentrations. The second disadvantage of indirect methods concerns specificity. On the one hand, it has been reported that β-glucuronidase treatment fails to completely hydrolyse the glucuronides of clofibric acid and some other aglycones [11]. Chemical methods which may liberate greater amounts of aglycone from a sample have the disadvantage that it is difficult or impossible to document their specificity. The approach outlined here overcomes both of the above problems, in that it allows the estimate of CAG in the presence of high concentrations of clofibric acid and has well documented specificity.

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CHROMBIO. 791

ANALYSIS OF CARMINOMYCIN IN HUMAN SERUM BY FLUOROMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is given for the determination of carminomycin (CMM) and a major metabolite carminomycinol (CMMOH) in serum from cancer patients after intravenous administration of carminomycin as the free drug.

CMM and CMMOH are extracted from serum with chloroform, the extract evaporated and the residue dissolved in methanol. High-performance liquid chromatography analysis utilized a C_{18} μBondapak reversed-phase column eluted with 0.1 mol/l acetate buffer (pH 4) — acetonitrile (60:40, v/v) with fluorescence detection. The assay is linear, reproducible, and precise with a limit of detection of 2 ng/ml. Representative serum levels of CMM and CMMOH in a cancer patient are presented.

INTRODUCTION

Carminomycin · HCl ($C_{26}H_{27}NO_{10}$ · HCl, free base MW 513.51, NSC-180024) is an anthracycline antibiotic with antineoplastic activity. It is isolated from the mycelia of *Actinomadura carminata* [1, 2]. The structure of carminomycin (CMM) (Fig. 1) is shown in relationship to the structures of adriamycin and daunomycin. All three compounds contain the amino sugar daunosamine and differ slightly at carbons 4 and 14. CMM is metabolized by reduction

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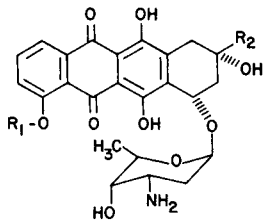

	R_1	R_2
ADRIAMYCIN	CH_3	$COCH_2OH$
ADRIAMYCINOL	CH_3	$CH(OH)CH_2OH$
DAUNOMYCIN	CH_3	$COCH_3$
CARMINOMYCIN	H	$COCH_3$
CARMINOMYCINOL	H	$CH(OH)CH_3$

Fig. 1. Structures of adriamycin (ADM) and metabolite adriamycinol, daunomycin, and carminomycin (CMM) and metabolite carminomycinol (CMMOH).

of the ketone at C_{13} to an alcohol, carminomycinol (CMMOH), a major metabolite. CMMOH has cytotoxic activity comparable with CMM at approximately equivalent optimal doses and similar maximum percentage increase in median survival times of treated vs. control mice on two different dose schedules [3, 4].

Although CMM is chemically similar to adriamycin (ADM), recent studies suggest it may have a mechanism of action different from ADM [5]. CMM is being considered as an alternative to ADM chemotherapy due to its reported lower incidence of cardiotoxicity [6]. Reports have been published on the analysis and kinetics of ADM and metabolites in human serum [7–9], but not on the analysis and kinetics of CMM and its metabolites.

This paper gives an analytical method for simultaneous determination of CMM and CMMOH in serum following intravenous injection of CMM as the free drug from the first dose of therapy. The development of the assay was based on the extraction properties and reversed-phase high-performance liquid chromatographic (HPLC) separation of other anthraquinone glycosides. The proposed assay includes extraction from the buffered serum sample into an organic phase, re-extraction into a slightly acidic aqueous phase and separation by reversed-phase HPLC. A high detection sensitivity and selectivity of the assay is achieved by using fluorescence detection.

EXPERIMENTAL

Drugs and reagents

Carminomycin 1 (Lot 78F581, 95% by assay) and CMMOH were from Bristol Labs. (Syracuse, NY, U.S.A.). ADM·HCl (batch No. 565), manufactured by Farmitalia (Milan, Italy), and outdated human serum, used for the standards, were supplied by SUNY/Upstate Medical Center (Syracuse, NY, U.S.A.). Clinical chemistry control serum was purchased from General Diagnostics (Avon, CT, U.S.A.).

Methanol, hexane, chloroform, and acetonitrile were of spectroquality Burdick and Jackson glass-distilled grade (Rainin Instrument, Brighton, MA, U.S.A.). Glacial acetic acid, monobasic potassium phosphate, sodium hydroxide solution, and ammonium hydroxide were of certified ACS grade. Deionized water filtered through a Millipore® apparatus was used throughout.

Preparation of stock aliquots

Ten mg of each drug were dissolved in 5 ml of distilled water. The CMM and CMMOH solutions were combined and quantitatively diluted to 0.2 mg/ml for each compound. The ADM solution was similarly diluted. Aliquots (0.2 ml) of each solution were transferred to disposable polypropylene tubes and stored frozen at −20° C or colder.

Preparation of working serum standards

Spiked serum standards were prepared in 13 × 100 mm PTFE-lined screw cap test tubes as follows.

A stock aliquot of CMM and CMMOH was diluted 1 : 20 with methanol in a polycarbonate tube and 50 µl and 20 µl of this solution were pipetted, in duplicate, to 2.0 ml of serum. A sample of the 1 : 20 dilution was diluted 1 : 10 with methanol and 100, 50, 20, and 5 µl of the resulting solution pipetted, in duplicate, to 2.0 ml of serum. The additions to serum were carried out rapidly prior to the addition of ADM. This results in final concentrations of 250, 100, 50, 25, 10, 7.5, 5 and 2.5 ng CMM and CMMOH per ml each. Standards were run with each set of patient samples.

Assay extraction procedure

A stock aliquot of ADM, the internal standard, was diluted 1:200 with methanol and 100 µl of the dilution were added to each 2-ml serum standard and 2-ml patient's clinical serum sample to give 50 ng ADM per ml. To each

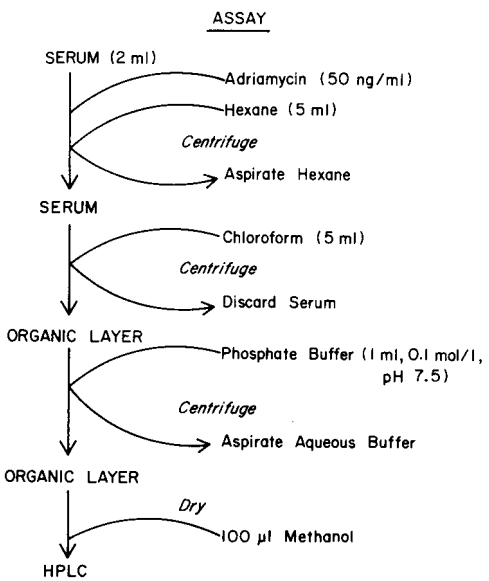

Fig. 2. Schematic flow diagram of the CMM assay in human serum.

standard and sample were added 5.0 ml of hexane. Each capped tube was shaken for 5 min on a rotorack at 35 oscillations per min and centrifuged at 2600 *g* for 15 min at ambient temperature. The hexane layers were aspirated and discarded. To each aqueous layer, 5 ml of chloroform were added; and each tube was capped, shaken, and centrifuged as before. The serum layer was aspirated and discarded. One ml of 0.1 mol/l phosphate buffer (pH 7.5), was added to each tube to wash the chloroform. The aqueous buffer was aspirated and discarded following mixing and centrifugation. Each extract was evaporated to dryness under a stream of filtered nitrogen in a water bath at 37°C, kept at ambient temperature overnight and reconstituted in 100 µl methanol, 80 µl of which were chromatographed. Fig. 2 is a schematic flow diagram of the assay.

High-performance liquid chromatography parameters

The HPLC system consisted of a U6K injector with a 2-ml loop (Waters Assoc., Milford, MA, U.S.A.), an Altex Model 110 pump (Rainin Instrument), and a Spectra Glo Fluorometer (No. 3301, Gilson Medical Electronics, Middletown, WI, U.S.A.). Fluorometric detection utilized a 15-µl glass flow-cell assembly (No. 3382) equipped with interference filters at 380—480 nm for excitation (No. 5-60) and an emission filter with a lower cut-off of 560 nm (No. 3-66). A scan of the intrinsic fluorescence spectrum of CMM, CMMOH, and ADM using a spectrophotofluorometer (Aminco-Bowman, American Instruments, Silver Springs, MD, U.S.A.) showed an excitation maximum of ca. 470 nm and an emission maximum at 545—550 nm for all three compounds. The column was a Waters Assoc. C_{18} µBondapak reversed-phase type (30 cm × 3.9 mm I.D., 10-µm particle size, used at ambient temperature. The mobile phase was 0.1 mol/l ammonium acetate buffer (pH 4.0)—acetonitrile (60:40, v/v). The buffer first was filtered through a 0.45-µm Millipore® filter and the eluent was de-aerated in a sonic bath. The column was conditioned with the mobile phase for approximately 1—2 h before use. The flow-rate was 1.9 ml/min, at a column head pressure of 130—150 bar with the mobile phase recycled.

Quantitation

Detector output was displayed on a Hewlett-Packard recorder Model 7123A and collected and processed by a Hewlett-Packard Model 3354/C Laboratory Automation System. Each chromatogram was interpreted using the Model 3354/C software system. The chromatographic data for each chromatogram were collected for each set of analyses and stored on a disc. The retention time, maximum signal and integral (signal × time) for CMM, CMMOH, and ADM were automatically obtained for each chromatogram. At the end of each series of analyses, the regression of peak height ratio (CMM or CMMOH response/ADM response) versus CMM or CMMOH concentration in the spiked standards was calculated by least-squares analysis and the concentration of CMM or CMMOH in each serum sample was estimated by inverse prediction [10].

RESULTS AND DISCUSSION

Chromatography

In preliminary work, reversed-phase chromatography on C_{18} and alkylphenyl μBondapak columns was compared with normal-phase adsorption chromatography on a microparticulate silica column, namely, Partisil 10 PAC PXS 10/25 (Whatman, Clifton, NJ, U.S.A.). Normal-phase chromatography had variable and skewed peak heights and variable retention times. In general, normal-phase columns can become deactivated by the extent of hydration from solvents. Also noted was loss of peak height when drug residues were reconstituted in the normal-phase eluent [chloroform—methanol—acetic acid—water (80:20:2:3)] and allowed to stand 1 h at room temperature. For the drugs studied here, reversed-phase was less sensitive to small changes in solvent composition. Our results correspond with recent publications recommending reversed-phase HPLC for ADM and metabolites [11], although normal-phase chromatography has been used for CMM measurement [12]. The results using a reversed-mode C_{18} column for the separation of CMM, CMMOH, and ADM showed improved resolution, recovery, linearity, and reproducibility over normal-phase HPLC.

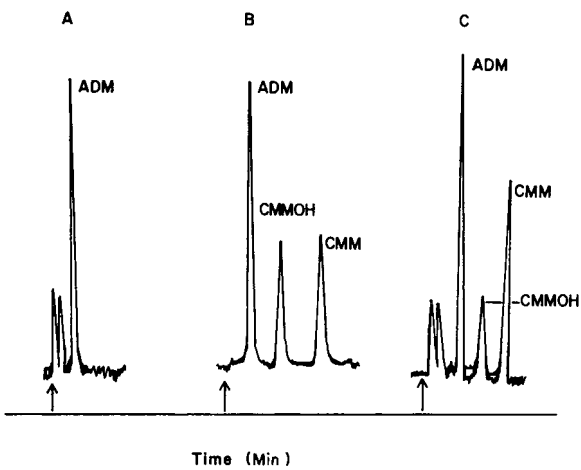

Fig. 3. (A) Elution profile of ADM, the internal standard of 50 ng/ml, extracted from a zero time patient serum sample. (B) Elution profile of serum supplemented with 25 ng/ml CMM and 25 ng/ml CMMOH with internal standard 50 ng/ml ADM. (C) Elution profile of serum sample from a patient administered 22 mg/m^2 CMM 30 min previously. Peaks represent: 50 ng/ml ADM, 14.16 ng/ml CMMOH and 29.5 ng/ml CMM.

ADM was chosen as the internal standard because of its similar structure and chromatographic behavior, enabling a short analysis time of 7 min per sample. Typical chromatograms, including spiked samples and a clinical sample, are shown in Fig. 3. Consistent retention times of 2.6 min for ADM, 4.0 min for CMM, and 5.8 min for CMMOH were obtained.

The composition of the mobile phase was influenced by CMM being a weak difunctional acid with a pK_{a1} of 8.00, requiring an acid modified in an organic solvent to reduce the elution time from a C_{18} column. After a day's

run, the column was flushed with acetonitrile—water (70:30, v/v) to avoid column degradation by long-term exposure to acidic pH.

Assay procedures

Water, methanolic HCl, chloroform—methanol—acetic acid—water (80:20:2:3, v/v), acetate buffer (pH 4.0)–acetonitrile (60:40, v/v), and methanol were examined as reconstitution solvents. The results (Table I) indicated that methanol was superior. The buffer wash at the final assay step was examined at pH 6.5, 7.5, and 8.5 without any significant differences in recovery. Schoeffel (Model SF970) and Waters fluorometers were compared in series to the Gilson fluorometer, but provided equivalent or less sensitivity than the Gilson. A commercial control serum was analyzed to check that interfering endogenous substances were excluded in the assay. The aglycone metabolites of CMM were not studied or quantified here. Typically, sample preparation requires 3—4 h of operator time for 40 samples, and 7—8 h for instrumental analysis. The reproducibility and stability of multiple CMM and ADM frozen aliquots were constant (Table II).

Linearity

The equations of the line of best fit for typical calibration curves for the CMM and CMMOH standards were $y=0.017X + 0.015$, and $y=0.020X - 0.008$,

TABLE I

STABILITY OF CARMINOMYCIN IN VARIOUS SOLVENTS

Concentration of carminomycin 1 ng/μl.

Reconstitution solvent	Area (K)	Time (h)	
100% Water	55.9	0	
	36.5	1	
	31.1	2	
	21.7	4	
	9.5	24	
Chloroform—methanol—acetic acid—water (80:20:2.3)	68.0	0	
	74.7	1	Separation into 2 phases
	69.9	2	
	71.6	4	
	68.9	24	
0.1 mol/l ammonium acetate (pH 4)–acetonitrile (60:40)	62.7	0	
	58.0	1	
	51.8	2	
	46.2	4	
	15.8	24	
100% Methanol	63.4	0	
	62.7	1	
	62.3	2	
	63.9	4	
	66.9	24	

TABLE II

CARMINOMYCIN AND ADRIAMYCIN ALIQUOTS

Aliquot	Injections	Average peak height			
		0 h	2 h	4 h	24 h
Carminomycin					
A	1	25.6	22.9	20.6	20.5
	2				
	3				
B	1	23.8	21.2	19.4	19.4
	2				
	3				
C	1	25.6	25.2	19.5	20.0
	2				
	3				
		0 h	2.5 h	5 h	24 h
Adriamycin					
A	1	32.9	29.9	30.5	33.7
	2				
	3				
B	1	32.0	33.4	29.3	33.0
	2				
	3				
C	1	34.1	31.4	36.3	33.5
	2				
	3				

respectively; the coefficient of correlation (r) for both regressions was 0.999. Within the range of 0—250 ng/ml no tendency of the calibration data to deviate from linearity was observed at either end of the range.

Limit of detection

Assuming that the mean minus three times the standard deviation is a reasonable limit for the extreme lower limit of response for spiked samples, only one blank response (Table III, No. 16) for CMM was greater than this limit for 2.5 ng/ml standards. All the CMMOH blank values fell below the limit for 2.5 ng/ml CMMOH standards. This separation of response indicates that the limit of detection for both compounds is slightly lower than 2.5 ng/ml serum at the 95% confidence level. Therefore, 2 ng/ml serum is a practical limit for the assay.

Precision and reproducibility

A set of 50 ng/ml spiked standards was prepared as soon as the assay was developed and subsets of five or six replicates assayed at intervals (Table IV). The precision within any subset was good, the mean coefficient of variation (C.V.) being 4.4%. However, the mean observed values were statistically different ($p \leqslant 0.05$) on each of four days during a month. The first day being extremely different from those following, probably can be laid to operator inexperience, but the remaining variability must be expected to be normal for this assay.

TABLE III

INSTRUMENTAL RESPONSE TO EXTRACTED BLANKS AND CARMINOMYCIN (CMM) AND CARMINOMYCINOL (CMMOH) STANDARDS NEAR THE LIMIT OF DETECTION

Solution No.	Peak height (arbitrary units)			
	4.0 min retention time; 2.5 ng CMM per ml serum	Blank serum	5.8 min retention time; 2.5 ng CMMOH per ml serum	Blank serum
1	772	0	698	0
2	498	0	459	0
3	689	181	632	0
4	661	146	546	0
5	503	0	629	0
6	722	196	587	101
7	606	0	493	0
8	615	0	391	0
9	967	0	633	0
10	901	0	559	0
11	790	0	510	135
12	772	0	537	0
13	522	237	474	0
14	864	173	577	0
15	744	236	625	0
16	765	593	567	0
17	779	192	484	0
18	705	0	499	82
19	987	184	699	0
Mean	730		558	
S.D.	142		82	
C.V. (%)	19		15	

TABLE IV

ASSAY PRECISION

Time (days)	Mean observed CMM concentration (ng/ml)	N*	S.D.	C.V. (%)
1	58.3	6	3.1	5.3
5	36.1	6	1.5	4.2
13	42.6	5	2.0	4.8
26	39.8	5	1.3	3.3

ANOVA table**

	Sum of squares	DF	MS	F	Pooled S.D.
Treatment	1701	3	567	135	
Error	75	18	4.19		
Total	177	21			2.05

*All means are significantly different based on ANOVA and Duncan's Multiple Range Test.

**DF = degrees of freedom; MS = mean square; F statistic value = treatment mean square/error mean square.

TABLE V

REPRODUCIBILITY OF CARMINOMYCIN ASSAY OF HUMAN SERUM
Dose = 22 mg/m^2.

Time (h)	Run 1 CMM concn. (ng/ml)	Run 2 CMM concn. (ng/ml)	Run 2 CMMOH concn. (ng/ml)
0.25	78.0	79.4	28.04
0.5	34.4	29.5	14.16
1.0	26.2	22.7	14.27
2	19.7	16.1	12.05
4	9.1	8.0	10.95
6	6.3	3.4	9.68
8	4.6	2.8	8.72
12	—	2.2	11.07
24	2.0	1.0	8.98

Reproducibility was assessed by analyzing a patient's sample set twice, at a one-month interval, with consistent results (Table V). Chronologically, regression equations were $y = 0.015X - 0.003$ and $y = 0.017X + 0.015$. Varying intercepts probably account for the slight concentration differences.

Application to biological samples

This assay has been employed in analyzing clinical serum samples for CMM and CMMOH. A typical serum concentration profile of CMM and CMMOH in man is shown in Fig. 4. The patient received 22 mg of CMM per m^2 intravenously. The serum CMM decline appeared biphasic with an elimination phase half-life of 12.5 h. CMMOH was detected within 15 min. After an initial decrease, the CMMOH concentration did not significantly change over

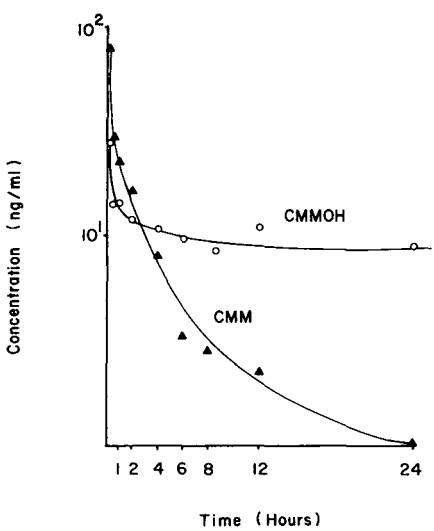

Fig. 4. Concentration vs. time curve of CMM and CMMOH after intravenous infusion of 22 mg/m^2 CMM from the same patient as in Fig. 3 and Table V. ▲ = CMM and ○ = CMMOH.

the 24-h collection period. The CMMOH concentration was higher than CMM from 4 h through 24 h. We have found different metabolite concentrations with CMM than reported pharmacokinetic values for ADM [13]. CMM kinetic results were similar to daunomycin metabolism [14].

These results show the applicability of the proposed assay to serum samples from patients administered therapeutic doses of CMM. The results also indicate a need to determine serum levels of CMM and especially of CMMOH for longer than 24 h after dosing. The pharmacokinetics of CMM and the activity of CMMOH suggest that CMMOH may be an important contributor to CMM administration and could be a useful drug itself.

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THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ANALYSIS OF NALIDIXIC ACID IN HUMAN PLASMA

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SUMMARY

A sensitive and highly selective thin-layer chromatographic method for determining plasma levels of nalidixic acid is presented. Plasma (50 μl) was acidified with 50 μl 1 *M* orthophosphoric acid and extracted with 100 μl toluene. A 40-μl aliquot of the extract was spotted onto the thin-layer plate with the aid of a Desaga Autospotter and, after irrigation, the nalidixic acid on the plate was converted into a fluorescent compound by exposing the plates to hydrogen chloride gas for 10 min and then to strong ultraviolet radiation from a mercury lamp for 10 min. The fluorescence was measured quantitatively using a spectrofluorimeter equipped with a thin-layer chromatogram scanning attachment.

INTRODUCTION

Nalidixic acid has been in use as an antibacterial agent in urinary tract infections since 1963. Numerous methods for the determination of nalidixic acid in pharmaceutical products and in biological specimens have been published. Among these are chemical methods based on acid—base titrations [1—3], microbiological [4], fluorimetric [5—7], UV spectrophotometric [8—10], gas—liquid chromatographic [11, 12], high-performance liquid chromatographic [13—16] and polarographic [17] methods. No quantitative thin-layer chromatographic (TLC) method has been established yet.

Whereas for previous assay methods at least 0.5 ml plasma was required we describe here a simple and specific TLC-densitometric method by means of which nalidixic acid can be determined in duplicate with good precision at therapeutic levels in a 100-μl plasma sample.

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EXPERIMENTAL

Reagents

All reagents used were of guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used without further purification. Nalidixic acid was a gift from Winthrop (Durban, South Africa), and was used as received.

Apparatus

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a TLC scanning attachment was used to measure the fluorescence of the spots on the plates using the following conditions: light source, xenon lamp; excitation wavelength, 375 nm; emission wavelength, 430 nm cut-off filter; excitation and emission slit widths, amplifier sensitivity and sample-adjustment were set so as to obtain approximately 80% of full-scale deflection on the recorder when the strongest spot was being scanned.

The other apparatus used consisted of silica gel 60 TLC plates (Merck), without fluorescent indicator; one 20 cm × 20 cm plate was cut into three plates of 6.7 cm × 20 cm; a Shandon S/P Chromatank; Hamilton dosing syringes (50 μl and 100 μl); 500-μl conical plastic microfuge tubes (Beckman Instruments, Johannesburg, South Africa); 10-μl disposable glass capillary micropipettes (Clay Adams division of Becton Dickinson, Parsippany NJ, U.S. A.); a Universal UV lamp, type TL/900 (Camag, Muttenz, Switzerland).

Plasma standards

An accurately weighed amount of nalidixic acid was dissolved in a weighed amount of fresh human plasma by shaking for 4 h. By using an average density of 1.027 for plasma [18] the nalidixic acid concentration can be calculated. Weighed amounts of this plasma stock solution were further diluted with weighed amounts of plasma to obtain standards with lower concentrations of nalidixic acid. Aliquots (0.5 ml) of the standard plasmas were stored frozen (−20°C) in 1-ml sealed glass ampoules.

Extraction

Volumes of plasma (50 μl), standard or unknown, were measured with the dosing syringe into the plastic conical microfuge tube followed by 50 μl 1 *M* orthophosphoric acid and 100 μl toluene. After shaking the stoppered microfuge tubes thoroughly by hand for 1 min, they were centrifuged briefly.

Spotting the plates

Aliquots of the toluene extracts (40 μl) were spotted 1 cm from the margin on the TLC plates using the Desaga Autospotter. Sixteen spots with unknown (*X*) and standard (*S*) amounts alternating in duplicate were applied to a 6.7 cm × 20 cm plate (*S*1 *X*1 *S*2 *X*2 *S*3 *X*3 *S*4 *X*4 etc.). The solutions were spotted at the maximum rate available (10 μl/min) with the autospotter while the fan speed was adjusted to evaporate the toluene at a rate which only just prevented the solvent fronts of the spots merging with each other.

Chromatography

Ascending development was carried out in an unsaturated tank up to the end of the plate at 6.7 cm with the mobile phase, dioxane—5 × diluted concentrated ammonia solution (3:1). The elution time was about 30 min at room temperature (ca. 23°C) and relative humidity of about 15%. After brief drying with a warm hairdryer the plates were dried by heating at 90°C for 5 min in a ventilated oven.

Induction of fluorescence and quantitation

The plates were allowed to cool down to room temperature and were then exposed for 10 min to hydrogen chloride gas generated in a chromatography tank by adding 2 ml concentrated hydrochloric acid to 10 ml concentrated sulphuric acid contained in a small beaker in the tank.

The hydrogen chloride treated plates were immediately exposed to UV radiation from an unfiltered mercury discharge lamp for 10 min. This procedure converted the nalidixic acid into a strongly fluorescent compound on the TLC plate (R_F = 0.4).

Each spot was scanned in the TLC scanning attachment of the MPF3 spectrofluorimeter in the direction of the solvent flow. Standard curves were constructed by plotting peak heights versus plasma concentrations of the known standards. The concentrations of unknown samples were then obtained by interpolation.

RESULTS AND DISCUSSION

Fig. 1 represents part of a densitogram showing peaks obtained for standard plasma containing the indicated amount of nalidixic acid, plasma samples of a

TABLE I

RECOVERY OF NALIDIXIC ACID FROM HUMAN PLASMA

In each case n = 4.

Method*	Nalidixic acid added (μg)	Mean recovery (μg)	C.V. (%)
a	2.34	2.32	9.5
	4.37	4.66	2.8
	8.72	9.08	2.1
	17.28	17.09	0.9
b	2.34	2.46	8.9
	4.37	4.88	6.6
	8.72	9.05	3.4
	17.28	16.50	6.0
c	2.34	2.18	11.9
	4.37	3.98	13.8
	8.72	8.85	6.1
	17.28	17.53	4.8

*(a) Spots scanned in direction of solvent flow; (b) spots scanned across direction of solvent flow; (c) spots applied manually (10 μl).

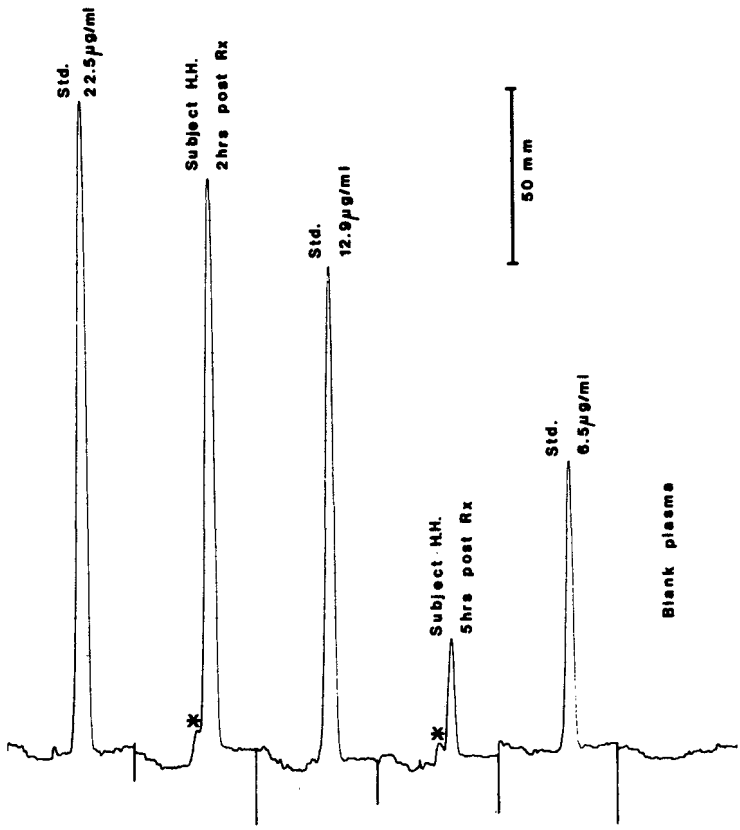

Fig. 1. Densitogram of nalidixic acid in plasma of a volunteer 2.5 h after ingestion of 1 g nalidixic acid; in plasma standards and blank plasma. Asterisk (*) indicates peak possibly due to hydroxynalidixic acid.

volunteer obtained 2 and 5 h after ingestion of 1 g of nalidixic acid as well as a blank plasma showing the absence of interfering endogenous compounds.

A summary of the recoveries of nalidixic acid added to normal human plasma is presented in Table I.

Little time could be saved by scanning the plates across the direction of solvent flow since the R_F values of the spots were found to be strongly dependent on the concentration of the nalidixic acid requiring frequent adjustment of the plate position while scanning. However, due to slight tailing producing elongated spots the detection limit is lower when the plates are scanned across the direction of solvent flow (characteristic of the slit geometry). A densitogram obtained of a plate scanned in such a way is shown in Fig. 2.

As an alternative to spotting the plates with the Desaga Autospotter, 10-µl aliquots of the toluene extracts were applied by means of calibrated 10-µl glass micropipettes allowing the whole volume to run into the plate by capillary action in a single application. Although the precision is lower using this method (see Table I) it is adequate for therapeutic monitoring. The main drawback of this manual spotting procedure is the time required to spot a large number of samples.

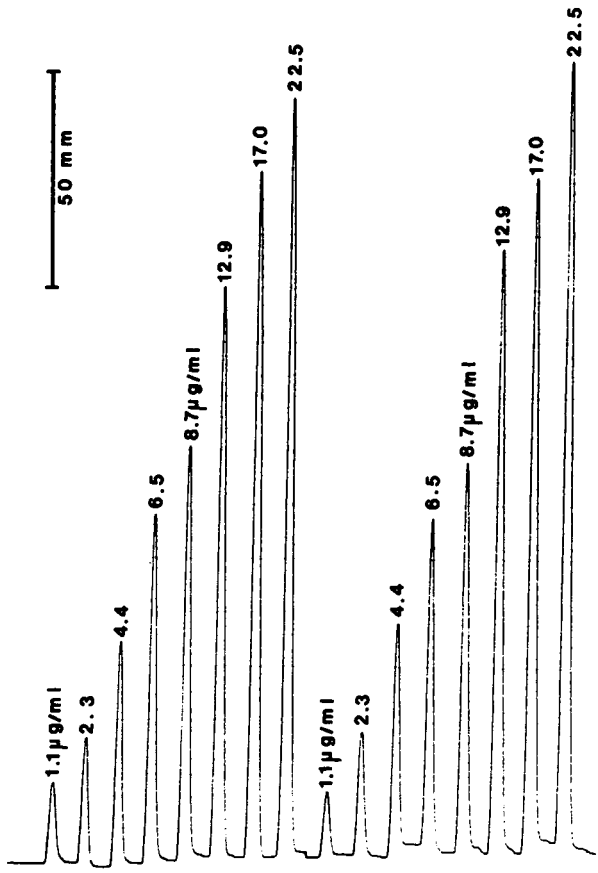

Fig. 2. Densitogram of spiked plasma samples scanned across the direction of solvent flow.

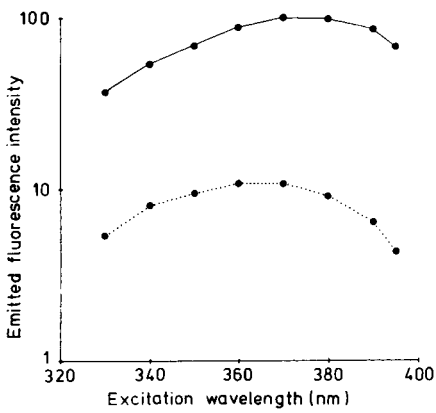

Fig. 3. Comparison of emitted fluorescence intensity obtained using two different types of silica gel plates. •——•, Silica gel 60 (without fluorescence indicator, Merck No. 5721); • . . . . . •, silica gel F60 (with fluorescence indicator, Merck No. 5715).

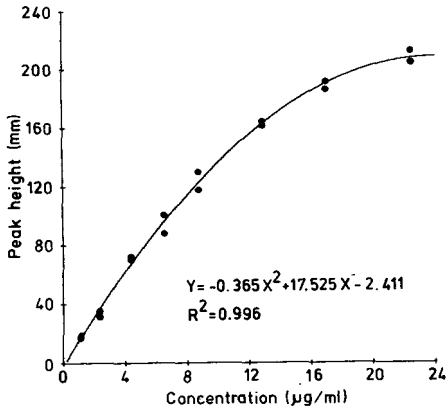

Fig. 4. Parabolic calibration curves obtained with spiked plasma standards.

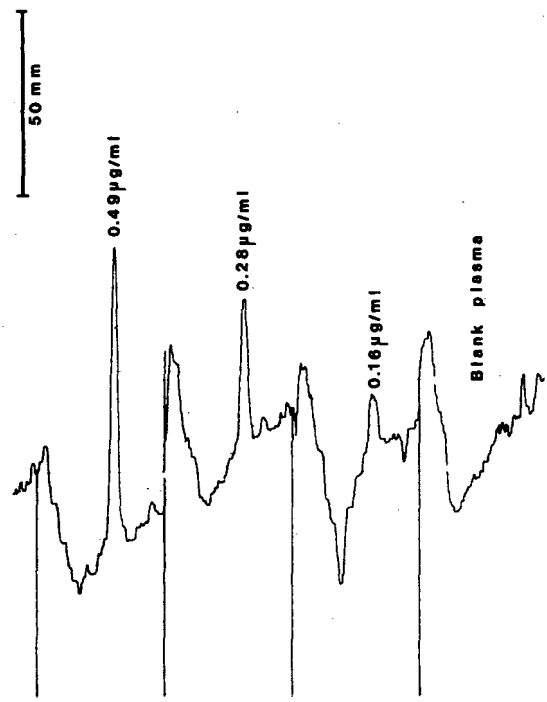

Fig. 5. Densitogram of plasma samples with very low concentrations of nalidixic acid.

The marked effect of the type of silica gel plates used on the fluorescence obtained is shown in Fig. 3 which shows the relative emitted fluorescence intensities obtained using silica gel plates F60 (with fluorescence indicator, Merck No. 5715) and silica gel 60 (without fluorescence indicator, Merck No. 5721). Note the logarithmic scale. These two types of plates were compared because, although fluorescence is being measured, we have in the case of other assays found it necessary on occasions to use plates with fluorescence indicator to be able to detect the presence of UV-absorbing compounds which may interfere

with the assay of the fluorescing compounds. In this case the marked difference in fluorescence intensity obtained was particularly noteworthy and is probably due to a relatively selective interaction between nalidixic acid and a component of the fluorescence indicator. The integrity of the fluorescence indicator is actually destroyed by the treatment with the hydrogen chloride gas.

Using the Autospotter to apply 40 μl of the toluene extract it was found by linear regression analysis that parabolic curves of the form $y = Ax^2 + Bx + C$ (y = peak height, x = plasma concentration) fitted the calibration data up to plasma concentrations of 22.5 μg/ml with correlation coefficients $r^2 = 0.99$ (n = 16) (Fig. 4). When concentrations higher than about 20 μg/ml are expected smaller volumes of the extracts should be spotted.

The lowest demonstrable concentration of nalidixic acid in plasma (spotting 40 μl of the toluene extract) was about 0.16 μg/ml. Fig. 5 shows a densitogram obtained for standard plasma samples with very low concentrations of nalidixic acid.

Selectivity

No interference with the determination was observed with the following compounds: mephenamic acid, flufenamic acid, niflumic acid, thiaprofenic acid, nalidixic acid, flubiprofen, fenoprofen, ketoprofen, ibuprofen, indoprofen, fenbufen, alclofenac, diclofenac, oxyphenbutazone, phenylbutazone, naproxen, probenecid, sulindac, indomethacin, carbamazepine and its two major metabolites carbamazepine epoxide and dihydroxycarbamazepine, penicillin G, furosemide, piretanide, all at a concentration of 20 μg/ml; paracetamol at 50 μg/ml; and salicylic acid at a concentration of 300 μg/ml.

Hydroxynalidixic acid, a known metabolite, was not available to be tested for possible interference although the small peak indicated by the asterisk in Fig. 1 which was obtained in the plasma of volunteers who had taken a single dose of 1 g of nalidixic acid in a bioavailability trial is probably the compound in question. In the trial this compound never attained concentrations which interfered with the nalidixic acid assay; however it is possible that during chronic treatment with nalidixic acid it may become troublesome.

This method was used to determine nalidixic acid in plasma samples obtained from volunteers participating in a comparative bioavailability trial. The results obtained in this trial are summarised in Table II.

TABLE II

PHARMACOKINETIC PARAMETERS FOR ORAL ABSORPTION OF 1 g OF NALIDIXIC ACID AS TABLETS IN THREE DIFFERENT FORMULATIONS

Mean (± S.D.) in six normal trial subjects.

Mean peak plasma concentration (μg/ml)	Mean time to peak plasma concentration (h)	Mean AUC* (0—32 h) (μg · h/ml)	Mean plasma halflife (min)
19.75 ± 6.14	2.25 ± 0.52	181 ± 65	
26.80 ± 11.57	2.58 ± 0.38	208 ± 96	93 ± 38
20.79 ± 16.21	2.08 ± 0.92	168 ± 101	

*AUC = Area under curve.

The halflife of 93 ± 38 min found is based on a one-compartment pharmacokinetic model and a blood sampling period of 10 h. This closely resembles the results obtained by other workers [5, 7]. However, with blood sampling time extended to include 24-h and 32-h samples, preliminary results indicate that the pharmacokinetics of nalidixic acid may be more accurately described by a two-compartment model with an alpha-phase constant of approximately 0.68 h^{-1} and a beta-phase constant of about 0.077 h^{-1}.

The main advantages of this TLC method lie in its simplicity, allowing a large number of samples to be processed per day, and its low sample-volume requirement which is a factor playing an increasingly important role in clinical trials where very often several parameters have to be monitored in each blood sample taken from the patient or volunteer.

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CHROMBIO. 801

Note

Myo-inositol levels in the cerebrospinal fluid of infants

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While developing multicomponent gas chromatographic methodology (metabolic profiling) for organic constituents of human cerebrospinal fluid (CSF) [1,2], we had an opportunity to analyze CSF samples of five infants with various suspected disorders. We found these samples to be very "dilute", except for the exceptionally high levels of myo-inositol and small amounts of glucose in the polyol profiles [1]. A comparison of infant samples with those of "normal" adults is given in Table I, whereas Fig. 1 compares typical profiles of an adult with an infant.

The "normal" values were recorded for adult patients who had disk injuries or arthritic conditions, but suffered from no known complicated illnesses. Because lumbar puncture is a procedure ethically and sometimes legally limited to clinically justified cases, no strictly normal values were available for infants.

The determinations of polyol profiles were performed using the previously described method [1] that consists of protein removal, sample derivatization and gas chromatography of the trimethylsilylated polyols, with use of glass capillary columns. Identity of chromatographic peaks was ascertained from mass spectra and retention of standards.

While myo-inositol levels can be either decreased or increased in certain clinically important cases such as bacterial meningitis, senile dementia [3], diabetic conditions, possibly heart disease and hypertension [3,4], chronic

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arachnoiditis and brain tumor [1], etc., such departures from the normal are considerably less substantial than the infant values reported here.

The metabolic importance of inositol is well established, while its lack could result in cerebral dysfunction. Myo-inositol is synthesized in the brain

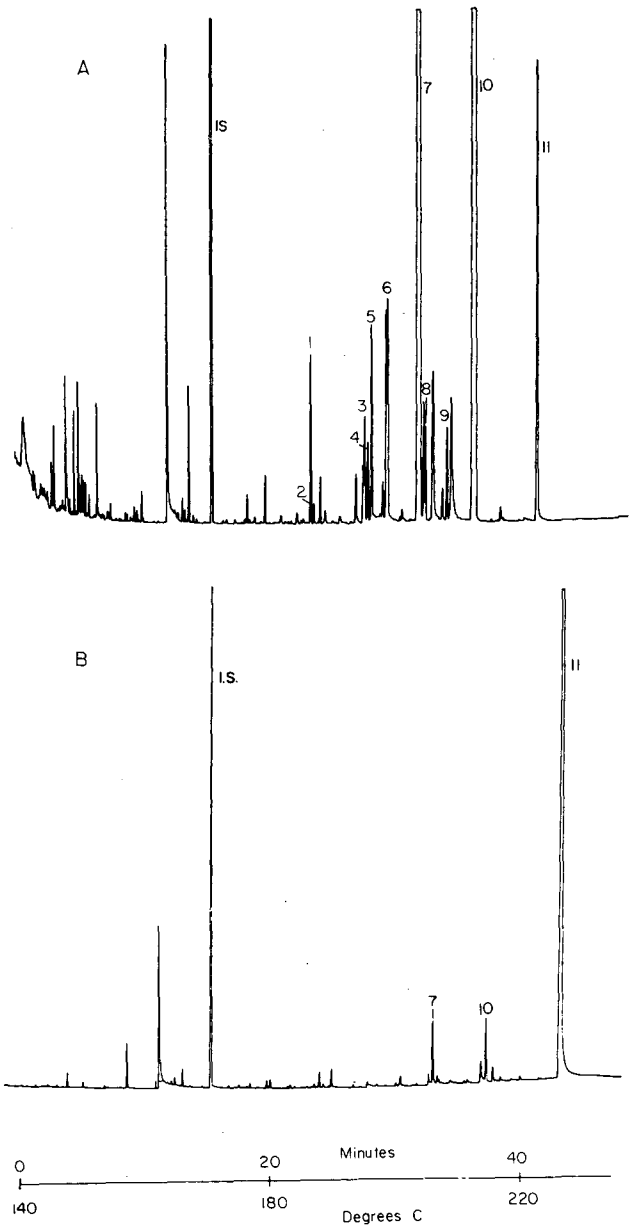

Fig. 1. Chromatograms of polyols in CSF. Conditions: 50 m × 0.25 mm I.D. glass capillary column, temperature programmed from 140—240°C at 2°C/min; flame ionization detector. (A) "Normal" adult sample; (B) infant sample. Peaks: I.S. = dodecanol (internal standard); 1 = arabinitol; 2 = ribitol; 3, 5 = fructose; 4, 8 = mannose; 6 = 1,5-anhydroglucitol; 7, 10 = glucose; 9 = glucitol (sorbitol); 11 = myo-inositol.

TABLE I

CONCENTRATION OF POLAR NEUTRAL COMPOUNDS IN THE CSF OF "NORMAL" ADULT PATIENTS AND INFANT PATIENTS

mg/l ± standard deviations, based on six normal and five infant samples.

Compound	"Normal" adult (mg/l)	Infant (mg/l)
Ribitol	3.4 ± 2.0	–
Fructose	7.6 ± 3.6	–
Mannose	8.5 ± 5.5	–
1,5-Anhydroglucitol	37.1 ± 16.5	–
Glucose	789 ± 275	9.8 ± 14.4
Glucitol	4.8 ± 3.0	–
Inositol	54 ± 12	612 ± 288

from glucose phosphate and incorporated into membrane lipid structures. Thus, the "overproduction" of this substance in the central nervous system of infants is not surprising in view of the intensive biosynthesis of new brain structures.

Finding high myo-inositol levels in this work is also consistent with the published chromatogram of Horning and Horning [5] and high urinary level of the same substance in a newborn.

ACKNOWLEDGEMENT

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Note

Determination of vanillylmandelic acid in urine by pre-column dansylation using micro high-performance liquid chromatography with fluorescence detection

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Vanillylmandelic acid (VMA) has been known as the final metabolite of catecholamines. Various analytical procedures for urinary VMA determination such as thin-layer chromatography [1], gas chromatography—mass spectrometry [2—4] and high-performance liquid chromatography [5, 6] have been reported. Recent investigations in our laboratory suggest a correlation between the content of VMA in urine and catecholamine metabolism. The importance of VMA determination compelled us to develop a simple and sensitive procedure for measuring the content of the metabolite in urine.

In our previous papers, benzoylation of urinary estrogen [7] and tosylation of noradrenaline in rat brain [8] were utilized for micro determination. In the present work, we applied the dansylation procedure for determination of urinary VMA. Dansyl derivatives have been very widely used in clinical analysis [9, 10]. In this paper, the phenolic hydroxyl group of VMA which was extracted from urine was dansylated as the fluorescent derivative and then separated and determined using micro high-performance liquid chromatography (MHPLC) [11] equipment with a fluorimeter.

MATERIALS AND METHOD

Standard VMA was obtained from Tokyo Kasei Kogyo (Tokyo, Japan).

All other chemicals used were of reagent grade purity. A Model Familic 100 (Jasco, Tokyo, Japan), a micro high-performance liquid chromatograph, equipped with a Model FP 110 fluorescence spectrophotometer was used for analysis. An SS-10 silica gel (particle size 10 μm) column 17 cm × 0.1 cm I.D. (Jasco) was used. The mobile phase was ligroin (boiling range 80—100°C)—chloroform—ethyl acetate (1:4:8). The flow-rate was 8 μl/min and the chromatographic separation was monitored with the fluorescence spectrophotometer set at 360 nm was the excitation wavelength and at 505 nm as the fluorescence wavelength. Chromatograms were recorded with a single-pen recorder (Rikadenki Kogyo, Tokyo, Japan). The chart speed was 12 cm/h.

According to the method of Felice and Kissinger [12], 1 ml of urine was transferred into a 10-ml glass tube containing 200 μg of *p*-hydroxybenzoic acid (PHBA) as an internal standard. The mixture was adjusted to pH 2.0 with 1 *N* hydrochloric acid and 0.3 g of sodium chloride was added to the solution from which VMA was extracted twice, each time with 3 ml of ethyl acetate and then back-extracted with 1 ml of 1 *M* potassium carbonate. An aliquot of 0.5 ml of the carbonate layer was transferred into a 25-ml glass-stoppered tube, and 5 ml of 0.01% dansyl chloride in acetone solution was added. After incubation at 40°C for 45 min, 2 ml of water were added, and the dansylated VMA was extracted twice, each time with 3 ml of chloroform. The combined extracts were evaporated to dryness under a stream of air and the residue was redissolved in 5 μl of ethyl acetate. A 0.01-μl sample of the solution was applied to the chromatograph. VMA was determined by the peak height ratio of VMA to PHBA. The standard curve of the peak height ratios showed good linearity within a range of injected sample amounts of 5—20 μg/ml.

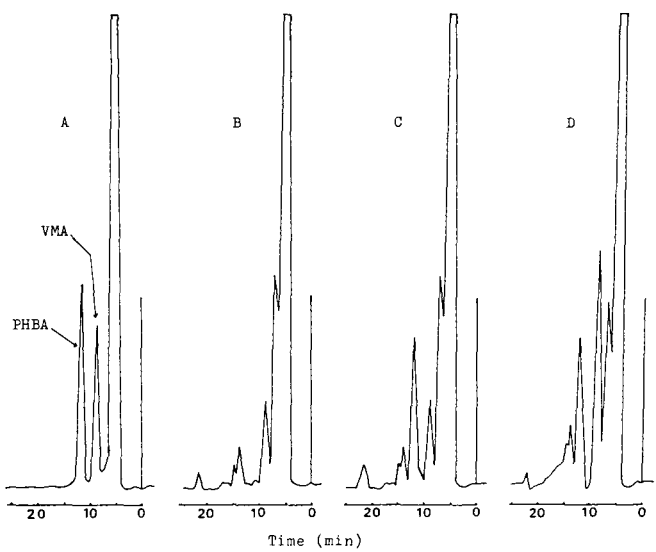

Fig. 1. Chromatograms of labeled samples: (A) VMA (10 μg/ml) and PHBA (200 μg/ml) (internal standard); (B) human urine blank; (C) human urine containing 200 μg/ml of PHBA; (D) human urine containing 200 μg/ml of PHBA and 20 μg/ml of VMA.

RESULTS AND DISCUSSION

MHPLC using micro-columns has been known to be a useful micro technique [11]. The combination of MHPLC and fluorescence detection is suitable for sensitive micro-determination of urinary VMA. Examination of the various procedures for the extraction, derivatization, and separation of VMA in biological samples suggested the optimized procedure described in the Experimental section. The results are shown in Fig. 1.

VMA content of urine can be measured down to 1 μg/ml. Under the present conditions, VMA and PHBA were eluted at 8.5 and 12.1 min, respectively, and were well separated from the other peaks of normal urine constituents. The method was reproducible with a standard deviation of less than ± 4% using 10 μg/ml samples of VMA. This technique seems to be promising for research concerning the influence of drugs on catecholamine metabolism and diagnostic analysis of related problems in both animals and humans. Human urinary VMA values obtained by this method were 1.3—19.5 μg/ml, with an average value of 7.6 ± 2.09 μg/ml (men, n = 10, age 25—37 years), which was in agreement with reports from other laboratories [1]. Application of this method for the investigation of the correlation between the VMA content and phaeochromocytoma will be reported in future papers.

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Note

Separation of taurine and glycerophosphoryl ethanolamine on amino acid analyzers

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Taurine is present in abundant levels in vertebrate brain and much evidence suggests it plays a neurotransmitter role [1]. The usual method for monitoring taurine levels is separation and analysis on an amino acid analyzer, where taurine elutes near the void volume of the commonly used buffer systems. Accurate analysis does depend upon resolution of taurine from all other ninhydrin-positive components; this separation has been assumed in all such analyses reported in the literature.

Recently, however, Tachiki and Baxter [2] reported that another compound, glycerophosphoryl ethanolamine (GPEA), co-elutes with taurine on a Durrum amino acid analyzer system containing the DC-4A resin and Pico Buffer IV mixture, a lithium citrate system for analyzing physiological fluid samples. Since GPEA is found in various levels in vertebrate brain samples, the co-elution of GPEA with taurine would introduce an element of uncertainty in most previous studies of taurine levels in brain tissue.

We have been analyzing the levels of several brain amino acids, including taurine, using two amino acid analyzers from Beckman Instruments. The report of Tachiki and Baxter [2] stimulated us to determine whether GPEA co-elutes with taurine in the analyzer procedures that we use. With pure preparations of GPEA and taurine, we found that there is a clear separation of these two compounds in three out of four systems checked by us.

MATERIALS AND METHODS

Glycerophosphoryl ethanolamine and taurine were obtained from Sigma (St. Louis, MO, U.S.A.). The four systems for separating and analyzing amino acids were all from Beckman Instruments; the first two are the newer

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accelerated systems. System A: Beckman 121MB, 200 × 2.8 mm column of AA-10 resin; flow-rate 10 ml/h; buffer 0.20 *N* Na^+, pH 2.8; column temperature 50°C. System B: same as System A except the eluting buffer was 0.2 *N* Li^+, pH 2.83 at 40°C column temperature. System C: Beckman 120C, 560 × 9 mm column of AA-15 resin; flow-rate 70 ml/h; buffer 0.2 *N* Na^+, pH 3.488; column temperature 55°C. System D: Beckman 120C, 550 × 9 mm column of Aminex A-6 resin; flow-rate 70 ml/h; buffer 0.3 *N* Li^+, 0.16 *N* citrate, pH 2.80; column temperature 40°C.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profiles for GPEA and taurine. GPEA eluted significantly prior to taurine in three out of the four methods tested: both of the Na^+ systems (usually employed for analyzing protein hydrolysates) and the slower of the physiological fluid (Li^+) systems. Only the accelerated physiological fluid System B failed to resolve the two compounds. Thus neither the Durrum [2] nor the Beckman standard accelerated lithium citrate system separated GPEA from taurine.

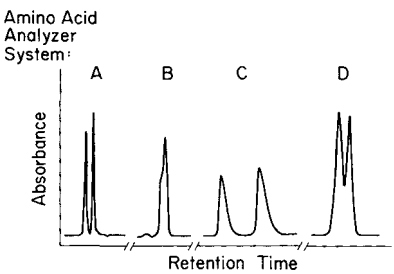

Fig. 1. Amino acid analyzer profiles for glycerophosphoryl ethanolamine (GPEA) and taurine. The four amino acid analyzer systems are described in Materials and methods. The first peak (or shoulder in system B) in each case is GPEA. Twelve nmol of GPEA and 8 nmol of taurine were applied in A and B, 72 and 50 nmol in C and D. The retention times (in minutes) for GPEA, taurine, and aspartic acid (the latter peak not shown in the figure) are in order: (A) 6.0, 7.6, 11.8; (B) 7.5 (GPEA + taurine), 20.6; (C) 26.6, 34.4, 42.6; (D) 28.3, 30.1, 54.0.

Our studies demonstrated that some, but not all, commercially available systems routinely used for the separation and analysis of amino acids do adequately separate GPEA from taurine. If GPEA is present in samples whose taurine content is to be monitored, care must be taken to choose an amino acid analysis technique that resolves these two compounds. It might be most advantageous to combine use of a Na^+ system for analysis of taurine in brain tissues and an accelerated Li^+ system for analysis of other ninhydrin-positive components in the samples. It is of course possible that, for a given amino acid analyzer system, other ninhydrin-positive compounds may overlap with GPEA or taurine; this was not tested in the present study.

An evaluation of previous reports on taurine levels must include consideration of the method used for resolution of taurine on an ion-exchange resin.

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Note

Determination of γ-carboxyglutamic acid excretion in urine

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The presence of γ-carboxyglutamic acid (GLA) in several calcium-binding proteins has been documented by many investigators [1, 2] and free GLA has been demonstrated to be excreted in urine [3] in quantities easily measurable with an amino acid analyzer.

Since the urinary GLA is a product of degradation of GLA-containing proteins, its measurement may be relevant to the study of metabolic diseases affecting bone metabolism and blood clotting mechanisms.

This paper describes a simple method suitable for the routine measurement of GLA in several urine specimens. The method affords a recovery of added GLA greater than 95% and has been used to follow the daily excretion of GLA in a quadriplegic patient.

EXPERIMENTAL

Materials

Pure GLA (monoammonium salt) was purchased from Calbiochem (San Diego, CA, U.S.A.); Dowex 50-X2 (H^+, 200—400 mesh) was purchased from BioRad Labs. (Richmond, CA, U.S.A.). The resin was washed with five bed volumes of 6 *N* hydrochloric acid and then washed with water to neutrality before use. Anion-exchange resin AG 1-X4 (Cl^-) was also purchased from BioRad Labs. It was converted to the OH^- form by stirring it with 1 *N* sodium hydroxide solution, and after removal of excess alkali was converted to the $HCOO^-$ form using 1 *M* formic acid.

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Sample preparation

Urine was collected daily from a 17-year old male, quadriplegic because of traumatic injury in the cervical portion of the spinal cord. Urine collections (24-h) were started 17 days after the accident and continued for nearly 70 days. Urine samples collected in periods of 5—7 days were pooled and the total volume of each pool was recorded. These urine samples were immediately frozen until analyzed. Urine samples (24-h) were also collected from normal children of both sexes (average age 15 years) and immediately frozen until analyzed.

GLA determination was done on aliquots of these urine samples after passing through either an anion-exchange resin or a cation-exchange resin, as described below, followed by analysis of the effluent with the amino acid analyzer.

Quantitation of GLA using an anion-exchange resin

The method of Fernlund [3] was suitably modified to estimate GLA in smaller volumes of urine. Urine samples were centrifuged at 3000 *g* for 15 min in a refrigerated centrifuge and 10 ml of the clear supernatant were applied on an AG 1-X4 (200—400 mesh) $HCOO^-$ column (10.5 × 0.6 cm). The column was then washed with 10 ml water. GLA was eluted with 88% formic acid—water (1:10). The first 2.5 ml of formic acid eluate were discarded and the next 8 ml were collected. The eluate was dried in a desiccator containing sodium hydroxide pellets. The residue was dissolved in 1 ml of the diluting buffer (0.2 *M* sodium citrate buffer, pH 2.2). After centrifugation at 3000 *g*, 15 min, 0.3 ml of the supernatant was mixed with 60 µl of 4 *M* sodium chloride solution and 0.24 ml of diluting buffer. A 0.5-ml aliquot of this sample (which corresponds to 2.5 ml of urine) was injected into an automatic amino acid analyzer.

Quantitation of GLA using a cation-exchange resin

An aliquot of the urine sample was acidified with 6 *N* hydrochlorid acid to pH 3.0. The urine was centrifuged at 3000 *g* for 15 min in a refrigerated centrifuge and 5 ml of the urine were applied on a 3.5 × 0.6 cm column of Dowex 50-X2 (H^+, 200—400 mesh). The eluate was collected; the column was washed with additional 5 ml of water which were added to the first eluate. The combined eluates were made alkaline (pH 10.0) by adding a few drops of 2.5 *N* sodium hydroxide (pH paper, ColorpHast). This solution was dried in a desiccator containing sodium hydroxide pellets and concentrated sulfuric acid. In order to remove completely the ammonia which interferes in the GLA determination, 1 ml of water was added twice to the residue and this was dried again. Finally 1 ml of 1 *M* acetic acid was added to neutralize the alkali and dried again. The residue was dissolved in 1 ml of diluting buffer (0.2 *M* sodium citrate buffer, pH 2.2). After centrifugation at 3000 *g* for 15 min, 0.3 ml of the supernatant was mixed with 60 µl of 4 *M* sodium chloride solution and 0.24 ml diluting buffer. A 0.5-ml aliquot of this sample (which represents 1.25 ml of urine) was injected into an automatic amino acid analyzer. Completion of the GLA elution from the cation-exchange column was determined by eluting the column with 5 ml of 1 *N* ammonium hydroxide, which elutes all retained amino acids. After complete removal of ammonia, this eluate was analyzed for GLA with the amino acid analyzer.

Amino acid analysis

An automatic amino acid analyzer (Beckman model 121) with automatic digital integrator (Infotronics) was used to analyze GLA in these urine samples. A satisfactory program is as follows. Beckman PA-28 resin (column 56 × 0.9 cm, temperature 51°C) was first regenerated with 0.2 *M* sodium hydroxide (1—40 min), and then equilibrated with 0.2 *M* sodium citrate buffer, pH 2.2 (40—80 min). Buffer flow-rate was maintained at 50 ml/h. The sample was injected between 80 and 86 min. The column was eluted with the same buffer for 110 min (86—196 min), after which 0.2 *M* sodium citrate buffer, pH 3.20 was started. Ninhydrin started at 159 min, flow-rate 25 ml/h. The GLA elutes at 190—200 min, i.e., 109 min after injection.

Color factor determination

The color yield of GLA is low compared to other amino acids [4] and hence an authentic GLA has been used as standard. A solution of GLA (5 μmole/ml) was made in diluting buffer. A 10—30 μl aliquot of this solution was mixed with 60 μl of 4 *M* sodium chloride solution and 510—530 μl of diluting buffer. A 500-μl aliquot of this solution (41.6—125 nmole GLA) was injected into the automatic amino acid analyzer. The color factor was calculated by dividing the amount of the GLA applied to the column by the area corresponding to the peak of GLA.

Recovery of GLA

GLA (0.25—0.5 μmole) was added to 5 ml of the urine sample and the urine was processed with the cation-exchange resin method. The same urine without any addition of GLA was processed in parallel. The amount of GLA was calculated in both cases using the color factor for pure GLA and the percentage recovery was calculated.

Other methods

Creatinine was determined by the Jaffe reaction [5]. Acid hydrolysis of GLA was done by heating the samples of urinary GLA containing the fraction with 6 *N* hydrochloric acid, at 110°C for 24 h.

RESULTS

Fig. 1 shows the elution profile of urinary GLA on the amino acid analyzer after prior purification by cation-exchange resin. Fig. 1A shows the analysis of the fraction not retained on Dowex 50-X2 H^+ column and Fig. 1B shows the analysis of the subsequent fraction eluted with 1 *N* ammonia. Clearly, all the GLA present in the urine passes through the Dowex 50-X2 H^+ column and none is retained. Acid hydrolysis of GLA obtained from Dowex 50-X2 completely destroyed it (Fig. 1C) due to its conversion to glutamic acid (not shown) [2].

The position of standard GLA in the amino acid analyzer effluent is shown in Fig. 2A. GLA from urine prepared by the cation-exchange resin appears in identical position (Fig. 2B). Mixing authentic GLA with the urinary GLA prepared by cation-exchange resin produces a single, augmented peak (Fig. 2C).

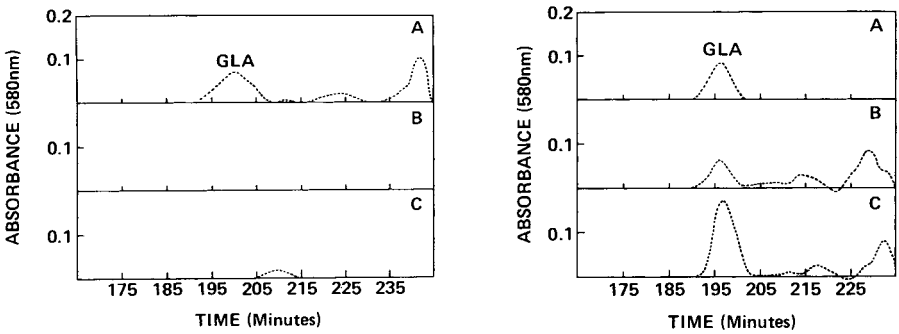

Fig. 1. GLA analysis in the urine after initial passage through a cation-exchange resin. (A) Pass through fraction; (B) ammonia eluate; (C) pass through fraction after acid hydrolysis.

Fig. 2. Elution pattern in the amino acid analyzer of (A) authentic GLA; (B) urinary GLA prepared by cation-exchange column; (C) urinary GLA mixed with authentic GLA.

TABLE I

RECOVERY OF γ-CARBOXYGLUTAMATE FROM URINE

	GLA (nmole)	
	Experiment 1	Experiment 2
Amount of GLA in 5 ml urine	222	222
Amount of externally added GLA to urine	250	500
Total GLA recovered	452	718
Recovery (%)	95.6	99.4

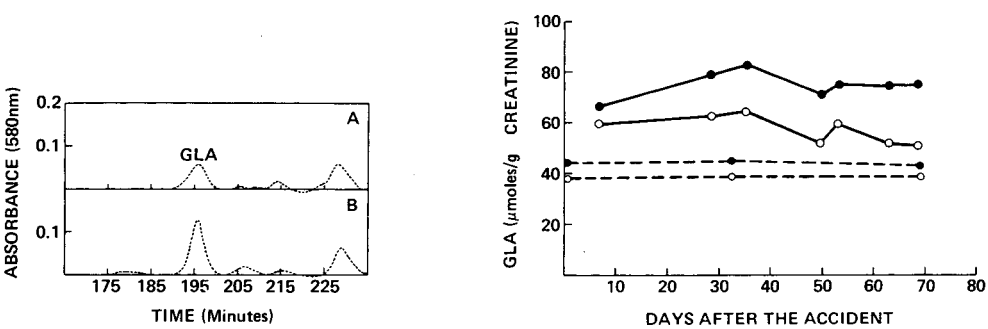

Fig. 3. Comparison of amino acid analyzer pattern of urinary GLA containing fraction prepared using (A) cation-exchange resin, and (B) anion-exchange resin.

Fig. 4. Urinary GLA in quadriplegic patient as measured by cationic procedure (●—●) and anionic procedure (○—○) . The dotted line shows the mean of the values for age-matched controls measured by cationic procedure (●- - - -●) and by anionic procedure (○- - - -○). The statistics of the values are as follows. Cationic method: average for paraplegic patient 69.2 ± 8.05 μmole/g creatinine, and controls 44.4 ± 17.8 μmole/g creatinine; $p < 0.01$. Anionic method: average paraplegic patient 50.7 ± 8.9 μmole/g creatinine, and controls 37.5 ± 8.9 μmole/g creatinine; $p < 0.015$.

Fig. 3 compares the amino acid analyzer profile of GLA prepared either by cation-exchange resin (Fig. 3A) or by anion-exchange resin (Fig. 3B). As seen in the figure both profiles are identical; the greater amount of GLA in the lower tracing reflects the larger size of GLA sample applied to the column (see Experimental).

Table I shows the percentage recovery of γ-carboxyglutamate from the urine by this procedure; 97.5% of the added γ-carboxyglutamate was recovered by this method.

Fig. 4 shows the pattern of excretion of GLA in one quadriplegic patient using the present method and the old one based on the use of the anion-exchange resin. The pattern of excretion is essentially the same when measured by the two methods. The mean daily excretion of GLA in this patient was found to be higher as compared to the age-matched controls when estimations were done by both procedures. However, it was found that the values are about 20—30% higher when measured by the new method.

DISCUSSION

We have described a rapid method for the analysis of GLA in urine samples. The method takes advantage of the highly negative charge of GLA; under conditions in which most other amino acids are held on the Dowex 50-X2 H^+ column, GLA is not retained and flows through the column. Analysis of this effluent fraction on an automatic amino acid analyzer provides a reliable quantitation of GLA in urine.

This method was found to be simpler than the previously described one, based on the use of an anion-exchange column [3]. It eliminates the preparation of AG 1-X4 ($HCOO^-$), elution with water and formic acid, and collection of fractions. With our simpler method, several urine samples may be processed at the same time.

The only precaution to be exercised is to remove the ammonia from the samples. Ammonia has been known to interfere in the analysis of GLA [4]. Complete removal of ammonia may be achieved by repeatedly drying the sample in alkaline conditions. Final addition of 1.0 *M* acetic acid and further drying facilitate the adjustment of the pH of the sample with diluting buffer. The other salts present in urine at normal concentration do not interfere in the analysis.

GLA prepared by either procedure eluted from the amino acid analyzer column at the same time as a pure GLA standard. With this method, recovery of GLA added to urine was found to be 95% or higher, while with the anion-exchange column the recovery of GLA ranges from 85—89% [3]. Moreover, the pattern of urinary excretion of GLA measured in one patient by both procedures was comparable, even though values were slightly higher with the proposed method (about 20—30%).

GLA is present mainly in proteins which have a role in calcium binding such as those found in blood clotting [6], bone proteins [2,7], and that involved in pathological calcifications [8]. Hence a disturbance of any of these systems could produce an increase in GLA excretion. It is also known that GLA is not degraded in the body to a great extent [9]. An increased GLA excretion in

conditions such as osteoporosis, scleroderma, dermatomyositis and in patients treated with warfarin has been reported [10]. In the present paper we have studied a patient with quadriplegia and have found that the GLA excretion in this patient is as high as that reported in osteoporosis [10]. These values probably reflect the increased turnover of connective tissue components of the bone during paralysis and complete bed rest.

ACKNOWLEDGEMENTS

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Note

Studies on dopamine-converting enzymes in human plasma

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The enzyme responsible for the biosynthesis of norepinephrine from dopamine, dopamine-β-hydroxylase (EC 1.14.17.1), has thus far been assayed by different procedures with a varying degree of complexity. One main approach is based on an enzymatic radiolabeling of the product using S-adenosyl-L-[methyl-^{14}C]methionine and a methyl transferase [1, 2]; another makes use of enzymatically liberated tritiated water from [7-$^{3}H_2$]dopamine [3]. In a third type of procedure, derivatization to form a fluorescent product, combined with thin-layer or liquid column chromatography and spectrofluorimetric detection, is utilized [4—7]. Recently a report describing an assay method based on the use of reversed-phase chromatography and electrochemical detection has appeared [8].

Our work, which was initiated before the latter study appeared, makes use of the same analytical principle. A quite different treatment of the reaction mixture prior to the chromatographic step was used, however. In our procedure no extraction on to aluminium oxide, a step which limits the information regarding product formation, was performed. With the technique described in this paper it has been possible, for the first time, to identify and study competitive enzymatic conversions of dopamine, due to the activity of other plasma enzymes, under the conditions used for the assay of dopamine-β-hydroxylase.

EXPERIMENTAL

Materials

All amine standards used were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) as were dopamine-β-hydroxylase and pargyline. Catalase was obtained from Serva (Heidelberg, G.F.R.); all other chemicals for the assay were from Merck (Darmstadt, G.F.R.).

A strong cation exchanger, Nucleosil SA 5 μm from Macherey, Nagel & Co. (Düren, G.F.R.), was used for the preparation of the analytical high-performance liquid chromatographic column.

Human plasma, obtained after blood sampling into heparinized tubes and centrifugation, was stored at −20° C if not used immediately.

Chromatographic procedure

The instrumentation and the chromatographic conditions used were the same as described previously [9] with the exceptions that a 170 × 4.6 mm analytical column was used and the detector cell was operated at an anode potential of +0.65 V (vs. Ag/AgCl) throughout.

Assay conditions

The reaction conditions were similar to those described by Wilcox and Beaven [3]. Addition of the plasma (1 ml) to the reaction medium (1.1 ml) which was also pre-thermostated to 40° C was taken as zero time. At different times a 100-μl aliquot was removed and mixed immediately with 250 μl of 0.8 *M* perchloric acid. The mixture was then centrifuged for 15 min at 9000 *g* to remove any protein precipitate. The clear supernatant was taken directly for analysis. The injected volume (20 μl) corresponded to 2.7 nmol of dopamine at 0% conversion and accordingly to 27 pmol of norepinephrine after 1% conversion to this product.

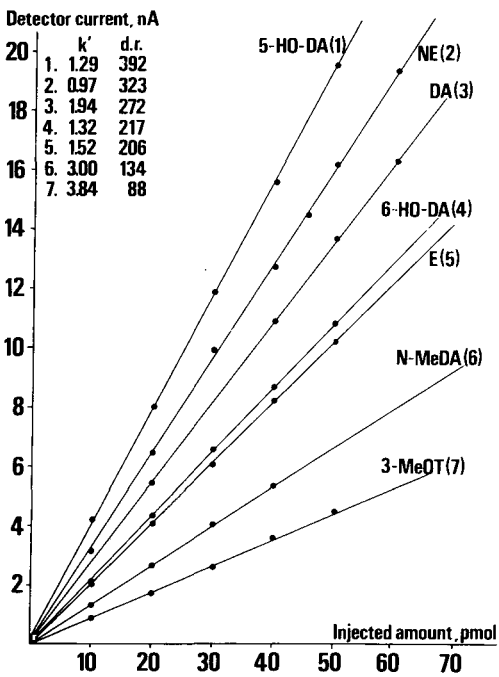

Fig. 1. Concentration dependence of the detector response for the various amine standards. Abbreviations: (1) 5-HO-DA = 5-hydroxydopamine; (2) NE = norepinephrine; (3) DA = dopamine; (4) 6-HO-DA = 6-hydroxydopamine; (5) E = epinephrine; (6) N-MeDA = N-methyldopamine; (7) 3-MeOT = 3-methoxytyramine.

Identification of reaction products

Identification of the reaction products was effected by rechromatography of the sample after the addition of an amine standard and observation of eventual peak superimposition. For an unequivocal identification, peak coincidence was verified in a different chromatographic system by the use of a reversed-phase analytical column.

RESULTS AND DISCUSSION

The evaluation of the chromatograms obtained from serial dilutions of a mixture of amine standards is summarized in Fig. 1. It shows the linear detector response for the compounds investigated, their capacity factors (k') and their detector response values (d.r.) obtained in the system expressed in pA/pmol at maximum peak height. The detection limit (signal-to-noise ratio = 2) can be as low as 0.1 pmol for norepinephrine under optimal conditions, which means that with the assay method described here as little as 0.02% conversion of dopamine can be measured, i.e. the true initial velocity can easily be computed.

In Fig. 2 typical chromatograms, illustrating the dopamine metabolites formed after a certain reaction time under the condition of the assay and ob-

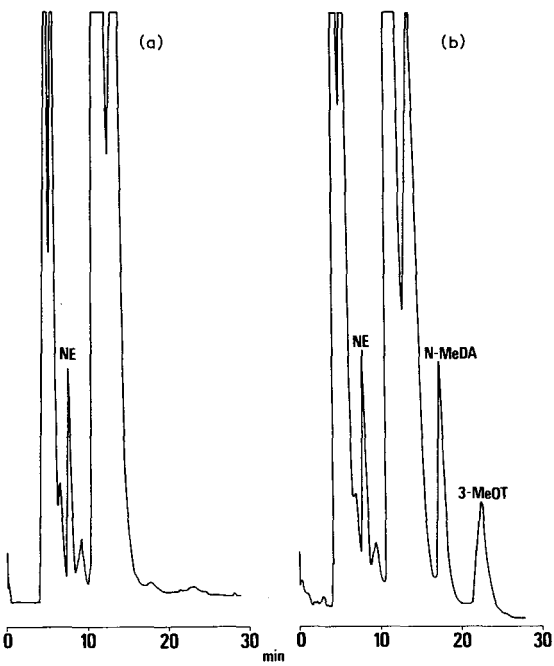

Fig. 2. Chromatograms showing the product composition (for abbreviations see Fig. 1) after a reaction time of 60 min. (a) Commercial enzyme preparation; (b) plasma sample. Buffer: 1 *M* sodium acetate (pH 5.2). Substrate concentration: 0.476 m*M*. The concentrations of all other reagents were as described in ref. 3. Pargyline was used as the sole monoamine oxidase inhibitor.

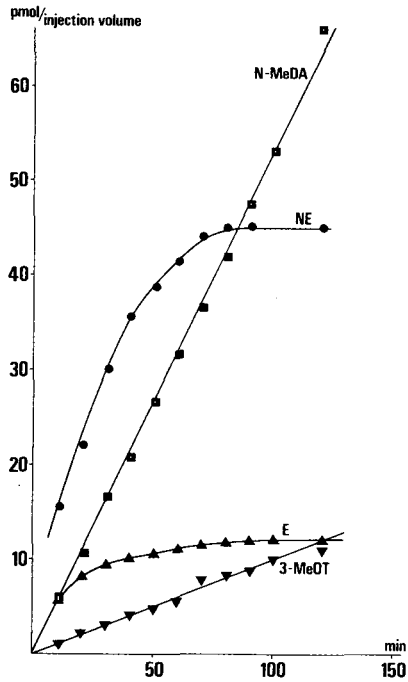

Fig. 3. Plasma-induced dopamine metabolite formation as a function of time. For reaction conditions see legend to Fig. 2. For abbreviations see Fig. 1.

tained with a commercial enzyme preparation (Fig. 2a) and with plasma as enzyme source (Fig. 2b), are shown. The chromatograms exhibited some peaks corresponding to electroactive species whose identity could not be established. The appearance of the identified metabolites as a function of time is demonstrated by Fig. 3. It is noteworthy that the formation of N-methyldopamine is completely linear with time, whereas norepinephrine formation is not. Similarly, 3-methoxytyramine increases linearly, while epinephrine appears soon to reach a steady-state concentration.

Blank experiments, carried out with boiled, centrifuged plasma, gave chromatograms showing the dopamine substrate as the only observable electroactive component.

CONCLUSION

Compared to other methods used for the determination of dopamine-converting enzymes such as dopamine-β-hydroxylase in biological samples, the method described here, in which electrochemical detection combined with high-performance liquid chromatography is utilized, appears to be superior for a variety of reasons. First, the extremely high sensitivity of the detector that can be achieved makes it possible to detect very low enzyme concentrations. Secondly, the ability of the detector to respond to a wide variety of compounds possessing an electro-oxidizable structural element (i.e. here the 3,4-dihydroxyphenyl or 3-methoxy-4-hydroxyphenyl moiety) means that metabolite formation due to the action of different enzymes can con-

veniently be studied kinetically as competitive reactions. Finally, the method does not rely on derivatization or extraction procedures, which obviates the need to use internal standards and calculations with respect to derivatization yields or recoveries.

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Note

The use of oestradiol-17β antiserum covalently coupled to Sepharose to extract oestradiol-17β from biological fluids

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Using radioimmunoassay, Glencross et al. [1] reported oestradiol-17β concentrations in bovine blood plasma, collected during the oestrous cycle, to be in the 1—10 pg/ml range. As the limit of reliable estimation of this steroid in the assay was 10—15 pg, it was necessary to use large samples of plasma (10—20 ml). This entailed extraction of the oestradiol-17β with solvent and extensive chromatographic purification of the extract to concentrate the steroid sufficiently for assay. Such preliminary purification procedures increase the error of the assay and also make it very laborious and time consuming.

A much simpler, yet efficient, method of extracting endogenous oestradiol-17β from large samples of bovine blood plasma and milk has now been devised, based on its binding to oestradiol-17β antiserum covalently coupled to Sepharose. The oestradiol-17β can be conveniently recovered for radioimmunoassay and the antiserum for re-use.

MATERIALS AND METHODS

Steroids and reagents

Steroids, assay buffer and dextran-coated charcoal suspension were as described previously [1].

Radioactivity counting

Radioactivity was measured by liquid scintillation counting (5 min) in glass vials in a 7-ml solution of 2,5-diphenyloxazole (POP; 5 g/l) and 1,4-di[2-(5-phenyloxazolyl)]-benzene (POPOP; 0.1 g/l) in toluene (MI 92 scintillation fluid; Packard Instruments, Reading, Great Britain), using a Packard Tricarb Spectrometer (Model B2450) and a ^{226}radium external standard.

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Plasma and defatted milk samples

Blood plasma samples (50 ml) taken by the method of Glencross et al. [1] from heifers in early pregnancy (7—25 days of pregnancy) when plasma oestradiol-17β levels are known to be low [2] were pooled.

Milk samples (30 ml) collected from non-pregnant cows and cows in the first 60 days of pregnancy were centrifuged (1900 *g*) for 20 min at 4°C and the cream layer removed. The defatted samples, which contained < 0.05% fat as measured by the Gerber method (British Standard 696), were pooled.

Antiserum

The antiserum (G510/6) was prepared against a 6-oxo-oestradiol-17β, carboxymethyloxime, bovine serum albumin conjugate provided by Dr. D. Exley [3] and raised in a castrated male goat (G510 of NIRD herd). It was highly specific for oestradiol-17β with a 6% cross-reaction to oestrone and less than 1% to oestradiol-17α.

Preparation of Sepharose-coupled antiserum

The antiserum was covalently coupled to Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) after activation with cyanogen bromide [4]. After washing with the buffers, the Sepharose-coupled antiserum was stood in 1 *M* ethanolamine, pH 9.0 for 2 h at room temperature to block any active sites remaining on the gel. The Sepharose-coupled antiserum was then washed with water and stored in gelatin-free assay buffer at 4°C in the dark.

Operation of columns of Sepharose-coupled antiserum

Sepharose-coupled antiserum in gelatin-free assay buffer was placed into columns having 13-mm sintered discs (porosity 1), obtained from Corning (Stone, Great Britain), to give 1-ml settled gel volumes. The columns of Sepharose-coupled antiserum were stored in the gelatin-free assay buffer at room temperature.

Before first use, the Sepharose-coupled antiserum column was washed with water (10 ml). An aqueous sample (water, diluted plasma or defatted milk) to which tritium-labelled oestradiol-17β had been added as tracer, was passed through the column, followed by water washings (3 ml, 10 ml) and the eluates discarded. Excess water was removed by briefly applying pressure from a nitrogen supply to the top of the column and also discarded. The antibody bound oestradiol-17β was then eluted with 3 ml water—acetone (5:95, v/v). The aqueous acetone, collected in a glass assay tube, was evaporated to dryness under nitrogen ready for radioimmunoassay.

The Sepharose-coupled antiserum column, after washing with water (10 ml) was ready for re-use. If not so required, it was stored at room temperature under gelatin-free assay buffer.

Radioimmunoassay

Oestradiol-17β, extracted and recovered by the above method, was then submitted to radioimmunoassay [1] using the oestradiol-17β antiserum (G510/6) at a dilution of 1:500,000.

RESULTS AND DISCUSSION

Specificity of the Sepharose-coupled antiserum

The specificity of the Sepharose-coupled antiserum was tested by submitting [^{3}H] oestradiol-17β and [^{3}H] progesterone dissolved in water (10 ml) to the extraction and recovery procedure and estimating the radioactivity in the water (10 ml) and aqueous acetone (3 ml) eluates. The results (Table I) show high recovery of [^{3}H] oestradiol-17β in the aqueous acetone fraction, whereas [^{3}H]-progesterone was not bound to the oestradiol-17β specific antibody.

TABLE I

RECOVERY OF [^{3}H]STEROIDS IN WATER AND AQUEOUS ACETONE FRACTIONS AFTER PASSING THROUGH SEPHAROSE-COUPLED ANTISERUM COLUMNS

Steroid added	% recovery (mean ± S.E.M., n = 3) of [^{3}H]steroid in water fraction	% recovery (mean ± S.E.M., n = 3) of [^{3}H]steroid in aqueous acetone fraction
[^{3}H] Oestradiol-17β (5.64 · 10^5 dpm; 665 pg)	4.2 ± 1.8	94.1 ± 3.2
[^{3}H] Progesterone (1.07 · 10^5 dpm; 165 pg)	90.6 ± 3.8	3.7 ± 0.3
[^{3}H] Progesterone and progesterone (1.02 · 10^4 dpm; 80 ng)	97.8 ± 0.4	3.9 ± 1.7

Efficiency of recovery of oestradiol-17β added to water, plasma or milk and extracted by the Sepharose-coupled antiserum; re-use of the antiserum columns

The efficiency of the procedure for extraction of oestradiol-17β from water, plasma or milk and the possible re-use of the Sepharose-coupled antiserum preparation were then investigated.

[^{3}H] Oestradiol-17β (3700 dpm; 4.4 pg) and oestradiol-17β standards (0, 5, 10, 13, 17, 20, 23, 28, 35, 45, 60 and 80 pg) were added to water (7 ml), vortex mixed (30 sec) and left to stand for 30 min at room temperature. Samples were passed through twelve Sepharose-coupled antiserum columns (each through a different column) and the oestradiol-17β eluted with aqueous acetone as previously described.

The same quantities of oestradiol-17β standards, including the [^{3}H] oestradiol-17β, after solution in plasma (5 ml) to which water (2 ml) had been added, were then extracted using the same twelve columns of Sepharose-coupled antiserum. The diluted plasma eluates as well as the oestradiol-17β containing eluates were collected. The procedure was repeated but with the same quantities of oestradiol-17β added to the diluted plasma eluates (each 7 ml) from the previous operation, again re-using the same twelve antiserum columns.

Standard quantities of [^{3}H] oestradiol-17β (3480 dpm; 4.1 pg) and oestradiol-17β (0, 5, 7, 10, 15, 25, 40, 65, 100 and 125 pg) added to samples of defatted milk (7 ml) were then submitted to extraction and recovery on ten of the same twelve columns of Sepharose-coupled antiserum. Similar quantities of oestra-

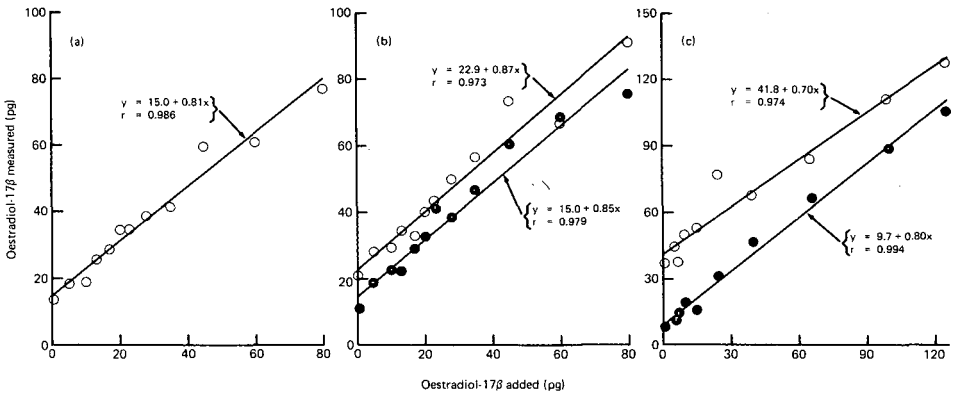

Fig. 1. Oestradiol-17β added to (a) water, (b) diluted bovine plasma or (c) defatted milk, extracted by Sepharose-coupled oestradiol-17β antiserum, recovered in aqueous acetone and estimated by radioimmunoassay. Oestradiol-17β standards added to samples from which endogenous oestradiol-17β had not been removed (○) or to samples from which oestradiol-17β had been removed (●) by prior passage through column.

diol-17β but added to the defatted milk eluates (each 7 ml) were then extracted and recovered on the same ten columns.

The application of oestradiol-17β containing samples in a batch of ten or twelve to the ten or twelve antiserum columns was randomized.

The extracts obtained from evaporation of all 56 aqueous acetone eluates were submitted to oestradiol-17β radioimmunoassay. The correlation between added and estimated oestradiol-17β was excellent (Fig. 1) with evidence of a "reagent blank", i.e. the estimate when no oestradiol-17β is present, of 10—15 pg and of an endogenous oestradiol-17β concentration of 2—3 pg/ml in the pooled plasma and defatted milk.

There was no evidence of deterioration of the Sepharose-coupled antiserum due to storing at room temperature in gelatin-free assay buffer or during successive use. In studies of the variation in the levels of oestradiol-17β in plasma and milk, to be reported elsewhere, it has been confirmed that the columns of Sepharose-coupled antiserum were stable at room temperature for twelve months and after at least 50 times of repeated use.

This extraction procedure using Sepharose-coupled oestradiol-17β antiserum is a simple, efficient and highly specific method of extracting and recovering oestradiol-17β from large volumes of bovine body fluid for accurate measurement by radioimmunoassay. The fact that the antibody preparation can be conveniently recovered for re-use adds to the overall value of the method.

Although this new procedure has been developed for the assay of oestrogens in bovine plasma and defatted milk, it may be generally valuable in analytical and possibly preparative procedures in which a compound (e.g. hormone, vitamin, drug) is present in biological fluid in very low concentrations, provided that the appropriate antiserum is available.

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S.A. Abeywardene (Veterinary Research Institute, Peradeniya, Sri Lanka) was an International Atomic Energy Agency fellow, while S.J. Corney and H.S. Morris undertook this work during their Industrial Training period as part-fulfilment of their degrees at Bradford and Bath Universities respectively.

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CHROMBIO. 800

Note

Determination of polyamines by ion-exchange thin-layer chromatography and video-densitometry

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(First received September 9th, 1980; revised manuscript received November 24th, 1980)

By the combination of thin-layer ion-exchange chromatography with video-densitometry [1, 2] several methods have recently been developed for the determination of amino acids and for the measurement of the activity of enzymes catalyzing amino acid transformation.

Since amino acids can be readily separated from polyamines by ion-exchange column chromatography [3,4], a thin-layer ion-exchange technique has been developed for the determination of basic amino acids and natural polyamines. Combined with video-densitometry [5] the method is suitable for the quantitative determination of polyamines in tissue extracts without any previous purification.

EXPERIMENTAL

Rat tissue samples were homogenized with a Potter-type Teflon homogenizer immediately after removal from the animals in 3 volumes (w/v) of 10% trichloroacetic acid, then centrifuged for 10 min at 5000 *g*. The clear supernatant was used for the determination of polyamines.

Tissue extracts of 20—40 μl were applied to Fixion 50X8 ion-exchange thin-layer chromato-sheets (Na^+) (Chinoin, Budapest, Hungary and Chromatronix, Palo Alto, CA, U.S.A.). As a reference 1--10-μl samples of a stock solution containing 50 nmole/l putrescine·2HCl, 20 nmole/l spermidine·3HCl, 10 nmole/l spermidine·4HCl, 25 nmole/l ornithine and 25 nmole/l arginine were also applied to the chromato-sheets. All the amino acids and polyamines were purchased from Sigma (Heidelberg, G.F.R.). The chromato-sheets were then run for about 4 h at room temperature in a solvent composed of 200 mmole/l potassium hydrogen phosphate and 2 mole/l sodium chloride. The pH of the solution was adjusted to 7.5 with sodium hydroxide. Trichloroacetic acid present in the

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extract does not alter the results of the determinations, because it runs to the top of the sheet. After chromatography the sheets were dried and developed with ninhydrin reagent containing cadmium acetate [6]. The concentration of polyamines and amino acids was then determined by measuring the density of the ninhydrin spots [1] in a Telechrom-S OE 976 video-densitometer (Chinoin). The detector of the equipment is a vidicon tube which performs electronically the three-dimensional integration of an illuminated chromatogram. In this process the x and y axes correspond to the geometry of the spot, whereas the z axis corresponds to the density. Thus, the density count of a large faint spot may be identical with that of a small darker spot.

RESULTS AND DISCUSSION

As it is seen in Fig. 1, the basic amino acids and natural polyamines (putrescine, spermidine and spermine) are readily separated on the cation-exchange chromato-sheet. The R_F values of the compounds are listed in Table I.

Resolution could be improved by over-run chromatography, i.e. by placing a filter paper strip horizontally on the top of the plate over its whole length. Care had to be taken for smooth contact of the paper and the plate. The optimal running time for the determination of natural polyamines was 8–10 h, but in this case the basic amino acids run off the sheet.

TABLE I

CONCENTRATION OF POLYAMINES IN RAT TISSUES

The data were controlled by measuring the dansyl derivatives of these compounds by fluorimetry. Less than 10% difference was found between the results of the two methods. Values are the means of 10 samples from CFY rats.

	R_F	Polyamines (nmole/g tissue)		
		Liver	Kidney	Brain
Methylamine	0.75	—	—	—
Ornithine	0.66	85–157	48– 63	33– 40
Arginine	0.38	Trace	108–159	29– 52
Putrescine	0.26	25– 34	11– 17	14– 32
Spermidine	0.11	470–620	390–460	630–750
Agmatine	0.08	—	—	—
Spermine	0.05	410–550	330–410	380–450

Under the conditions described in Experimental, the method is suitable for the separation and quantitation of 2–60 nmoles of natural polyamines and basic amino acids. With the original optics of the video-densitometer (ENK TV lens, 12.5 mm, 1:1.3) the error of the determination is within ± 3%. The relationship between concentration and density is illustrated in Fig. 2.

Sensitivity could be increased up to 0.1 nmole of polyamines by using a Fujinon C 6× 17.5 zoom optics (Fuji Photo Optical Ltd., Tokyo, Japan) attached to the Bosch TV camera of the equipment. In this case the error increases to about ± 5%.

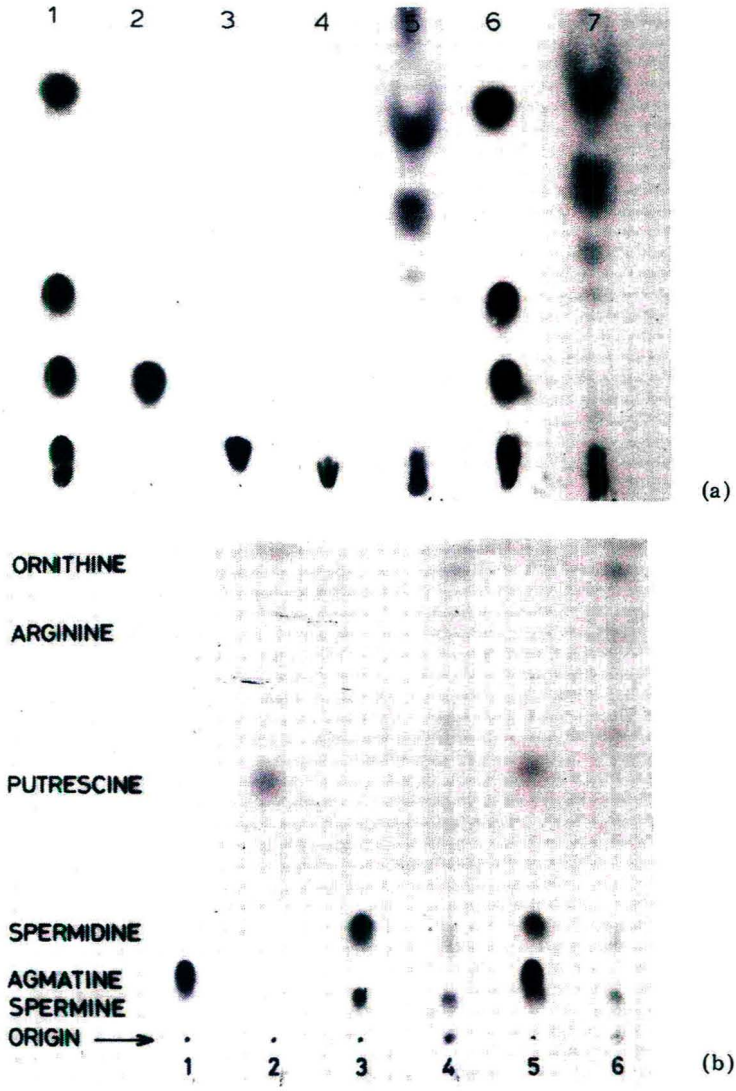

Fig. 1. Ion-exchange chromatography of basic amino acids and natural polyamines from rat liver extracts at room temperature. Solvent: 200 mmol/l potassium phosphate buffer containing 2 mole/l NaCl (pH 7.5). (a) Determination of basic amino acids and polyamines. Running time = 4 h. 1 and 6, mixture of reference compounds; 2, putrescine; 3, spermidine; 4, spermine; 5 and 7, liver extract. (b) Determination of natural polyamines. Running time = 8—10 h. 1, agmatine; 2, ornithine and putrescine; 3, spermidine and spermine; 4 and 6, liver extract; 5, mixture of reference compounds.

The data in Table I demonstrate the results of determining the polyamine content of various rat tissues. The measurements were carried out with individual tissue extracts obtained from ten CFY rats weighing between 150 and 200 g.

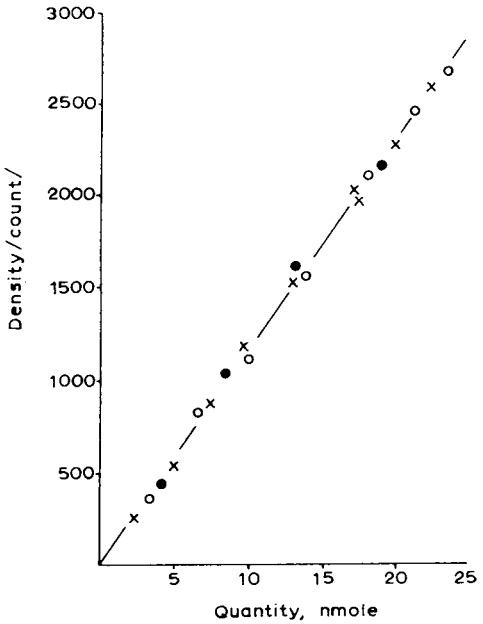

Fig. 2. The change in density of ninhydrin spots of various polyamines as a function of the concentration of polyamine standards. •, putrescine; ○, spermidine; ×, spermine.

The relatively wide range of these values can be attributed to biological variation, as has also been found in other laboratories [7,8].

Recently a similar method has been published by Pongor and Kramer [9] for the determination of biogenic amines on Fixion 50X8 chromato-sheets.

Our preliminary data convincingly suggest that the analytical technique reported in the present paper may supply satisfactory data both on the diamine and the polyamine content of other tissues than those described here.

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CHROMBIO. 786

Note

Determination of flurbiprofen in human plasma using gas chromatography—mass spectrometry with selected ion monitoring

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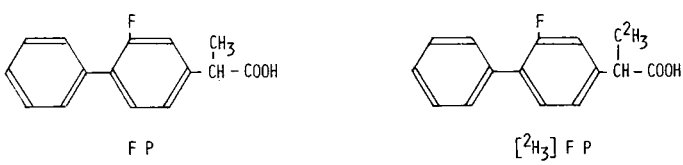
Flurbiprofen (FP), 2-(2-fluoro-4-biphenylyl) propionic acid, provides a wide range of antiinflammatory, antipyretic and analgesic activities [1—3], and has the chemical structure shown in Fig. 1.

Kaiser et al. [4] and Kawahara et al. [5] have reported gas chromatographic (GC) methods for the determination of FP in human plasma after oral administration of FP. The minimum detectable concentration in each method was 50 ng/ml.

In a preliminary experiment of this study, it was found that the plasma levels of FP following application of FP plaster were lower than the limits of GC determination. This paper describes a highly sensitive gas chromatographic—mass spectrometric (GC—MS) method using selected ion monitoring (SIM) which allows the quantitation of plasma levels as low as ng/ml. The method established is applicable to pharmacokinetic and bioavailability investigations with respect to percutaneous absorption of FP in humans.



F P [$^2H_3$] F P

Fig. 1. Chemical structures of FP and [$^2H_3$]FP.

EXPERIMENTAL

Reagents and materials

Benzene, diethyl ether (for pesticide residue analysis), hydrochloric acid, sodium carbonate, sodium hydrogen carbonate and sodium hydroxide (ana-

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lytical grade) were obtained from Nakarai Chemical (Kyoto, Japan). Diazomethane ether solution (alcohol-free) was obtained from N-methyl-N-nitroso-*p*-toluenesulfonamide. A carbonate buffer (pH 9.5) was prepared with sodium carbonate and sodium hydrogen carbonate solutions (0.1 *M*).

[$^2H_3$]FP (see Fig. 1) was synthesized in this laboratory and 0.001 *N* sodium hydroxide solution containing 50 ng/ml [$^2H_3$]FP was used as the internal standard solution.

n-Tetracosane (TC), used as a secondary internal standard, was obtained from Applied Science Labs. (State College, PA, U.S.A.).

The plaster (10 cm × 13.6 cm), containing 40 mg of FP per sheet, was obtained from Lead Chemical (Toyama, Japan).

Gas chromatography—mass spectrometry conditions

GC—MS—SIM was carried out on an Hitachi 6MG gas chromatograph—mass spectrometer with an electron impact source and a multiple ion detector. The glass column (1 m × 3 mm I.D.) was packed with 1.5 % OV-17 on Chromosorb W AW DMCS (80—100 mesh). The column, injection port and molecular separator temperatures were 175°C, 220°C and 240°C, respectively. The carrier gas (helium) flow-rate was 50 ml/min. The ionizing energy and trap current were 20 eV and 60 μA, respectively. The ions of *m/e* 258 (FP) and *m/e* 261 ([$^2H_3$]FP) were chosen for SIM.

Sample preparation

In a glass-stoppered test-tube are placed 0.5—2 ml of plasma, 1 ml of the internal standard solution and 1 ml of 3 *N* hydrochloric acid solution. The mixture is extracted with 15 ml of benzene by shaking for 5 min and centrifuging for 5 min at 1660 *g*. The benzene layer is transferred to another test-tube and back-extracted with 5 ml of carbonate buffer solution (pH 9.5) by shaking for 5 min. After centrifuging for 5 min at 1660 *g*, the benzene layer is removed by aspiration. A 0.5-ml aliquot of 1 *N* sodium hydroxide solution is added to the remaining basic aqueous layer. The mixture is washed with 5 ml of benzene, acidified (pH 1) with 1 ml of 3 *N* hydrochloric acid solution and extracted with 10 ml of diethyl ether by shaking for 5 min and centrifuging for 5 min at 1660 *g*. The ether layer is evaporated to dryness in a flask under reduced pressure at 40°C. The residue is treated with 0.5 ml of diazomethane—ether solution. After reaction for 5 min at room temperature, the excess amount of reaction reagent is removed by evaporation under reduced pressure (21 mmHg) at 15°C. The residue is dissolved in 50 μl of methyl alcohol, and a 1—6-μl portion is injected into the GC—MS system.

Plaster application to volunteers

Six healthy male adults, 29—36 years, 57—63 kg, applied two sheets of FP plaster (80 mg/man) on both arms for 8 h.

A 5—10-ml volume of blood was withdrawn using a heparinized syringe at 0, 2, 4, 6 and 8 h after application of the plaster and 0.5—2 ml plasma, depending on the concentration of FP, was used for analysis.

RESULTS AND DISCUSSION

Selected ion monitoring

Trimethylsilylation and methylation have been appreciated as simple and common derivatization methods for GC analysis of carboxylic acids. Although both methods are also applicable to FP, this study employed methylation with use of diazomethane, because it gave rather cleaner chromatograms for plasma specimens. Fig. 2 shows the mass spectrum of the methyl ester of FP (Me-FP). The base peak of Me-FP at *m/e* 199 is about two times as intense as the molecular ion peak at *m/e* 258. The molecular and base ions of the methyl ester of [$^2H_3$]FP (Me-[$^2H_3$]FP) shifted, as expected, by three mass units from those of Me-FP; namely, from *m/e* 258 to 261 and from *m/e* 199 to 202 (Fig. 3). Although the base peak at *m/e* 199 seemed favorable for the

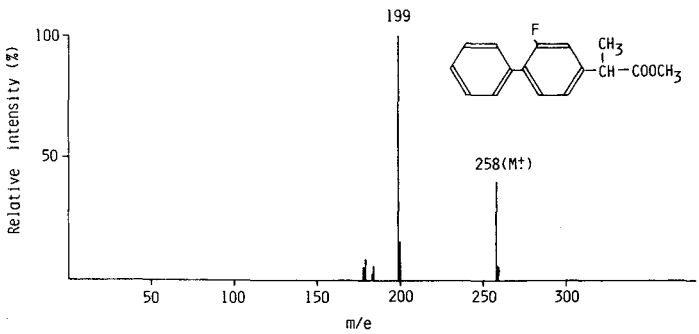

Fig. 2. Mass spectrum of methyl derivative of FP.

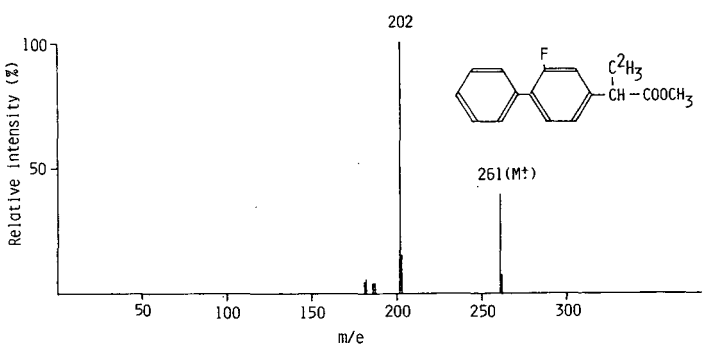

Fig. 3. Mass spectrum of methyl derivative of [$^2H_3$]FP.

SIM of FP, the particular mass fragmentogram suffered from interference by peaks of plasma components; one peak interfered with the separation of the FP peak and another peak had a long retention time. Such disadvantages were avoided by using *m/e* 258 for SIM. Fig. 4 shows a typical mass fragmentogram by SIM at *m/e* 258 and *m/e* 261 for a plasma specimen following application of FP plaster.

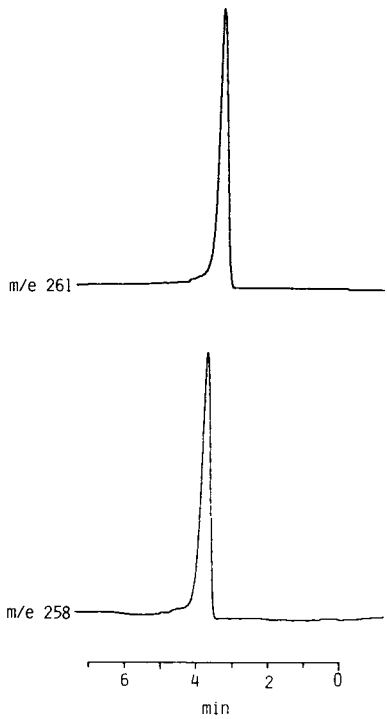

Fig. 4. Mass fragmentogram of plasma extract obtained after application of FP plaster (80 mg/man).

Sample preparation

The extraction procedure involving solvent extraction to organic phase, back-extraction to aqueous phase followed by re-extraction with organic solvent is tedious but often advantageous in providing clean samples of body fluids. The use of benzene for the extraction of FP from human plasma has the advantage of easy separation of the organic layer, whereas the diethyl ether used in the previous study [5] sometimes gave rise to an emulsion. The pH of the alkaline solution with which FP was back-extracted has an influence on the purity of the plasma extract. Carbonate buffer solution (pH 9.5) gave cleaner extracts than sodium hydroxide solution.

Time and temperature dependencies of the methylation of FP and [$^2H_3$]FP using ethereal diazomethane were examined by varying the reaction time (2, 5 and 10 min) and reaction temperature (0°C, 25°C and 35°C). TC was used as a secondary internal standard to evaluate the effect of those conditions on the yields of Me-FP and Me-[$^2H_3$]FP. The peak-height ratios of methyl esters vs. TC monitored at *m/e* 267 indicate that the yields of the methyl esters were independent of time and temperature, and the reaction was completed within 2 min at room temperature.

It was feared that Me-FP and Me-[$^2H_3$]FP might be partly lost during the evaporation of excess ethereal diazomethane under reduced pressure. This was examined by varying the evaporation temperature (15°C, 25°C and 35°C) and evaporation period (5, 10 and 15 min) at 21 mmHg. TC was again used as a secondary internal standard to estimate the loss of methyl esters. The results,

as shown in Fig. 5, indicate that the peak-height ratios of Me-FP and Me-[$^2H_3$]FP vs. TC remained constant when the evaporation temperature was 15°C or 25°C, but decreased with about 20 % loss of each product during evaporation between 5 and 15 min at 35°C.

These observations confirmed that methylation for 5 min at room temperature followed by evaporation of excess ethereal diazomethane at 15°C under reduced pressure (21 mmHg) is sufficient for the determination of FP.

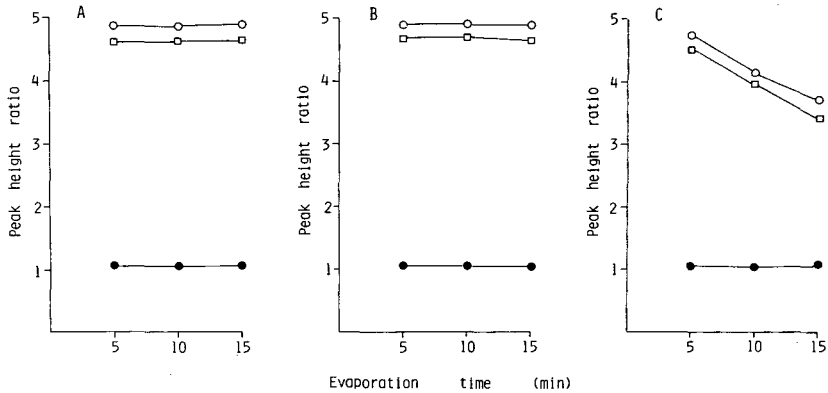

Fig. 5. Influence of evaporation time and temperature on the recoveries of methyl derivatives of FP and [$^2H_3$]FP. Evaporation temperatures: A, 15°C; B, 25°C; C, 35°C. ○, Peak-height ratio of FP to TC; □, peak-height ratio of [$^2H_3$]FP to TC; ●, peak-height ratio of FP to [$^2H_3$]FP.

Calibration graph

It is advantageous to use a stable isotope derivative as an internal standard for the GC—MS—SIM determination of the parent compound because retention times of both compounds are very close and, therefore, their response ratio is almost independent of the GC conditions. The calibration graph of FP against [$^2H_3$]FP was drawn using 2 ml of control plasma spiked with several known amounts of standard FP. The peak-height ratio (FP/[$^2H_3$]FP) thus obtained was linear over the FP concentration range 1.25—150 ng/ml with a correlation coefficient of 0.999. The statistical evaluation of the accuracy and precision of the present method is given in Table I, indicating satisfactory reproducibility over a wide range of plasma levels of FP.

Application of the method

Fig. 6 illustrates the time course of plasma levels of FP in six volunteers after application of two sheets of plaster (80 mg/man). Kaiser et al. [4] reported that the maximum mean level (1.32 ± 0.25 ng/ml) of FP in human plasma was observed at 1 h after a single oral administration of FP (10 mg/man) as a compressed tablet. However, the present results (Fig. 6) indicate a very slow and slight percutaneous absorption of FP with maxima at 6 h or more after application of the FP plaster. Since the present method allows specific and reproducible determinations of plasma levels of FP as low as ng/ml, the pharmacokinetics and bioavailability of FP following percutaneous absorption could be discussed by completing the time courses of Fig. 6. This will follow in the near future.

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF FP IN PLASMA

Amount of FP added to plasma (ng/ml)	Amount of FP found (ng/ml; mean of 3 experiments)	Precision (coefficient of variation, %)
2.5	2.4	10.5
5	5.1	3.3
12.5	12.4	6.0
25	25.2	1.1
50	50.1	1.3
100	101.2	4.5
150	149.4	3.3
300	296.6	5.8

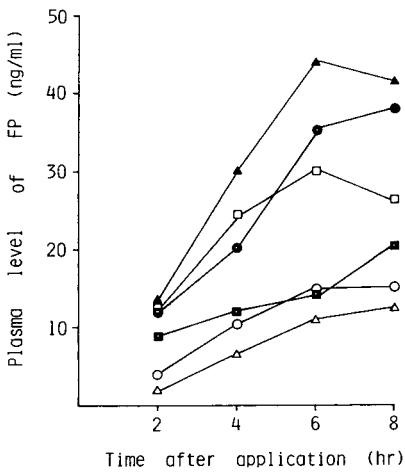

Fig. 6. Plasma levels of FP in human subjects after application of FP plaster (80 mg/man). The symbols specify the volunteers.

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CHROMBIO. 780

Note

Rapid method for the determination of chlorpheniramine in urine

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Chlorpheniramine maleate is a widely used antihistaminic drug [1—3]. Numerous reports have been published on its disposition, metabolism and pharmacokinetics [4—10].

Various methods have been reported in the literature for the analysis of chlorpheniramine in biological fluids; for example, chloroform extraction followed by spectrophotometric determination [5], gas—liquid chromatographic (GLC) analysis of ethereal extracts [6,7], thin-layer chromatography followed by fluorimetric determination [11] and GLC determination of ketones produced by oxidation of the compound (prior to GLC analysis) [7,12,13]. However, the use of any of these methods has been limited by one or more factors such as lack of sensitivity, non-specificity, non-reproducibility and lack of speed.

Peet et al. [9] described a radioactive tracer procedure for the analysis of chlorpheniramine in urine and blood. Although radioactive tracer techniques are very sensitive, their use in biopharmaceutical studies is also limited.

Single-extraction methods using solvents heavier than water have been reported for various drugs [14—17]. These methods were claimed to be sensitive, specific and fast. We now report a single-extraction method for the analysis of chlorpheniramine in urine.

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EXPERIMENTAL

Compounds and materials

The compounds used were kindly supplied by various firms: chlorpheniramine maleate, mono- and di-desmethylchlorpheniramine by Allen and Hanburys Ltd. (London, Great Britain), and brompheniramine and mono-desmethylbrompheniramine [5] by A.H. Robins Co. (West Sussex, Great Britain). Chlorpheniramine N-oxide was not available as an authentic material.

Gas—liquid chromatography

A Perkin-Elmer F33 gas chromatograph with a flame ionization detector and linked to an Hitachi Perkin-Elmer 1-mV Model 56 recorder was used. Column A (glass column, 2 m × 0.64 cm O.D.) was packed with Chromosorb Q (AW DMCS), 100—120 mesh, and coated with 3% OV-17, and operated under the following conditions: nitrogen, 1.68 kg/cm^2; hydrogen, 0.96 kg/cm^2; air, 2.1 kg/cm^2; oven temperature, 210°C; injection point temperature, 250°C. The column was conditioned at 260°C for 24 h and was silanized with 2 × 5 μl of hexamethyldisilazane (HMDS) before use. The retention times of chlorpheniramine, mono-desmethylchlorpheniramine, di-desmethylchlorpheniramine, brompheniramine and mono-desmethylbrompheniramine were 6.0, 7.5, 8.5, 9.0 and 11.0 min, respectively. Chlorpheniramine N-oxide was analyzed as chlorpheniramine after reduction with titanous trichloride [7].

Determination of chlorpheniramine in urine

To 5 ml of the urine sample in a centrifuge tube were added brompheniramine maleate (10 μg of base per ml, 1 ml) as the internal standard, chloroform (100 μl) and sodium hydroxide (20%, 0.5 ml). The solution was mixed thoroughly on a Whirlimixer (2 min) and centrifuged (5 min). A small globule of clear chloroform solution forms at the bottom of the tube. A 10-μl syringe was inserted through the aqueous phase into the chloroform layer and 5 μl of the solution extracted carefully so that no aqueous solution entered the syringe. The syringe barrel was wiped carefully with a clean tissue and the 5 μl of solution injected on to column A.

The concentration of chlorpheniramine in the urine sample was determined by measuring the ratio of the chlorpheniramine peak (t_R = 6 min) to the brompheniramine peak (t_R = 9 min) and reading the corresponding concentration of chlorpheniramine from a calibration curve. The calibration curve was constructed by repeating the above procedure using solutions of chlorpheniramine of known concentration (5—0.4 μg/ml) in urine and plotting the peak height ratio (PHR) against concentration of chlorpheniramine. The data were subjected to linear regression analysis to give the appropriate calibration factors.

Comparison between chloroform and ether extraction of chlorpheniramine in urine

Twelve samples (5 ml of each sample) of chlorpheniramine maleate solution in urine (4 μg of base per ml) were prepared. Half of these samples were extracted and analysed by the method described above. To each of the other

samples were added brompheniramine maleate (10 μg of base per ml, 1 ml) and sodium hydroxide (20%, 0.5 ml) and the mixture extracted with ether as previously described by Khan [7]. The ethereal extracts were concentrated (42°C, ca. 50 μl) and injected (5 μl) on to the GLC column A. The mean values of the PHR obtained from each extraction method were calculated and the amount (μg) of chlorpheniramine in each sample was determined.

RESULTS AND DISCUSSION

Using the single-extraction method we were able to detect chlorpheniramine in urine (from subjects receiving a 4.0-mg dose of the drug) in amounts of less than 60 ng. Mono-desmethylchlorpheniramine, di-desmethylchlorpheniramine and chlorpheniramine N-oxide could be detected within the limit of 60—70 ng. Sharp and symmetrical peaks were obtained for chlorpheniramine and brompheniramine using the GLC conditions described in the Experimental section (see Fig. 1). The compounds were well separated from each other on the column (see Experimental section). None of the expected metabolic products of chlorpheniramine (mono-, di-desmethylchlorpheniramine and the N-oxide)

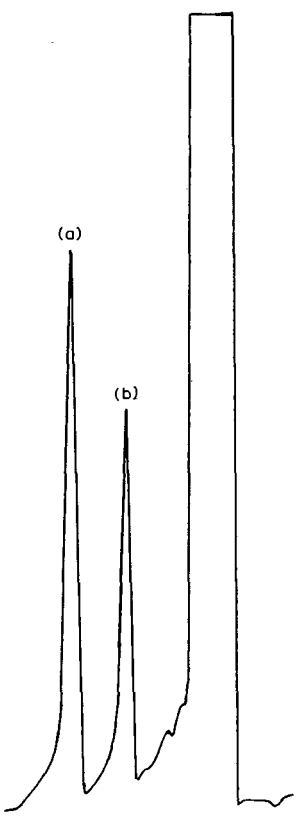

Fig.1. Gas—liquid chromatogram of the chloroform extract of urine obtained from a subject receiving an oral dose of chlorpheniramine maleate (a). Brompheniramine (b) was added to the urine as internal standard.

TABLE I

COMPARISON BETWEEN CHLOROFORM (A) AND ETHER (B) EXTRACTION METHODS FOR CHLORPHENIRAMINE IN URINE

Sample	Amount of chlorpheniramine extracted (μg)	
	Method A	Method B
1	21.1	19.6
2	20.2	20.0
3	20.7	21.6
4	22.1	19.9
5	20.5	20.1
6	21.5	19.8
Mean (± S.E.)	21.0 ± 0.26	20.1 ± 0.27

were detected in the urine of the subjects receiving the drug. Only 20—30% of the dose was excreted unchanged in the urine, in agreement with previous findings [6—9].

The recovery of chlorpheniramine from urine using the ether extraction method (method B, Table I) was more than 90% [7,12]. The amounts of chlorpheniramine extracted using the single-extraction method (method A, Table I) were identical to those obtained using the ether extraction method. This indicates that a recovery of more than 90% can be obtained using the single-extraction method. This extraction method is superior to the ether extraction method because (1) there is no loss of drug due to multiple extractions, evaporation, or adsorption to glass, and (2) it is economical.

The single-extraction method gave reproducible results (Table I). When four calibration curves were constructed (on different occasions) using this method, the corresponding calibration factors were almost identical. The average calibration factor ($\bar{X}$ ± S.E.) was 1.147 ± 0.0133. Linear calibration curves were obtained over the range 0.5—4.8 μg/ml (correlation coefficient = 0.9997).

This method has advantages over the previously described methods for the analysis of chlorpheniramine in urine since it offers the required specificity, sensitivity, reproducibility and speed [5—7,9,11,12]. It is especially useful in routine analysis when a large number of samples are involved and information is required urgently in poisoning cases.

We conclude that the present method is applicable to metabolic, disposition and pharmacokinetic studies of chlorpheniramine, and related compounds (e.g. brompheniramine) and in general to most compounds of high lipid solubility (i.e. with a high chloroform—water partition coefficient).

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CHROMBIO. 781

Note

Quantitative gas—liquid chromatographic method for the determination of phenoperidine in human plasma

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The 4-phenylpiperidine synthetic analogues of narcotic analgesics, for instance pethidine and phenoperidine (ethyl-4-phenyl-1-(3-hydroxy-3-phenylpropyl)-piperidine-4-carboxylate) have been the subject of some major research programmes in our laboratories. Recent studies on the use of narcotics in hospital practice showed that pethidine, morphine, diamorphine and phenoperidine were the four most widely used potent analgesics [1]. In particular, phenoperidine which is 50 times more potent than pethidine, was used exclusively in surgical theatres and intensive care units.

The metabolic fate and disposition of pethidine, the oldest synthetic narcotic, in both animal and human subjects have been extensively studied [2, 3]. However, very little is known about the biotransformation and pharmacokinetics of its closely related analogue, phenoperidine. The purpose of this paper is to present a report on the choice of some phenoperidine analogues and the development of one for use as an internal standard to determine plasma levels of phenoperidine after intravenous administration to healthy volunteers.

EXPERIMENTAL

Apparatus

A Sigma 3 gas chromatograph, all glass system, fitted with a phosphorus—

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nitrogen detector and linked to a Hitachi chart recorder Model 56 (Perkin-Elmer, Beaconsfield, Great Britain) was used. This instrument was operated with coiled glass column (1 m × 4 mm O.D.) silanised with hexamethyldisilazane (HMDS; Chromatography Services, Merseyside, Great Britain) before use and packed with various stationary phases. The operating temperature for the injector and detector was 350° C, and also a suitable one for the column. Gas flow-rates were nitrogen (carrier gas), 30 ml/min; hydrogen, 5 ml/min; and air, 100 ml/min. The temperature of the rubidium bead in the phosphorus—nitrogen detector was adjusted to optimum condition before use. Other apparatus used: 10-ml and 15-ml capacity centrifuge tubes with well fitting screw caps (Sovirel, Levallois-Perret, France); 15-ml capacity stoppered evaporation tubes with finely tapered bases. All this glassware was cleaned by soaking overnight in a 2% solution of RBS 25 (Chemical Concentrates, RBS, London, Great Britain) in water, then rinsed thoroughly with methanol and hot tap water followed by distilled water. These tubes were subsequently silanised by rinsing with HMDS in re-distilled chloroform (3%, v/v), and dried at 250° C overnight. This treatment of glassware was found to be necessary to eliminate possible loss of drug due to adsorption to the glass wall [4].

Materials

The following materials were used: chloroform, dichloromethane, diethyl ether, methanol and *n*-hexane, all of AnalaR grade and freshly re-distilled; sodium hydroxide solutions (5 *N* and 0.1 *N*, ether-washed); phenoperidine hydrochloride and R3757 hydrochloride, a phenoperidine analogue (Janssen Pharmaceutica, Beerse, Belgium); practolol hydrochloride and propranolol hydrochloride (I.C.I., Macclesfield, Great Britain); oxprenolol hydrochloride (Ciba Geigy, Macclesfield, Great Britain) a *n*-butyl reversed ester analogue of phenoperidine (synthesised in own laboratory).

Gas—liquid chromatography

Methanolic solutions of phenoperidine and possible internal standards were injected onto the following chromatographic systems at various oven temperatures.

System 1. Supelcoport (80—100 mesh) coated with 3% OV-1, in a coiled glass column (1 m × 4 mm O.D.).

System 2. Supelcoport (80—100 mesh) coated with 10% SP-2100, in a coiled glass column (1 m × 4 mm O.D.).

The columns were conditioned at 20° C below that of the maximum recommended temperature of the relevant stationary phase for 24 h. Each column was then silanized twice in situ with 20-μl aliquots of HMDS before use.

Compounds which were investigated as possible internal standards were: practolol, propranolol, oxprenolol, metoprolol, a reversed ester analogue of phenoperidine [1-(3-hydroxy-3-phenylpropyl)-4-phenyl-4-piperidinol-*n*-butyl ester] and R3757, a phenoperidine analogue with longer side chain [ethyl-4-phenyl-1-(5 hydroxy-5-phenylpentyl)-piperidine-4-carboxylate].

Retention times, resolution and symmetry factors of the analytical peaks of these compounds for various gas—liquid chromatography (GLC) systems were determined and the most suitable compound for use as internal standard was then established.

General procedure for the determination of phenoperidine in plasma

Blood samples were obtained by venous puncture after intravenous administration and collected in heparinised polythene tubes. The red blood cells were separated from the plasma by centrifugation (3000 *g* for 10 min). A plasma sample (2.5 ml) diluted with distilled water (2.5 ml) in a 15-ml glass centrifuge tube was made alkaline (pH 10—12) with 20 μl 5 *N* sodium hydroxide solution and the internal standard R3757 (60 μl of a methanolic solution equivalent to 50 ng/ml) was added. The alkaline solution was extracted with 12 ml of diethyl ether—dichloromethane (4:1) using an automatic shaker at a speed of 40 rpm for 10 min. The organic layer was then separated from the aqueous by centrifugation (2500 *g* for 15 min) and was transferred carefully into a 15-ml evaporation tube. The extract was then evaporated to dryness at 50—55°C on a water bath. An aliquot of re-distilled *n*-hexane (20 μl) was added to dissolve the dried extract. The stoppered tubes were then stored at −20°C before GLC analysis. An aliquot (1—2 μl) of the final concentrate was injected into the GLC system. The concentration of phenoperidine present in the plasma sample was determined from the ratio of the peak height of phenoperidine to that of the internal standard. Calibration graphs were prepared as follows: methanolic standard solutions of phenoperidine and internal marker were added to the drug-free plasma to cover a concentration range of 5—80 ng/ml).

Recovery, selectivity, reproducibility and storage

Recovery. Eight replicate samples of phenoperidine in plasma (20 ng/ml) were extracted and evaporated to dryness as described in General procedure. The internal standard, R3757 (50 ng/ml in methanol), was then added to each tube and evaporated to dryness and 1.5 μl aliquots injected onto GLC System 2 as described earlier. These results were then related to the 100% value obtained from standard methanolic solutions containing phenoperidine 20 ng/ml and R3757 50 ng/ml.

Selectivity. Samples of plasma from patients on a variety of drugs were analysed to find out if they produced peaks after chromatography which interfered with those of phenoperidine and R3757.

Reproducibility. Seven replicate samples of phenoperidine in plasma (20 ng/ml) were assayed by the General procedure and the peak height ratios of the drug to the marker were calculated.

Storage. Samples of plasma were analysed immediately and after storage at −20°C for seven days.

RESULTS AND DISCUSSION

Choice of GLC system

Two GLC systems were investigated and their performance is summarised in Tables I and II and Fig. 1. Both System 1 (3% OV-1) and System 2 (10% SP-2100) were satisfactory for the analysis of phenoperidine. The reversed ester analogue of phenoperidine was not resolved by any of the GLC systems investigated, and considered unsuitable for use as an internal standard. Compounds such as propranolol, practolol and metoprolol were considered un-

TABLE I

PERFORMANCE OF GLC SYSTEM 2

Glass column (1 m × 4 mm O.D.) packed with 10% SP-2100 on Supelcoport (80—100 mesh); column temperature, 275° C.

Drug	Retention time (min)	Symmetry factor (limit 0.95—1.05)	Resolution between marker (> 1.0)
Phenoperidine	6.0	1.03	—
Propranolol	1.2	1.00	2.8
Practolol	2.1	0.98	2.7
Metoprolol	0.8	1.01	3.1

TABLE II

PERFORMANCE OF GLC SYSTEM 1

Glass column (1 m × 4 mm O.D.) packed with 3% OV-1 on Supelcoport (80—100 mesh); column temperature, 235° C.

Drug	Retention time (min)	Symmetry factor (limit 0.95—1.05)	Resolution between marker (> 1.0)
Phenoperidine	3.0	1.00	3.8
R3757 (internal standard)	5.3	0.98	—

suitable as internal standards because their retention times in the systems investigated were much shorter than that of phenoperidine (Table I).

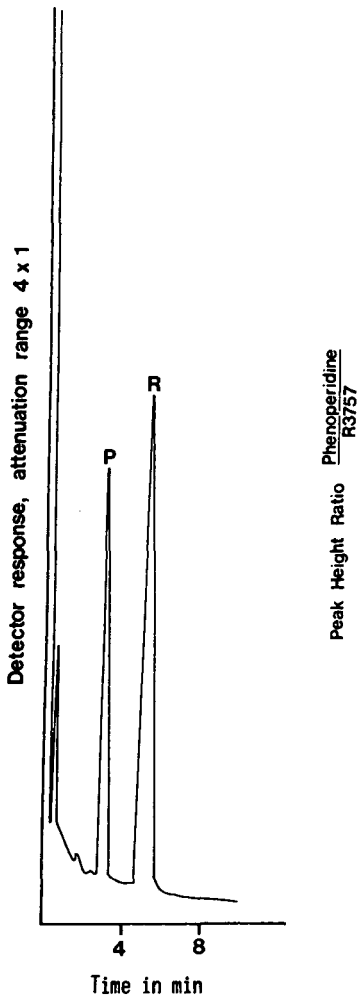
Recovery, selectivity, reproducibility and storage

The GLC System 1 (3% OV-1 on Supelcoport, 80—100 mesh) was chosen for routine analysis because phenoperidine had a shorter retention time at a lower operating column temperature (Tables I and II).

The relative recovery of phenoperidine from human plasma was 88.8 ± 2.7%. During the course of these studies, it was established that substances in samples obtained from patients on a variety of drugs, particularly morphine, diamorphine, pentazocine, pethidine and methadone did not interfere in the analysis of phenoperidine.

Repeated assays of the same plasma samples containing phenoperidine indicated that the reproducibility of the peak height ratio of phenoperidine to internal standard was 100 ± 1.42%. Calibration graphs for phenoperidine were linear over the range of 2—80 ng/ml (Fig. 2). The graphs were found to be reproducible when repeated ten times during the studies.

Samples of plasma whether fresh or stored at −20° C for seven days did not give peaks that would interfere with the measurement of peaks corresponding to phenoperidine and the internal standard in the chromatogram (Fig. 1). There was no appreciable loss of the drugs from the samples after storing at −20° C for seven days.



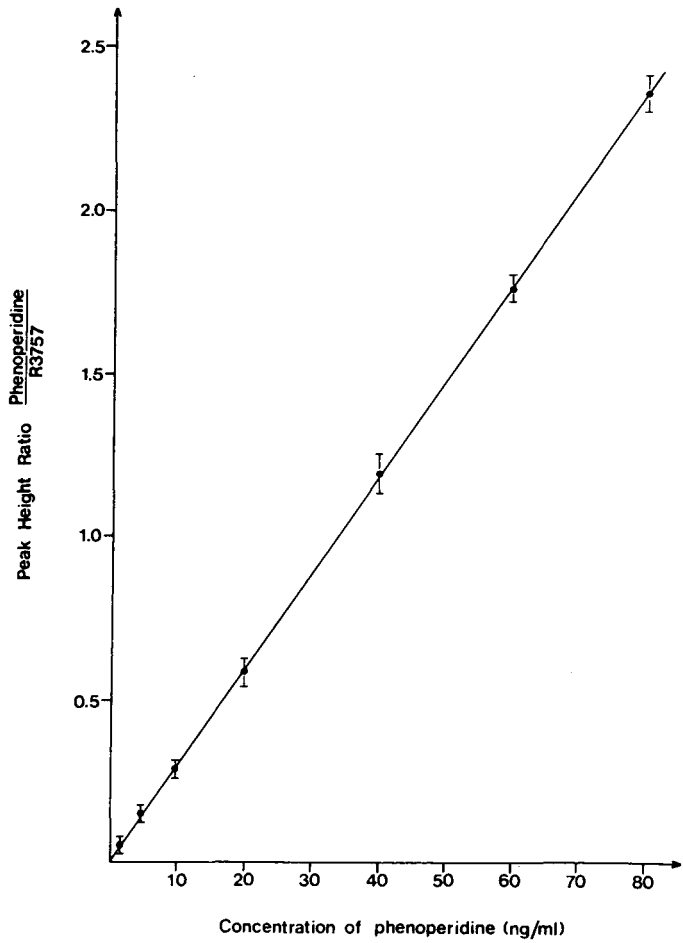


Fig. 1. Chromatogram of phenoperidine (P) 25 ng per ml and internal standard, R3757 (R) 50 ng/ml after extraction from plasma, using GLC System 1 (3% OV-1) with column temperature at 235° C.

Fig. 2. Typical calibration graph of phenoperidine in plasma.

APPLICATION

This procedure has been used in preliminary studies on the plasma concentration of phenoperidine in volunteer subjects. These studies were carried out during the administration of repeated doses of ammonium chloride since previous investigations have shown that the disposition of pethidine is influenced by urinary pH [5]. After overnight fasting, subjects were given a bolus dose of phenoperidine (15 μg/kg, intravenous) and blood samples (approximately 8 ml) were removed at frequent intervals from an intravenous cannula. The concentration of phenoperidine in plasma was measured by the General procedure.

An example of the plasma profile of a male volunteer subject (age 26 years,

weight 65 kg) obtained in this preliminary study is shown in Fig. 3. There is a rapid decline in the plasma concentration of phenoperidine followed by a secondary rise between 30 and 40 min. The concentration of the drug then declined more slowly and phenoperidine was still detected after 120 min. A separate study has shown that the secondary rise was largely abolished by concurrent treatment with antacid (Andursil, 10 ml at 10-min intervals for at least 1 h during the course of the study). It is probable that the secondary peak may be due to the elimination of the drug into the acidic gastric fluid, followed by its subsequent re-absorption in the small intestine.

A more detailed report of this work is in preparation.

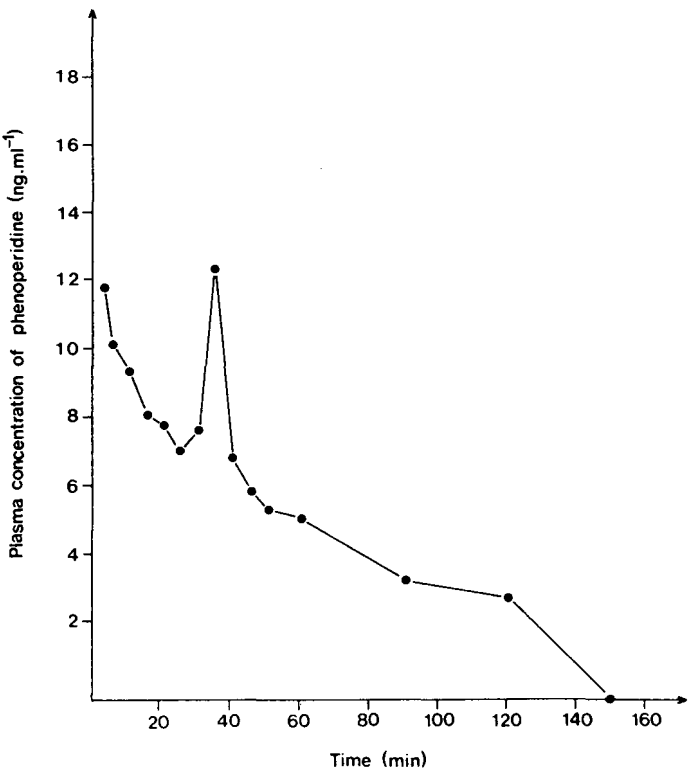


Fig. 3. Profile of plasma concentration of phenoperidine with time after an intravenous dose of 15 μg/kg to a male subject.

ACKNOWLEDGEMENT

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Note

Trace determination of almitrine in plasma by gas—liquid chromatography using a nitrogen—phosphorus detector

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The response of the nitrogen—phosphorus (N—P) detector is roughly proportional to the number of nitrogens contained in the compound, but the response is also highly dependent on the type of nitrogen group the compound contains. In general, those compounds having structures that would be expected to be favorable for the formulation of cyan radicals following pyrolysis on the surface of the rubidium bead would be expected to give the greatest response. The principle of operation of the N—P detector has been discussed [1]. This type of detector has found increasing application in the analysis of pharmaceutical compounds and has been used to determine low concentrations of drugs. Review articles have been presented for drugs applications [2, 3].

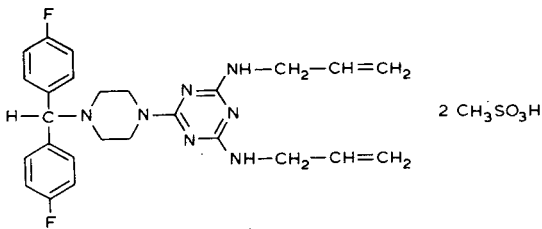
This paper describes a method for the determination of almitrine in plasma and erythrocytes. This compound exhibits agonist peripheral chemoreceptor properties through peripheral chemoreceptor stimulation; its formula is given in Fig. 1. Almitrine contains seven nitrogen atoms and gives a very good response in a N—P detector. Preliminary studies carried out on almitrine using a flame ionisation detector gave a limit of detection (100—200 ng/ml of plasma) that was insufficiently sensitive to follow the kinetic profile of the drug in blood or plasma.

MATERIALS AND METHODS

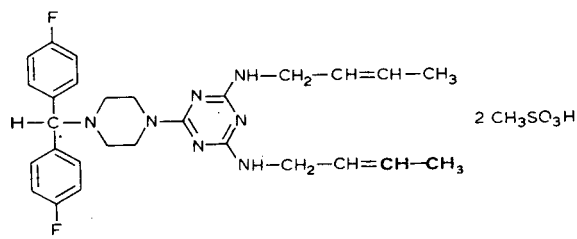
Reagents

Almitrine bis mesylate or bis-(allylamino)-4,6-{[bis(fluoro-4-phenyl)methyl]-4-piperazinyl}-2-triazine bis mesylate and 2082 (internal standard) were supplied by Science Union et Compagnie, Suresnes, France (Fig. 1).

Acetone, cyclohexane, 1 *N* sodium hydroxide and methanol were supplied by Merck (Darmstadt, G.F.R.); pro analysis quality was used.



Almitrine bis mesylate



2082 (Internal standard)

Fig. 1. The structure of almitrine bis mesylate and its internal standard (2082).

Apparatus

A Perkin-Elmer Sigma series gas chromatograph equipped with a N–P detector using a rubidium glass bead, or a Girdel gas chromatograph equipped with a N—P detector using rubidium salt were utilised. A glass column (0.5 m × 2 mm I.D.) previously washed with methanol and acetone and silanized by dimethylchlorosilane in toluene was subsequently packed with Chromosorb W HP (80—100 mesh), coated with 5% OV-1 (methyl silicone from Applied Science Labs., State College, PA, U.S.A.). The column was initially conditioned by the following temperature programme: 1 h at 100°C with nitrogen flow-rate of 20 ml/min, then from 100°C to 280°C at 2°/min with nitrogen flow-rate then for 4 h at 280°C without nitrogen flow, and finally at 280°C overnight with a nitrogen flow-rate of 5 ml/min. The final chromatographic conditions were nitrogen flow-rate 40 ml/min, oven temperature 270°C and injection port and detector temperatures 300°C.

Taking of blood samples

Blood (5—12 ml) was collected in heparinized glass tubes and was centrifuged for 8 min at 1000 *g* immediately after collection. The plasma layer was quickly removed and transferred to a separate tube using a Pasteur pipette. The

erythrocyte layer was further centrifuged for 5 min at 1000 *g* and the remaining supernatant liquid was discarded. The plasma and erythrocyte samples were stored at −20°C prior to analysis.

Extraction procedure for plasma

A 1-ml volume of plasma was placed in a 20-ml tapered glass tube and 7 ml of cyclohexane were added. The tube was shaken vigorously for 15 min on a horizontal shaker and centrifuged for 5 min at 3000 *g*. The cyclohexane phase was transferred to a separate tapered glass tube and the aqueous phase was extracted a further two times by cyclohexane under the same conditions. The combined cyclohexane phases were evaporated in a water-bath at 50°C under a gentle stream of nitrogen to a volume of 5—7 ml. To the concentrated cyclohexane phase were added 2 ml of 1 *N* sodium hydroxide. The tube was shaken horizontally for 15 min and centrifuged for 5 min at 3000 *g*. The cyclohexane phase was transferred to a tapered glass tube and 100 µl of internal standard solution (2 µg/ml in acetone) were added. The tube was shaken for 1 min on a vortex mixer. The solvents were evaporated under a gentle stream of nitrogen to dryness in a water-bath at 50°C. The residue was dissolved in 100 µl of acetone and 1 µl or 2 µl were injected into the gas chromatograph. Retention times of about 2 min and 4 min for almitrine and its internal standard, respectively, were obtained. Fig. 2 shows the chromatographic response of plasma containing almitrine and internal standard and of a plasma blank.

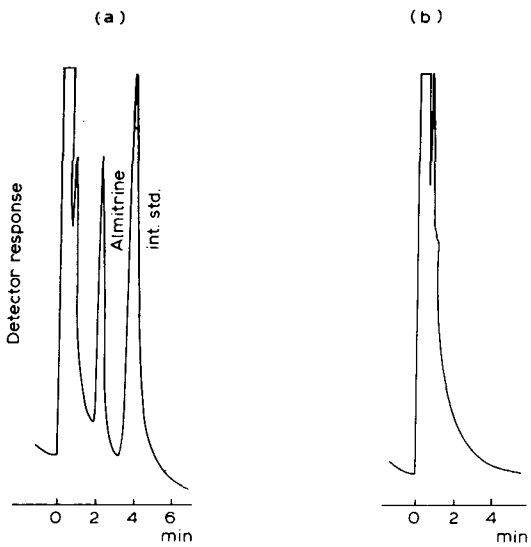


Fig. 2. Chromatograms of (a) plasma, containing almitrine and internal standard, and (b) plasma blank.

Determination of almitrine in erythrocytes or in whole blood

One gram of erythrocytes or whole blood was poured into a tapered glass tube. Redistilled water (1 ml) was added to haemolyse the red cells. The tube was shaken for 1 min on a vortex mixer and extracted by the method described for plasma.

Calibration curve

A standard curve for almitrine was obtained by adding 25, 50, 75, 100 and 150 ng of almitrine in acetone (1 μg/ml) and 100 μl of internal standard solution (2 μg/ml in acetone) to 1-ml samples of drug-free plasma. To reproduce protein binding as closely as possible, the plasma samples thus spiked with almitrine were kept overnight before extraction. The samples were extracted according to the method described above.

The ratio of the peak areas of almitrine/internal standard was used to construct a calibration curve. The coefficient of correlation was calculated and the curve was used for the quantitation of almitrine in plasma and erythrocytes samples. Table I shows the results obtained for the calibration curve.

Identification of almitrine

The structure of the compound analysed by gas—liquid chromatography (GLC) was confirmed as almitrine by coupling the GLC system to a mass spectrometer (Ribermag R 10.10 system). This demonstrated a molecular peak at m/e = 477 and the characteristic moieties: $C_{26}H_{29}F_2N_7$ (m/e = 477), $C_{13}H_{20}N_7$ (m/e = 274), $C_{10}H_{16}N_7$ (m/e = 234), $C_{10}H_{15}N_6$ (m/e = 219), and $C_{13}H_9F_2$ (m/e = 203).

To be sure that we were only measuring the unchanged drug without its metabolites, two complementary methods were used: first, by measuring the different m/e ratio characteristics for almitrine and for the analysed peak, and

TABLE I

CALIBRATION CURVE OF ALMITRINE-SPIKED PLASMA

	Concentration of almitrine (ng/ml)					
	0 (plasma blank)	25	50	75	100	150
Peak area ratio almitrine/internal standard	0.04, 0.06	0.26, 0.25	0.39, 0.39	0.60, 0.56	0.72, 0.73	1.05, 1.07
Mean of the two values	0.05	0.255	0.39	0.58	0.725	1.06

TABLE II

COMPARISON OF PEAK AREA RATIO ALMITRINE/INTERNAL STANDARD RESULTS FROM MASS FRAGMENTOGRAPHY AND GLC WITH THE N—P DETECTOR

Sample	Mass fragmentography	GLC + nitrogen detector
1	1.77	1.74
2	2.06	1.98
3	2.78	2.56
Correlation coefficient	0.996	0.997

secondly by using the Biemann method which demonstrates the homogeneity of the peak [4]. The results obtained confirm that this assay is specific for almitrine.

In addition, we analysed a calibration curve and plasma samples from three patients using both fragmentography and gas chromatography with N—P detector and we found the results to be in agreement (Table II).

RESULTS AND DISCUSSION

Limit of sensitivity

If the limit of sensitivity is defined as that signal which is three times higher than the background signal, the method described can be used to determine plasma containing about 1 ng of almitrine per ml.

Reproducibility

To determine the reproducibility of the procedure, six 1-ml aliquots of plasma from the same subject (containing about 100 ng of almitrine per ml) were extracted and analysed as described above. The ratio of the peak areas for almitrine and the internal standard was calculated giving a result of 100 ± 2.5 ng/ml (mean ± standard deviation). The reproducibility was further determined under the same conditions using plasma containing about 20 ng of almitrine per ml; the result was found to be 20 ± 1.7 ng/ml (mean ± standard deviation).

Comment: Under conditions of the quantitative extraction of almitrine (pH 3—12) from aqueous solutions, the internal standard is not sufficiently extracted. More-reproducible results were obtained using the method described than by adding internal standard at the first stage of the extraction.

Difficulties in extracting almitrine from plasma

Almitrine is strongly bound to plasma proteins. In vitro assays have shown that this binding increases with time and reaches a maximum after several hours. For this reason and to reproduce as closely as possible in vivo protein binding, plasma samples were kept overnight after spiking and before the extraction procedure. The binding is so strong that other solvents used for liquid extraction do not effectively break the binding, and thus cannot be used with efficiency for this drug. Cyclohexane was chosen as the extraction solvent since it is capable of easily breaking the protein bond. With cyclohexane, the extraction from in vivo samples is five times higher than with chloroform and three times higher than with diethyl ether.

Patient profiles

The method described has been used to determine plasma and whole blood concentrations of almitrine in patients treated for chronic obstructive lung disease. The study was carried out after oral administration of the drug. Fig. 3 shows the results obtained on seven subjects dosed at 3 mg/kg. The plasma concentration found was generally three times higher than the whole blood concentration. This observation becomes important for those patients with higher than normal packed cell volume levels. In order to obtain sufficient plasma from these patients for analysis, it is necessary to collect 10—12 ml of blood to obtain 2 ml of plasma.

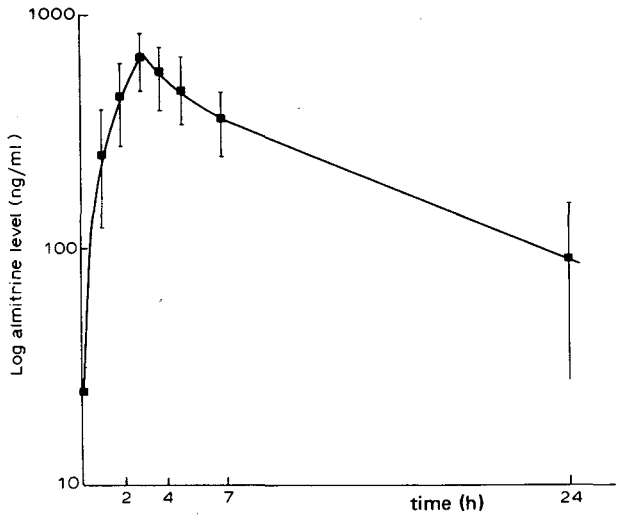


Fig. 3. Mean almitrine profile of seven subjects dosed orally at 3 mg/kg, expressed as mean plasma curve ± S.D.

CONCLUSION

A N—P detector has been used successfully for the determination of almitrine in plasma and erythrocytes at the ng/ml level. The sensitivity of the method described will allow us to follow the plasma kinetics in hospitalised patients with respiratory insufficiency treated by almitrine where plasma concentrations may range from 10 to 500 ng/ml.

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Note

Rapid and simple method for the measurement of methotrexate and 7-hydroxymethotrexate in serum by high-performance liquid chromatography

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Methotrexate (MTX, amethopterin, 4-amino-N^{10}-methylpteroylglutamic acid), a folate antagonist which acts by inhibition of dihydrofolate reductase, is used as an anti-neoplastic agent alone or in combination with other anti-neoplastic drugs. Toxic reactions have become more likely with the recent introduction of high dose MTX therapy [1], one study [2] quoting a 6% mortality rate using this regimen. Many of the side-effects are due to delayed excretion of MTX and adverse reactions depend upon the duration of exposure to, as well as the magnitude of, excessive MTX concentrations [3]. The monitoring of serum MTX concentrations is essential to detect those patients at risk of developing toxicity and allow appropriate action to be taken [4]. Measurement of the less soluble major metabolite 7-hydroxymethotrexate (7-OH MTX) may also be important as it has been implicated in the development of renal toxicity [5].

Methods for measuring MTX concentrations have been reviewed [6]. Techniques using high-performance liquid chromatography (HPLC) include ion-exchange [7—10] and reversed-phase chromatography after prior extraction and concentration [11, 12] or pre-column derivatisation [13]. Some of these also measure the major metabolite 7-OH MTX [8—12]. This paper

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describes a reversed-phase technique which allows direct injection of supernatant after protein precipitation.

MATERIALS AND METHODS

Equipment

An Applied Chromatography Systems reciprocating pump was used with a Cecil CE 212 variable-wavelength UV monitor fitted with an 8-μl chromatography cell. A 120 mm × 4 mm I.D. stainless-steel analytical column and a 45 mm × 4 mm I.D. pre-column were packed with the 5-μm particle size stationary phase Hypersil—octadecylsilane (Shandon, London, Great Britain), using a slurry technique. Injections were performed with a Specac loop injector, fitted with a 50-μl loop.

Reagents

Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, Great Britain). The perchloric acid (PCA) used was 2 *M* in distilled water.

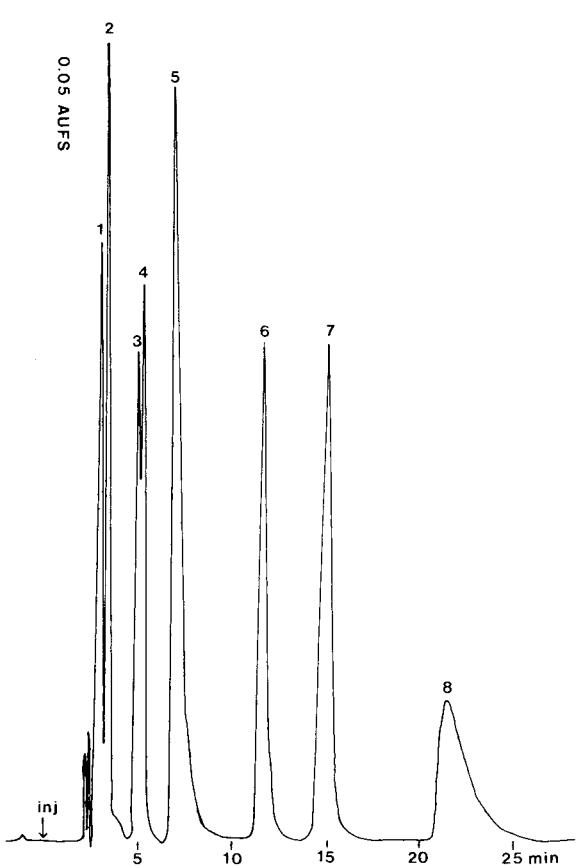
Non-formulated (USP) methotrexate (94.1% nominal purity) and analogues were obtained from Lederle Labs. (Hampshire, Great Britain). Stock standards of 1 mg/ml in 0.1 *M* sodium hydroxide were prepared (stored at 4°C and found to be stable over several months). Subsequent dilutions for working standards were prepared in either mobile phase buffer or pooled serum. 7-OH MTX (90% pure) was kindly donated by Dr. D.G. Johns (N.C.I., Bethesda, MD, U.S.A.). The internal standard (I.S.), N-[4[[(2,4-diamino-6-quinazolinyl) methylamino]benzoyl]] aspartic acid, was a gift from Dr. L.H. Kedda (N.C.I.), and was used at a concentration of 10 μg/ml in 2 *M* perchloric acid. All other chemicals were of AnalaR grade.

Procedure

Serum (500 μl) and 300 μl I.S. (10 μg/ml in 2 *M* PCA) were vortex mixed and centrifuged for approximately 5 min at 2000 rpm (approximately 1300 *g*). A 50-μl aliquot of the supernatant was injected onto the column. The eluting solvent was 20% methanol in Tris—sodium dihydrogen phosphate (both 0.1 *M*), pH 6.7, at a flow-rate of 1 ml/min, corresponding to a pressure drop of approximately 75 bars. Chromatography was performed at ambient temperature with detection at 305 nm. The ratios of peak heights of MTX and 7-OH MTX to I.S. in chromatograms from serum samples were compared with those obtained from a working standard. Results obtained with HPLC were compared with those obtained by radioimmunoassay (RIA) [14] using ^{125}I-labelled MTX [15].

RESULTS

Fig. 1 shows the separation of MTX, 7-OH MTX, I.S. and five related compounds chromatographed using this sytem. Chromatography of serum taken from a patient 4 h after a 6-h infusion of 3.3 g MTX is shown in Fig. 2. A blank sample of pooled serum showed no significant interference (Fig. 3).



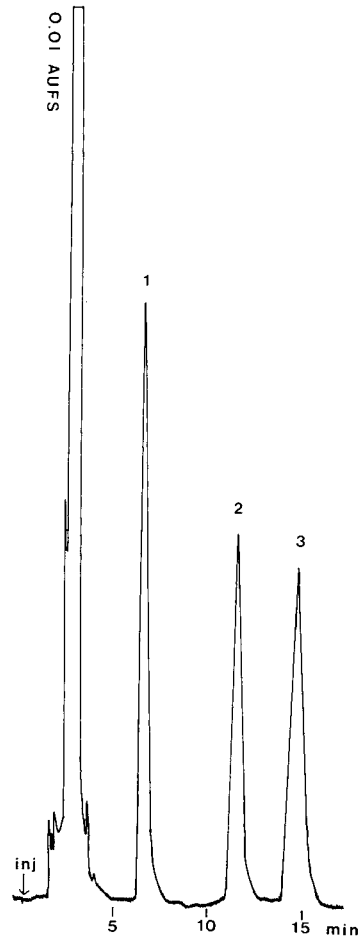


Fig. 1. Separation of (1) folinic acid, (2) folic acid, (3) aminopterin, (4) N^{10}-methylfolic acid, (5) internal standard, (6) MTX, (7) 7-OH MTX, (8) 2,4-diamino-N^{10}-methylpteroic acid. Conditions as in text.

Fig. 2. Chromatogram of sample from patient 4 h after termination of a 6-h infusion containing 3.3 g MTX. (1) Internal standard; (2) MTX; (3) 7-OH MTX.

The recoveries of MTX, I.S. and 7-OH MTX from pooled serum estimated by comparison with external standards were 82.2%, 87.5% and 98.2%, respectively. Standard dilutions of MTX and 7-OH MTX in Tris—phosphate buffer and pooled serum were assayed in duplicate over the range 0.5—20 μg/ml ($1.1 \cdot 10^{-6}$—$4.4 \cdot 10^{-5}$ *M*). The standard curves obtained were linear. MTX and 7-OH MTX were added to serum at two different levels and assayed five times during a single run, and on five different days. The within- and the between-batch variations are shown in Table I.

Table II lists those drugs that might commonly be administered with MTX.

TABLE I

PRECISION DATA

Concentration (μg/ml)		Coefficient of variation (n = 5)	
		Within-batch (%)	Between-batch (%)
MTX	1	3.4	10.3
	10	1.9	7.3
7-OH MTX	1	3.2	12.6
	10	2.9	8.6

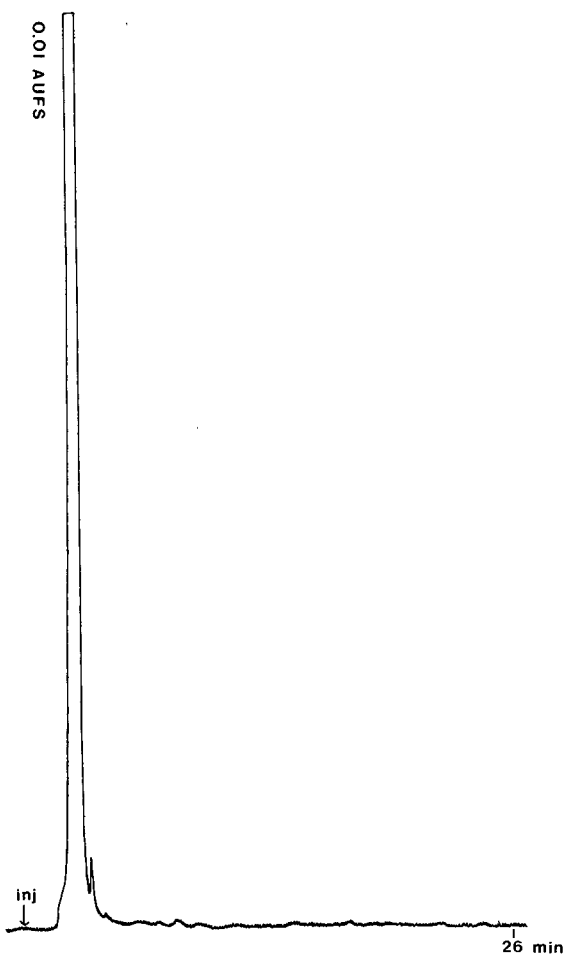


Fig. 3. Chromatogram of blank sample from patient not receiving therapy.

TABLE II

DRUGS TESTED FOR CHROMATOGRAPHIC INTERFERENCE

Caffeine	Imipramine
Salicylate	Desipramine
Cortisol	Trimipramine
Prednisone	Diazepam
Prednisolone	Nitrazepam
Dexamethasone	Chlordiazepoxide
Morphine	Phenobarbitone
Cytosine arabinoside	Hydrallazine
Vincristine	Folinic acid
Daunomycin	

Only cytosine arabinoside produced an interfering peak (k' = 4.8, retention relative to MTX = 1.12). The others were either retained indefinitely or not at all.

The lowest measurable levels of MTX and 7-OH MTX were considered to be 100 ng/ml ($2.2 \cdot 10^{-7} M$) and 200 ng/ml ($4.2 \cdot 10^{-7} M$) respectively at which concentrations the peak heights were five and three times the noise level of the system respectively.

Fifty-two samples were obtained from patients during the 48 h following high dose MTX (approximately 3 g per square metre body surface area infused in 6 h). The samples were analysed by HPLC and RIA for MTX and 32 of the samples were analysed for 7-OH MTX by HPLC. The concentrations of MTX measured by HPLC ranged from 0.1—216 μg/ml ($0.2—476 \cdot 10^{-6} M$) and correlated well with the results obtained by RIA (r = 0.99, $y = 0.77x + 0.99$, where x and y are RIA and HPLC values respectively measured in μg/ml). RIA gave overall higher values ($p < 0.01$, Wilcoxon Matched-pairs Signed-Ranks test [16]).

7-OH MTX concentrations ranged between 0.2—56 μg/ml ($0.4—119 \cdot 10^{-6} M$). Up to 2 h post infusion the 7-OH MTX concentrations were always less than the MTX concentrations but after 4 h post infusion 7-OH MTX always exceeded MTX, being up to ten times greater at 24 h post infusion. Serial samples from four patients showed that the maximum concentration of 7-OH MTX occurred 2 h post infusion and was 35—56 μg/ml.

DISCUSSION

This reversed-phase system gives good baseline resolution of MTX, 7-OH MTX and I.S.; a similar reversed-phase system using 10-μm RP-8 [13] failed to achieve this baseline separation although the authors did not illustrate the improved performance claimed for 7-μm particles. The method has a high degree of specificity since these compounds are well separated from endogenous compounds, N^{10}-methylfolic acid and aminopterin (contaminants of commercial MTX preparations and likely to be measurable after high dose infusions), folinic acid (citrovorum factor, used as antidote in high dose regimens) and the minor metabolite 2,4-diamino-N^{10}-methylpteroic acid (DAMPA).

Of nineteen drugs likely to be administered simultaneously, only cytosine arabinoside interfered, although its presence would be detected by a shoulder on the MTX peak; however when co-administered its much shorter half-life and lower dosage diminish the likelihood of significant interference.

There was a close correlation between results for MTX obtained using HPLC and RIA although the latter gave overall higher values; this was not due to a lower recovery as the use of the I.S. compensates for this but suggests that RIA measures some substance not present in the chromatography peak. Discrepancy between the RIA and competitive dihydrofolate reductase binding assay has been reported [17], and this may be due to the nominal 30% cross-reaction of the antibody with DAMPA. Further studies are in progress using HPLC to determine the effects of DAMPA on RIA.

Sample preparation is simple and rapid and gives a high recovery of MTX, 7-OH MTX and I.S.; a batch of samples can be prepared for chromatography in about 10 min and a single sample can be chromatographed in about 20 min. The sensitivity of the assay is 100 ng/ml ($2.2 \cdot 10^{-7}$ *M*) for MTX which enables toxic levels to be measured up to 48 h post infusion.

Methods using an organic solvent extraction step [8, 10--12] are considerably more time-consuming and have only a modestly increased sensitivity, their overall recovery being often less than 50%. Methods offering about a tenfold increase in sensitivity use either pre-column fluorescence derivatisation [13] or an on-line column concentration step [7, 9].

Later than 4 h post infusion, 7-OH MTX concentrations are greater than those of MTX and 24 h post infusion we have found them up to ten times higher, similar to those reported by Lankelma and Van der Klein [9]. Cohen et al. [12] state that their 7-OH MTX concentrations measured in MTX equivalents due to lack of an authentic standard were probably only about one quarter of the "true" concentrations, and if their MTX/7-OH MTX time course relationship is corrected accordingly it agrees well with our findings. Measurement of 7-OH MTX may be clinically important due to its probable effect upon renal function [5] and because it competes with MTX for entry into cells thereby decreasing the therapeutic efficiency of MTX [9].

This method would be particularly useful in a department where the workload is small but the results are required quickly. It is simple to operate and cheap to run.

ACKNOWLEDGEMENTS

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Note

Estimation of ICI 35,868 (Diprivan^{R}) in blood by high-performance liquid chromatography, following coupling with Gibbs' reagent

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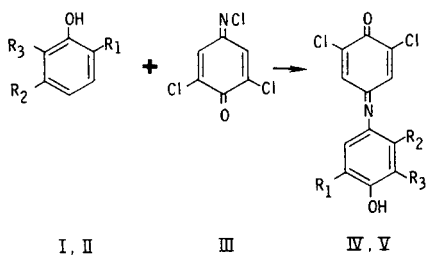
(First received August 21st, 1980; revised manuscript received October 29th, 1980)

ICI 35,868 (Diprivan^{R}*, 2,6-diisopropylphenol; I, Fig. 1) is a new intravenous anaesthetic agent. In animals [1, 2] and man [3, 4] it rapidly produces anaesthesia of short duration devoid of excitory side effects.



I, II III IV, V

	R_1	R_2	R_3
I and IV	$(CH_3)_2CH$	H	$(CH_3)_2CH$
II and V	$(CH_3)_2CH$	CH_3	H

Fig. 1. Structural formulae of ICI 35,868 (I), thymol (II), Gibbs' reagent (III) and the indophenols IV, V.

Pharmacokinetic studies in animals have shown that anaesthesia was maintained by concentrations in the range 1—4 μg/ml [5]. These studies were monitored by a fluorescence assay procedure with a limit of detection of 300 ng/ml. To define fully the pharmacokinetic parameters in man and thus allow predictions for the use of ICI 35,868 for maintenance of anaesthesia, a more sensitive assay procedure is required.

*Diprivan is a trademark, Property of Imperial Chemical Industries Ltd.

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This paper describes a procedure, based on coupling to Gibbs' reagent [6] and high-performance liquid chromatography (HPLC) of the resultant dye, which can quantify concentrations of ICI 35,868 down to 25 ng/ml. Thymol was included as internal standard to allow for variations occurring during extraction and derivatisation.

MATERIALS AND METHODS

Reagents

Cyclohexane (Spectrosol grade), isopropanol, sodium chloride, potassium dihydrogen orthophosphate and ammonia solution (35%, w/w) were all Analar grade from Hopkin and Williams (Chadwell Heath, Great Britain), Gibbs' reagent was from Koch-Light (Colnbrook, Great Britain).

Thymol and tetramethyl ammonium hydroxide (Fluka, Buchs, Switzerland) trifluoroacetic acid (Fluorochem, Glossop, Great Britain) and acetonitrile and methanol (both HPLC grade from Rathburn Chemicals, Walkerburn, Great Britain) were all used without further purification. Diethyl ether (Analar grade from Hopkin and Williams) was redistilled before use. ICI 35,868 is a liquid at room temperature and was stored in sealed glass ampoules at −20°C. For day-to-day use the contents of an ampoule were transferred to a glass vial fitted with a screw cap containing a PTFE liner. This vial was also stored at −20°C and allowed to come to room temperature before use.

Standard solutions

Stock solutions of ICI 35,868 and thymol (1 mg/ml in methanol) were diluted as necessary with methanol to ensure that a total of not more than 100 μl of methanol was added to blood to give the required concentrations of ICI 35,868 and internal standard. The stock solutions were stable for at least seven days at −20°C but were prepared fresh each day.

Sample preparation

To oxalated whole blood (1 ml) was added internal standard (as described under Quantification) and the sample vortex mixed (Rotamixer, Hook and Tucker, Croydon, Great Britain). After addition of aqueous potassium dihydrogen orthophosphate (0.1 *M*, 1 ml) and cyclohexane (5 ml) the mixture was tumbled (60 rpm, 10 min) in a rotary tumbler (TM/V100, Luckham, Burgess Hill, Great Britain). After centrifugation (700 *g*, 2 min) to separate the phases, an aliquot of the organic layer (4.5 ml) was transferred to a clean tube. To this was added, with gentle agitation, Gibbs' reagent in isopropanol (1 mg/ml, 60 μl) and tetramethylammonium hydroxide solution [1 part 24% (w/v) solution in methanol and 9 parts isopropanol, 50 μl]. The reaction was allowed to proceed at room temperature (20 min) before water (1 ml) was added and the mixture tumbled (15 min) and centrifuged (700 *g*, 3 min). The organic layer was aspirated and discarded, aqueous sodium chloride [25% (w/v), 1 ml] and diethyl ether (5 ml) were added and, after further mixing and centrifugation, an aliquot of the ethereal layer (4.8 ml) was transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in a mixture of acetonitrile—water—ammonia (80 : 20 : 0.05; 500 μl).

Towards the limit of quantification of the procedure, i.e. in the range 20—100 ng/ml, background interference can be minimised by reduction of the amount of Gibbs' reagent, to that corresponding to a two-fold molar excess of the anticipated maximum ICI 35,868 concentration.

High-performance liquid chromatography

An Altex pump (Model 110A) was used in conjunction with an automatic injection system (WISP Model 710, Waters Assoc., Milford, MA, U.S.A.) and a column (20 × 0.5 cm) packed with 5-μm Hypersil ODS (Shandon Southern Products, Runcorn, Great Britain) in the manner described by Bristow et al. [7]. The eluting solvent was acetonitrile—water—trifluoroacetic acid (80 : 20 : 0.1) with a flow-rate of 1.5 ml/min. The ultraviolet detector (Spectromonitor III, Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used at 276 nm.

Quantification

Calibration standards were prepared in control whole blood by addition of known amounts of ICI 35,868 and an amount of thymol corresponding to the median concentration of the calibration series under examination. The standards were treated in an identical manner to the unknown samples. The ratios of the peak heights derived from ICI 35,868 and thymol in the standards were used to construct calibration curves from which unknowns were quantified.

RESULTS AND DISCUSSION

ICI 35,868 absorbs in the ultraviolet (λ_{max} 275 nm) but its molar absorptivity is insufficient to allow quantification at the levels occurring in biological fluids after therapeutic doses. The compound also possesses a natural fluorescence, which formed the basis of the procedure previously described for measurement in biological samples [5]. However, the limit of quantification of this method was 300 ng/ml. To allow a full pharmacokinetic evaluation in man it was anticipated that a more sensitive analytical procedure would be required.

Since ICI 35,868 (I) has no *para* substituent, it reacts with Gibbs' reagent (2,6-dichloroquinone-4-chloroimide, III) in the presence of base to yield an intensely blue "indophenol" [2,6-dichloroquinone-4-(2,6-diisopropyl-1-hydroxyphenol)-imide, IV]. The leuco form of this derivative has greatly enhanced UV molar absorptivity compared to the parent compound and when quantified by UV detection after HPLC separation, permits estimation of ICI 35,868 in biological fluids down to 25 ng/ml, which is adequate for monitoring human pharmacokinetic profiles. Thymol (2-isopropyl-5-methylphenol, II) has a similar structure to ICI 35,868 and forms the corresponding "indophenol" (V). Compounds IV and V were stable for at least a week when stored in acetonitrile—water—ammonia.

When these blue dyes are injected under the acidic HPLC conditions they are converted instantly to the colourless leuco forms which have a high absorbance at 276 nm. These reduced forms are unstable under acid conditions, releasing the free phenols. However, the half-life of this decomposition was 30—40 min for both compounds and, since the HPLC column transit time was less than 6 min and the flow-rate was kept constant, this was found not to affect the re-

producibility of the procedure. HPLC chromatograms from blood extracts are shown in Fig. 2.

The results of nine calibration series in the range 25—1000 ng/ml were treated by linear regression analysis, and the y-axis intercept (c) and the S.E. calculated. A value of c plus twice its S.E. was taken as the limit of detection for each series. The mean of these values was found to be 25 ± 3 ng/ml (S.E.M., n = 9). This corresponded to the lowest concentration used routinely in calibration series and confirmed that 25 ng/ml was a reasonable lower limit for the estimation of ICI 35,868.

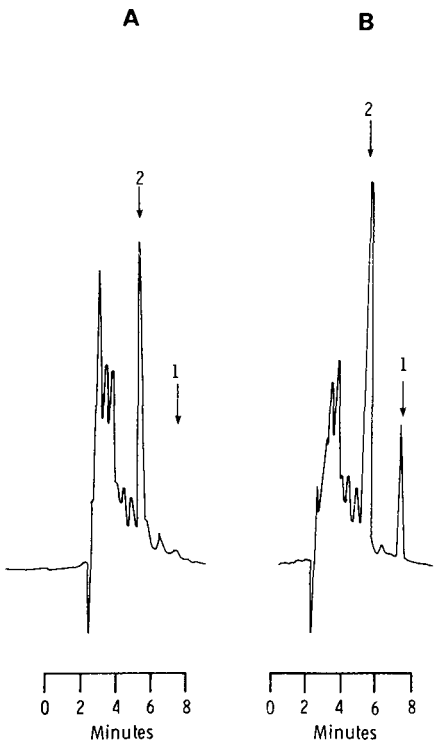

Fig. 2. Typical chromatograms of (A) control blood to which internal standard (100 ng/ml) has been added and (B) control blood to which has been added ICI 35,868 (40 ng/ml) and internal standard (100 ng/ml). The arrows 1 and 2 indicate the retention times of ICI 35,868 and internal standard respectively.

The present HPLC procedure was compared with the fluorimetric procedure in (a) a series of standards prepared by the addition of known amounts of ICI 35,868 to control blood and (b) in samples from animals which had received ICI 35,868. For (a) the concentration range was 0.6—7 μg/ml, for (b) 0.07—2.0 μg/ml. The results of these parallel determinations are presented in Fig. 3 with the exception of those which were less than 0.30 μg/ml which could not be quantified by the fluorescence assay. The regression line $Y = 0.97X + 0.10$ (r^2 = 0.96) obtained from the data is also presented. This figure shows that, within the range where both assay procedures are applicable, they produce compa-

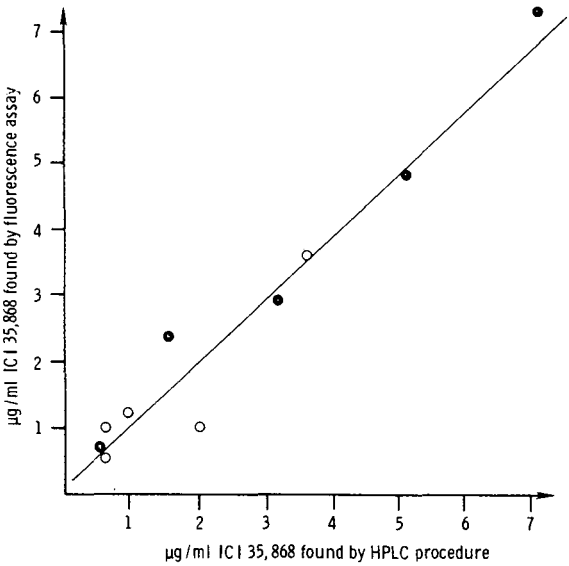

Fig. 3. Comparison of assay results by fluorimetric and HPLC procedures on known samples (0.6, 1.5, 3.0, 5.0 and 7.0 µg/ml, •) and unknown samples (○).

rable results. The accuracy of the HPLC procedure was found to be better in that, for the samples whose ICI 35,868 content was known (series a), the mean percentage found or added was 100 ± 4 (S.E.M., n = 5). For the fluorescence assay the corresponding value was 114 ± 12%.

Within-batch variation of the HPLC procedure was examined in greater detail by quintuplicate analyses of samples to which known amounts of ICI 35,868 had been added. The results obtained are shown in Table I. These show that the HPLC procedure gives an accurate assessment of the ICI 35,868 concentration with an overall mean result of 106 ± 2% (S.E.M., n = 20) of the added amount.

The between-batch variation was assessed by analysis of the same samples on four separate occasions. The mean coefficient of variation was 4.4 ± 0.8% (S.E.M., n = 4), showing excellent agreement between results obtained on different days.

This procedure was used to assess the stability of ICI 35,868 in blood samples stored under various conditions. No decrease in drug concentration was

TABLE I

DETERMINATION OF ICI 35,868 ADDED TO CONTROL BLOOD; WITHIN-BATCH VARIATION

Added (µg/ml)	Found* (µg/ml)	Mean recovery (%)
0.2	0.21 ± 0.01	104
1.0	1.11 ± 0.04	111
4.0	4.32 ± 0.42	108
8.0	7.87 ± 0.32	98

*Each value is mean ± S.E.M. (n = 5).

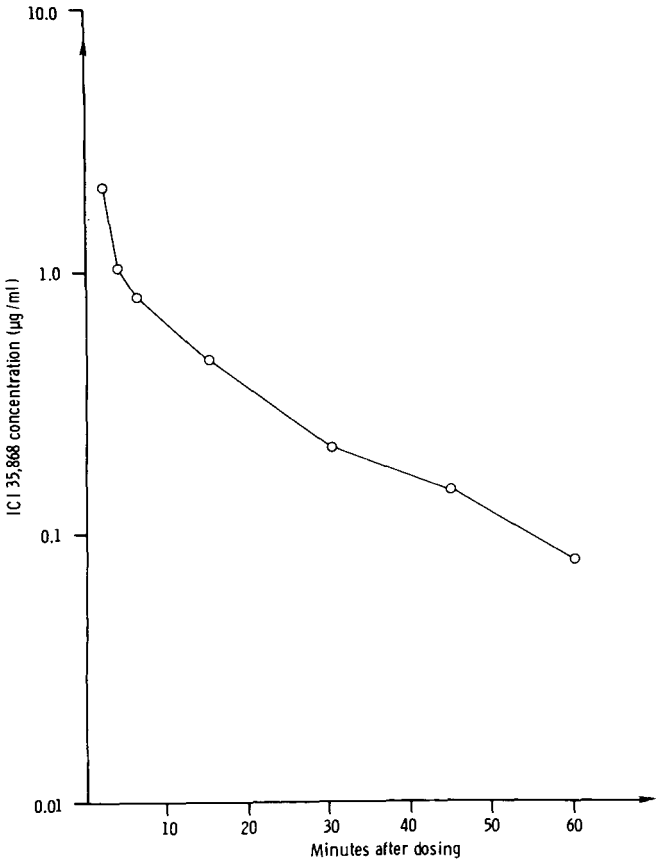

Fig. 4. Concentrations of ICI 35,868 in the blood of a dog after an intravenous dose of 5 mg/kg.

found in samples stored one week at room temperature in the light, one week at 4°C or nineteen weeks at −20°C in the dark. Since diazepam is frequently given as premedication for administration of intravenous anaesthetics, we have subjected to this procedure blood spiked with up to 400 ng diazepam per ml. There was no interference in the measurement of ICI 35,868.

Fig. 4 shows the profile of ICI 35,868 concentrations obtained in a dog after a single intravenous dose of 5 mg ICI 35,868 per kg.

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Note

Determination of alinidine in human plasma by high-performance liquid chromatography

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The N-allyl derivative of clonidine (alinidine, ST 567) produces substantial bradycardia in several species [1, 2] and in man [3, 4]. This bradycardic effect is not mediated by alpha- and beta-adrenoreceptors or by muscarinic receptors [3, 5]. Unlike beta-adrenoreceptor blocking agents and calcium antagonists this bradycardia is not accompanied by a depressant effect on the AV-nodal conduction [3]. A substance of such a pharmacological profile may be of clinical interest. Preliminary studies in patients with coronary heart disease indicate that the use of alinidine may represent a new kind of antianginal therapy [4].

We describe here an assay for alinidine using high-performance liquid chromatography (HPLC). The assay permits studies of the pharmacokinetics of this agent in man.

EXPERIMENTAL

Reagents and materials

All chemicals and solvents were analytical grade quality (E. Merck, Darmstadt, G.F.R.) and were used without further purification. Alinidine · HBr, [^{14}C] alinidine · HBr (specific activity 36 μCi/mg), and 2-[N-(thienyl-2-methyl)-N-(2,6-dibromophenyl)-amino]-imidazoline · HCl (STH 2199-Cl) which is shown in Fig. 1 were generously supplied by C.H. Boehringer Sohn (Ingelheim, G.F.R.). Aqueous solutions were prepared in double distilled water. All glassware used during the sample preparation was silanized with dichloromethylsilane, rinsed with toluene—methanol and several times with methanol and

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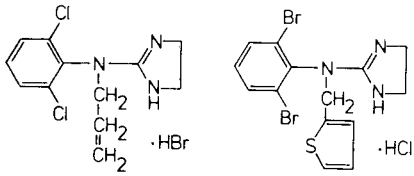

Alinidine
ST 567

STH 2199

Fig. 1. Chemical structures of alinidine · HBr and STH 2199-Cl.

double distilled water. The PTFE linings of the screw-capped culture tubes were ultrasonicated in methanol and double distilled water and rinsed extensively.

Sample preparation

The plasma samples (1 ml) were transferred to PTFE-lined screw-capped culture tubes containing 5 μl of internal standard STH 2199-Cl (about 300 ng). Fifty microlitres of 5 *N* sodium hydroxide and 5 ml of diethyl ether were added. The samples were extracted for 25 min on a rotary mixer at 25 rpm and centrifuged at 1000 *g* for 10 min. The ether phase was separated from the aqueous phase and extracted twice with 2 ml of 0.01 *N* hydrochloric acid under the same conditions as before. The combined aqueous phases were alkalinized with 50 μl of 5 *N* sodium hydroxide and extracted with chloroform under the same conditions. The aqueous layer was aspirated. The chloroform phase was transferred to a conical glass tube and evaporated under a gentle nitrogen stream. The walls of each tube were rinsed with 200 μl chloroform and evaporated again. The procedure was repeated with 100 μl chloroform. The residues were dissolved in 25 μl mobile phase, agitated on a Vortex mixer for 60 sec and centrifuged briefly. A 20-μl aliquot of the extract was injected into the chromatograph.

Chromatography

A Waters Model 6000A high-pressure solvent delivery system was used equipped with a Model U6K injector and a Model 450 variable-wavelength detector fitted with a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm) (Waters Assoc., Milford, MA, U.S.A.). The absorbance was measured at 210 nm. The mobile phase was 50 m*M* sodium phosphate in methanol—water (50:50) adjusted to pH 5.0 with hydrochloric acid and filtered through an 0.45-μm filter before use. The detector output was recorded at 10 mV at a chart speed of 5 mm/min. All chromatography was performed at ambient temperature.

Calibration

Calibration curves were constructed by adding known amounts of alinidine · HBr (10, 20, 30, 50, 75, 100, 125 and 150 ng/ml) to pooled human plasma. The peak height ratios of alinidine · HBr to the internal standard were plotted against the concentration of alinidine · HBr. The least squares regression line was fitted through the data points. The alinidine concentrations of the un-

known samples were determined by using the regression equation of the calibration curve which was assayed concurrently with the unknown samples. Plasma samples containing concentrations higher than 150 ng/ml were adequately diluted with blank plasma.

Application of the method

A healthy volunteer received 80 mg alinidine · HBr intravenously and as a tablet. The crossover studies were carried out four weeks apart. The subject had taken no drugs for at least two days before the study, refrained from smoking on the days of the study, and had fasted overnight. Blood samples were taken frequently after dosing from an antecubital vein using silanized plastic syringes and heparin (5 U/ml blood) as anticoagulant. The plasma was separated by centrifugation and stored at −20°C until assayed.

RESULTS AND DISCUSSION

The resolution of the chromatographic system was checked daily by injection of 20 µl of a mixture containing alinidine · HBr and the internal standard. Alinidine and STH 2199 showed retention times of 4.3 and 5.9 min, respectively (Fig. 2). Using the extraction method described above endogenous plasma components did not interfere with alinidine or the internal standard (Fig. 2). The detection limit for alinidine (using a signal-to-noise ratio of 4) was about 5 ng/ml.

The linearity of the detector response was assessed by injecting 20 µl of alinidine solutions with concentrations ranging from 10—500 ng/ml. The absolute peak height of alinidine was plotted against the concentration. The relationship was linear and passed through the origin. Calibration curves were obtained by plotting the peak height ratios of alinidine and STH 2199 versus the alinidine concentrations. In the investigated concentration range (10—150 ng/ml) the regression line was linear (r = 0.995) with an intercept on the y-axis close to the origin (0.0064).

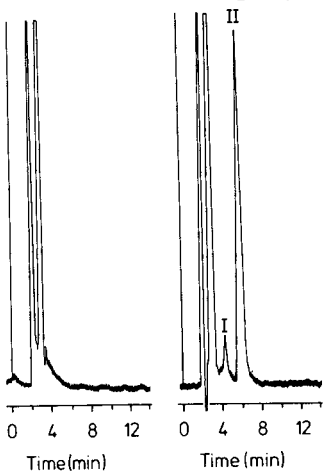

Fig. 2. Chromatograms of control plasma (left panel) and plasma containing 10 ng/ml of alinidine · HBr (I) and 295 ng/ml of STH 2199-Cl (II) (right panel).

The precision of the method was evaluated in a blind study in the concentration range of 10—500 ng alinidine per ml. The experimentally determined concentrations agreed sufficiently with the theoretical concentrations (Table I). The day-to-day variation of the assay stayed in an acceptable range. The slope of the standard curve showed a coefficient of variation of 7.4% (n = 10) within a time period of six weeks.

The recovery of the extraction procedure as described above was estimated by using [^{14}C] alinidine. The recovery was 77.2 ± 5.9% (n = 24) in the concentration range 10—400 ng/ml.

TABLE I

PRECISION OF THE ASSAY

Theoretical alinidine ◦ HBr concentrations (ng/ml)	Experimentally determined alinidine • HBr concentrations* (ng/ml)	n	Accuracy** (%)
6.06	6.96 ± 2.92	6	14.8
94.29	89.17 ± 4.16	6	5.4
258.80	254.81 ± 22.94	12	1.5
553.13	530.41 ± 12.45	7	4.1

*Mean ± S.D.

**Calculated according to ref. 6.

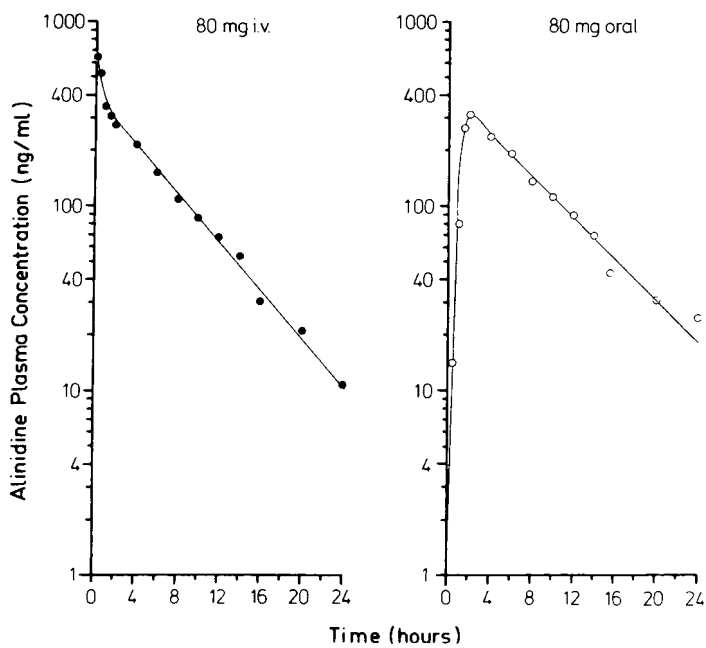

Fig. 3. Semi-logarithmic plot of plasma concentrations after an intravenous (•) and an oral (○) dose of 80 mg alinidine.

The application of the assay is demonstrated for one subject who received 80 mg alinidine intravenously and orally. Fig. 3 shows the plasma concentration—time curves for the intravenous and the oral administration of alinidine. In this particular subject the biological half-life of alinidine was 4.6 h for the intravenous dose and 5.8 h for the oral dose. The plasma clearance was 484 ml/min and the volume of distribution was 3.87 l/kg. A nearly complete bioavailability (94%) was calculated. Studies in man using radiolabeled alinidine gave a bioavailability of 100% and a mean biological half-life of 3.5 h [7]. Preliminary results from subjects who received 40 mg of alinidine gave pharmacokinetic parameters in a similar range. The data shown in this paper demonstrate that the HPLC method can be applied to pharmacokinetic studies of this new compound.

ACKNOWLEDGEMENTS

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Note

Determination of osalmid in plasma by high-performance liquid chromatography

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(First received September 30th, 1980; revised manuscript received November 18th, 1980)

Osalmid, 4'-hydroxysalicylanilide, [1] is currently used clinically as a choleretic drug [2]. In the course of investigations on its absorption, distribution, metabolism, and excretion, a rapid and sensitive method of assay was required. High-performance liquid chromatography (HPLC) was found to meet the requirement.

The present paper describes the HPLC determination of osalmid in the plasma. The dog and human plasmas were assayed by the same procedure. The method can be applied to other body fluids.

EXPERIMENTAL

Materials

Osalmid was the product of Yoshitomi Pharmaceutical Ind., Fukuoka, Japan. 2,7-Dihydroxynaphthalene was the product of Wako, Osaka, Japan. Isopropyl ether was distilled immediately before use in the extraction procedure. All solvents and chemicals were of reagent grade.

Instrumentation

An Hitachi Model 638 liquid chromatograph equipped with a universal in-

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jector and an Hitachi variable-wavelength ultraviolet effluent monitor operated at 300 nm was used. The column was a LiChrosorb Si-100 (Merck, Darmstadt, G.F.R.; particle size, 5 μm; 150 × 4 mm I.D.). The temperature of the column was maintained at 20°C. The flow-rate of the mobile phase was 0.8 ml/min.

Extraction procedure

Plasma (2.0 ml), water (1.0 ml), and phosphate buffer (pH 7.0; 15.0 ml) were mixed. Isopropyl ether containing 5% isoamyl alcohol (25.0 ml) was added to the mixture and the sample was extracted for 10 min with shaking. The organic layer (20.0 ml) was separated by centrifugation (ca. 1000 *g*, 10 min) and shaken for 5 min with 0.4 *M* NaOH (5.0 ml). After most part of the organic layer was discarded by aspiration, the residual solution was transferred to a glass-stoppered test tube. The organic layer was completely removed by centrifugation (ca. 1000 *g*, 5 min). To the aqueous layer (4.5 ml) were added 15% HCl (1.0 ml) and 10% isoamyl alcohol—*n*-butyl acetate (0.1 ml) containing 2,7-dihydroxynaphthalene (15 μg/ml) as a reference standard. The mixture was shaken for 5 min and then allowed to stand for 5 min in a water-bath (19°C). After most part of the aqueous layer was removed by centrifugation (ca. 1000 *g*, 5 min), the mixture was again allowed to stand for 5 min at 19°C. After the complete removal of the aqueous layer by centrifugation (ca. 1000 *g*, 2 min), a 10-μl volume of the organic layer was injected into the chromatograph.

For the preparation of the calibration curve, a mixture of drug-free plasma (2.0 ml), aqueous solution of osalmid (1.0 ml; 300—900 ng/ml), and phosphate buffer (15.0 ml) was carried through the procedure described above.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of an extract of drug-free plasma and that of plasma containing osalmid (581 ng per 2 ml of plasma). The mobile phase was dichloromethane—*n*-hexane—methanol—5% acetic acid (66 : 30 : 4 : 0.35, v/v). The retention times were 5.4 min for osalmid and 6.9 min for the reference standard. The solvent system was found most suitable for the present purpose.

Increasing the concentration of acetic acid in the mobile phase made the retention times longer. When the proportion of 5% acetic acid was 0.30, the peaks of osalmid and the reference standard partially overlapped. Without the added acid, the osalmid peak was extremely broad because of its adsorption on the column.

With a solvent of a higher dichloromethane—*n*-hexane ratio, both peaks became closer, and with a solvent of lower dichloromethane concentration the peaks shifted to longer retention times. Increased methanol concentration in the solvent caused the peaks to overlap.

Using the most suitable solvent system described above, the ratio of the peak height of osalmid to that of the reference standard was plotted against the amounts of osalmid added to the standard. The calibration curves thus obtained passed through the origin and were linear up to at least 900 ng.

When the reference standard was added to plasma sample just before the

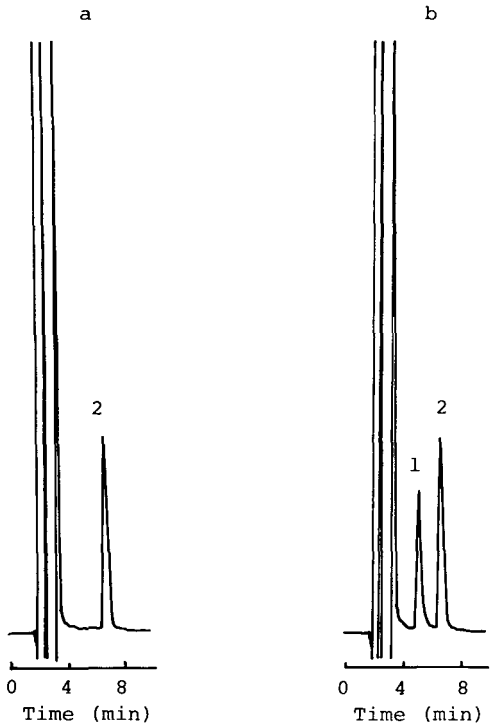

Fig. 1. Chromatograms of (a) an extract of drug-free plasma of a dog and (b) that of plasma containing osalmid (581 ng per 2 ml). Peaks: 1 = osalmid; 2 = 2,7-dihydroxynaphthalene (internal standard).

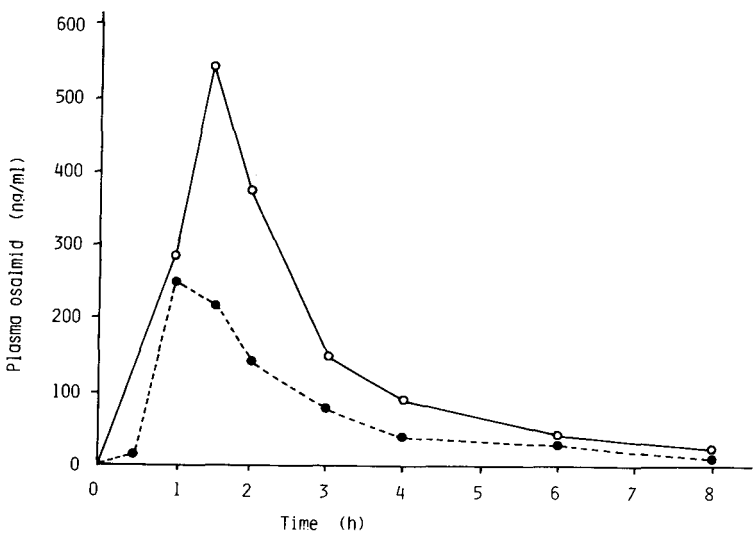

Fig. 2. Plasma concentration of osalmid at various times after oral administration to dogs. Doses of osalmid per animal were 500 mg (closed circle) and 1000 mg (open circle).

extraction, only 62% of the added amount was extracted and the results were sometimes irregular.

The precision was examined using plasma containing 212, 318, and 624 ng/ml (n=10). The coefficients of variation did not exceed 2.0%. The recovery of osalmid was checked by adding known amounts of osalmid (204, 301, 408, and 602 ng) to 1.0 ml of plasma (n=10). Recoveries were 98 ± 3% (mean ± S.D.). The method permits the accurate determination of osalmid in plasma at concentrations as low as 9 ng/ml.

Fig. 2 shows examples of the time—concentration curves in the plasma of dogs administered osalmid orally. The osalmid concentration in plasma increased immediately after the administration, reached a maximum in 1—1.5 h, and then decreased at a first-order rate. The biological half-life was about 1.5 h. Treatment of the plasma with 2 M NaOH or β-glucuronidase prior to the assay procedure resulted in the enhancement of the analytical values. This indicates that a considerable amount of osalmid is present in conjugated forms. In the plasma of dogs administered osalmid intravenously, the conjugated forms were hardly present.

Details will be reported elsewhere of the results of studies of the bioavailability of various pharmaceutical preparations containing osalmid.

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Note

Simultaneous determination of chloramphenicol and its succinate ester by high-performance liquid chromatography

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Since its introduction in 1949, chloramphenicol (CAP) has been extensively used as an antibacterial and an antirickettsial agent [1]. Currently, it is the only drug that can be used effectively in patients with meningitis secondary to *Hemophilus influenzae* type b resistant to ampicillin [2]. However, CAP has been associated with a variety of adverse effects [3]. Two types of hematological toxicity have been reported: a rare idiosyncratic reaction [4] and a relatively common dose-related toxicity [5, 6]. Serum CAP concentrations of greater than 25 μg/ml and prolonged courses of therapy have been associated with dose-related bone marrow suppression [5, 7, 8]. Fatal toxicities such as "gray syndrome" in neonates [9—11] and "gray toddler syndrome" in infants [12] have been reported with CAP serum concentrations above 50 μg/ml. In humans, CAP is extensively metabolized. However, in premature infants, who lack an effective glucuronidation pathway, normal CAP doses can lead to accumulation causing serious side effects [11].

Although CAP pharmacokinetics in infants and children have been studied only recently, two reports [13, 14] have described an increase rather than decrease, in CAP concentrations with time following an intravenous dose of chloramphenicol sodium succinate (CAPS). This finding suggested the need for measuring both CAP and CAPS to account for incomplete or delayed hydrolysis of CAPS to CAP. CAP pharmacokinetic studies strongly suggest

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that CAP therapy should be individualized based on serum concentration measurements using a reliable, accurate and specific assay.

Several methods have been reported for the analysis of CAP in biological fluids [16—18], but high-performance liquid chromatography (HPLC) is the method of choice [19]. As yet, none of the published HPLC procedures provides an optimal method for the simultaneous measurement of CAP and CAPS in small sample volumes. Two methods [19, 20] did not employ an internal standard placing too much reliance on accurate volume transfers and on comparing the peak height of CAP to the standards for quantification. One of these methods [19] measured CAP and CAPS but required a relatively large sample (500 μl) of plasma or serum making it unsuitable for pediatric use. Other HPLC assays [21—26] used an internal standard but did not measure CAPS. Column temperature had to be maintained at nonambient conditions in two methods [20, 24]; no chromatogram was shown in one of the reports [22].

The purpose of this communication is to describe a simple and rapid HPLC procedure for simultaneous determination of CAP and CAPS in small volume samples using N-acetylchloramphenicol (NACAP) as an internal standard. To demonstrate its clinical utility serial blood samples were collected from a patient receiving CAPS for the treatment of *H. influenzae* meningitis. Simultaneous CAP and CAPS concentrations at steady state are described.

EXPERIMENTAL

Chemicals and reagents

Chloramphenicol, chloramphenicol monosuccinate ester and N-acetylchloramphenicol were obtained from Parke-Davis (Ann Arbor, MI, U.S.A.). Ampicillin and gentamicin sulfate were obtained from Sigma (St. Louis, MO, U.S.A.) and penicillin G from Squibb (Princeton, NJ, U.S.A.). Methanol and acetonitrile (glass-distilled) were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Chromatographic equipment and conditions

The reversed-phase HPLC system consisted of Consta Metric pump II G (Laboratory Data Control, Riviera Beach, FL, U.S.A.), μBondapak C_{18} column, 30 cm × 3.9 mm, 10 μm (Waters Assoc., Milford, MA, U.S.A.), analytical fixed-wavelength UV detector, Model 153 (Beckman Instruments, Fullerton, CA, U.S.A.) and a recorder, Series 5000 (Fisher Recordall, Houston Instruments, Houston, TX, U.S.A.).

Methanol—water—acetic acid (37:62:1) was used as the mobile phase at a flow-rate of 1.5 ml/min. All chromatography was performed at room temperature. Chart speed of the recorder was set at 0.2 in./min.

Standards

CAP (10 mg) was dissolved in phosphate buffer saline, pH 7.2 (10 ml) while CAPS (10 mg) and NACAP (10 mg) were prepared in distilled water (10 ml) to give a concentration of 1 mg/ml. Appropriate amounts of these standard solutions were added to the serum samples to yield concentrations

of 0.5, 1, 3, 6, 10, 15, 20, 25, 30, 37, 45 and 60 μg/ml of CAP and CAPS. All samples were stored at −20°C as suggested by Nilsson-Ehle et al. [19].

Assay procedure

Known amounts of CAP and CAPS were added to 50 μl of serum placed in polypropylene 400-μl microcentrifuge tubes. Acetonitrile, 100 μl containing 10 μg of the internal standard was then added to the mixture. The resulting mixture was vortexed for 5 sec and then centrifuged at 9380 *g* for 5 min. The supernatant (50 μl) was injected into the HPLC instrument with the detector set at 0.01—0.02 a.u.f.s. (wavelength 254 nm).

Calculations

The concentrations of CAP and CAPS in the unknown plasma samples were calculated by comparing its CAP:NACAP and CAPS:NACAP peak height ratios with those obtained from CAP and CAPS standard curves.

Recovery and precision

CAP and CAPS were added to drug-free serum and then analyzed by the procedure described above but without any added internal standard. Fifty microliters of the supernatant were injected and peak heights corresponding to each compound measured. Absolute recovery was calculated by comparing these peak heights with peak heights obtained by direct injection of pure drug standards.

Precision of the method was evaluated by analysis of serum standards containing both CAP and CAPS at concentrations of 1, 10 and 40 μg/ml. These samples were analyzed nine times by one individual and eight times by another.

Clinical application

An 8-month-old infant with *H. influenzae* meningitis was receiving intravenous CAPS (Chloromycetin®, Parke-Davis) 100 mg/kg/day divided into four equal doses. A total of 185 mg CAPS was infused over 30 min during each 6-h interval. At steady state, serial blood samples were collected during a 6-h dosing interval. Serum samples were stored at −20°C and analyzed within four days.

RESULTS AND DISCUSSION

A typical chromatogram of NACAP, CAP and CAPS is shown in Fig. 1. The peaks are sharp and symmetrical allowing use of peak heights rather than peak areas to quantitate detector response. Detector response (peak height) was linear over 0.5—60 μg/ml range for both CAP and CAPS, with both curves passing through the origin. Peak height ratios of CAP:NACAP and CAPS: NACAP from extracted samples were also linear over 0.5—60 μg/ml concentration range.

The limits of detection were 0.5 and 0.2 μg/ml for CAP and CAPS respectively. Recovery of both compounds ranged from 96—103% while precision of CAP and CAPS varied from 2.5—6%. Daily variations in CAP and CAPS

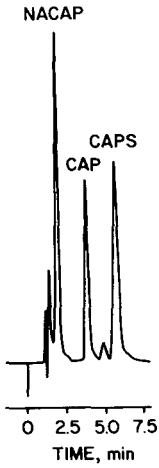

Fig. 1. Chromatogram obtained from serum sample containing NACAP (17.2 μg/ml); CAP (20 μg/ml) and CAPS (43 μg/ml), with retention times of 1.87, 3.9 and 5.75 min respectively (0.02 a.u.f.s.).

concentrations were < 5%. The retention times for NACAP, CAP and CAPS were 1.87, 3.90 and 5.75 min respectively. CAP and CAPS determination did not interfere with ampicillin and gentamicin which are commonly used with chloramphenicol clinically. Although stability of CAP in serum has been reported [19] no data were available for CAPS. In our experiments in serum stored at −20°C, CAP and CAPS were stable (> 95% activity) for at least

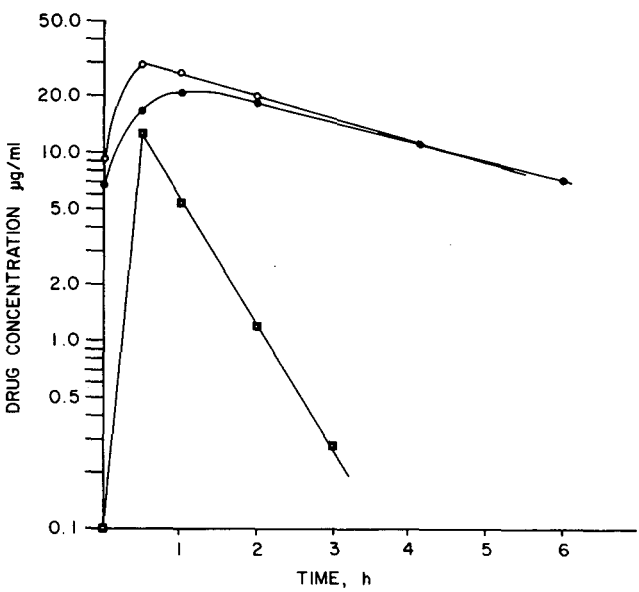

Fig. 2. Steady-state CAP (•), CPAS (▪) and CAP + CAPS (○) serum concentration—time curve obtained from a patient receiving 185 mg CAPS intravenously every 6 h.

two months and four days respectively. At −50° C, CAPS was stable for at least two weeks.

Steady-state CAP and CAPS serum concentration data from a patient receiving multiple doses of intravenous CAPS are shown in Fig. 2. CAP peak concentration of 20.66 μg/ml was achieved at 0.5 h after stopping the infusion. CAPS peak concentration of 12.58 μg/ml which accounted for about 26% of total drug (CAP + CAPS), was reached at the end of infusion. CAPS was detectable up to 3 h. The elimination of both CAP and CAPS appeared to be first-order demonstrating half-lives of 3.2 and 0.45 h respectively. Although the CAPS half-life has not been reported before, our CAP half-life value is consistent with the previously reported range of 0.87—17.8 h [13].

This assay system has proven simple, rapid, sensitive, specific and reproducible in simultaneously measuring CAP and CAPS. The small sample size required in this procedure makes it suitable for individualizing chloramphenicol therapy in pediatric population.

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Book Review

Introduction to protein sequence analysis, by L.R. Croft, Wiley, Chichester, New York, Brisbane, Toronto, 1980, XI + 157 pp., price £ 4.95, ISBN 0-471-27710-X.

This paperback is advertised as an up-to-date review of current methods for the determination of amino acid sequences of proteins. It is, indeed. It covers both enzymic and chemical methods of protein cleavage (Ch. 1 and 2), deals with purification of peptides (Ch. 3), describes the sequenator and the solid phase sequencer (Ch. 4 and 5); considerable attention is paid to the identification of amino acid phenylthiohydantoins (Ch. 6) and it refers to manual methods of sequencing (Ch. 7). The last chapter of this tiny volume is devoted to the application of mass spectrometry to the sequence analysis of peptides and proteins.

The advantage of this volume can be seen in the clear text and in the fact that the book is short (sometimes too short), which is certainly an advantage for newcomers. Those who have heard something about peptide analysis will probably look for new methods of peptide separations — but these are lacking here, though they are quite perspective both in separation efficiency and speed of operation. Keeping in mind how difficult it is to write a book on such a wide problem like protein sequencing, one comment is probably a very good score.

The book is reasonably priced and because of its conciseness one would expect it to appear even on shelves of those involved only marginally in protein sequencing.

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NEWS SECTION

MEETINGS

MIDWEST REGIONAL MEETING OF THE A.Ph.A. ACADEMY OF PHARMACEUTICAL SCIENCES

The Annual Midwest Regional Meeting of the American Pharmaceutical Association Academy of Pharmaceutical Sciences will be held on Monday, May 18, 1981, at the Marriott O'Hare in Chicago, IL, U.S.A., beginning at 9.00 a.m. For information contact Dr. Thomas B. Marriott, D-463, Abbott Laboratories, 1400 Sheridan Road, North Chicago, IL 60064, U.S.A.

4th INTERNATIONAL BIOANALYTICAL FORUM

The 1981 Forum will be held September 7–10, in Guildford, Great Britain. The main topic will be the separation and determination of metabolites of drugs and other foreign molecules in biological fluids. In addition there will be 'Advances' sessions, e.g. on automatic sample preparation and on TLC, including HPTLC and densitometry, and analytical case histories. For information, contact Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.

COURSES

Some Approaches to the Analysis of Biological Specimens for Drugs (advanced workshop course), Guildford, September 10–12, 1981. In this course techniques such as HPTLC, ion-pair HPLC, ultramicro methods and automatic sample preparation will be treated. The course is aimed at workers already experienced in classical analytical approaches and will include practical work. For information, contact Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.

Introduction to Determination of Drugs in Biological Fluids (workshop course), Guildford, September 15–19, 1981. This course is aimed especially at drug company staff, e.g. graduate recruits whose initiation into the relevant arts, particularly sample preparation, will be facilitated by the course. It will include a range of practical work. For information, contact Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford, GU2 5XH, Great Britain.

CALENDAR OF FORTHCOMING MEETINGS

May 3–7, 1981 Hindelang (Bavarian Alps), F.R.G.	**4th International Symposium on Capillary Chromatography** Contact: Dr. J. Rijks, Laboratory of Instrumental Analysis, University of Technology, P.O. Box 513, NL-5600 MB Eindhoven, The Netherlands
May 4–7, 1981 Brussels, Belgium	**XXIXth Annual Colloquium Protides of the Biological Fluids** Contact: Dr. Hubert Peeters, Colloquium secretariat, c/o Lipid and Protein Dept., Institute for Medical Biology, Alsembergsesteenweg 196, B-1180 Brussels, Belgium. Tel. (32-2) 344.19.50. (Further details published in Vol. 222, No. 2)
May 5–8, 1981 Gatlinburg, TN, U.S.A.	**Separation Science and Technology for Energy Applications** Contact: A.P. Malinauskas, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A. (Further details published in Vol. 198, No. 3)
May 11–15, 1981 Avignon, France	**5th International Symposium on Column Liquid Chromatography** Contact: Professor G. Guiochon, Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, Route de Saclay, 91128 Palaiseau, France. (further details published in Vol. 194, No. 3).
May 18, 1981 Chicago, IL, U.S.A.	**The Annual Midwest Regional Meeting of the A.Ph.A. Academy of Pharmaceutical Sciences** Contact: Dr. Thomas B. Marriott, D-463, Abbott Laboratories, 1400 Sheridan Road, North Chicago, IL 60064, U.S.A.
May 18–20, 1981 Jekyll Island, GA, U.S.A.	**11th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. R.W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
May 19–22, 1981 Munich, F.R.G.	**11th German Hospital Conference** Contact: Joint Working Committee of German Hospitals, Tersteegenstraat 9, D-4000 Düsseldorf 30, F.R.G. Tel. (02 11) 43 46 83.
May 20–22, 1981 Eger, Hungary	**Symposium on the Analysis of Steroids** Contact: Prof. S. Görög, c/o Hungarian Chemical Society, 1061 Budapest VI, Anker köz 1, Hungary.
May 21–25, 1981 Perth, Australia	**4th International Conference on Trace Element Metabolism in Man and Animals** Contact: Australian Academy of Science, P.O. Box 783, Canberra City, ACT 2601, Australia.
May 26–28, 1981 Washington, DC, U.S.A.	**3rd International Symposium on Rapid Methods and Automation in Microbiology** Contact: Dr. Richard C. Tilton, Chairman, ISRMA Planning Committee, University of Connecticut Health Center, Department of Laboratory Medicine, 263 Farmington Avenue, Farmington, CT 06032, U.S.A.
June 16–17, 1981 Venice, Italy	**1st International Symposium on Chromatography in Biochemistry, Medicine and Environmental Research** Contact: Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546. (Further details published in Vol. 198, No. 3)

June 18–19, 1981 Venice, Italy	**8th International Symposium on Mass Spectrometry in Biochemistry, Medicine and Environmental Research** Contact: Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546.
June 22–26, 1981 Veldhoven, The Netherlands	**4th International Symposium on Affinity Chromatography and Related Techniques** Contact: Secretariat, 4th Int. Symp. on Affinity Chromatography and Related Techniques, Department of Organic Chemistry, Faculty of Sciences, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands, (Further details published in Vol. 222, No. 2).
June 28–July 3, 1981 Kiryat Anavim, Israel	**The Sixth International Symposium on Bioelectrochemistry and Bioenergetics** Contact: Aharon Katzir-Katchalsky Center, Weizmann Institute of Science, Rehovot, Israel.
July 22–24, 1981 Manchester, Great Britain	**Biochemical Society Annual General Meeting and Joint Society/Nucleotide and Nucleic Acid Group Colloquium on Biochemical Interactions of Plasmids with their Hosts** Contact: The Biochemical Society, 7 Warwick Court, High Holborn, London WC1R 5DP, Great Britain.
Aug. 23–28, 1981 Espoo, Finland	**Euroanalysis IV – Triennial Conference of the Federation of European Chemical Societies** Contact: Professor L. Niinistoe, Department of Chemistry, Helsinki University of Technology, SF-02150 Espoo 15, Finland.
Aug. 30–Sept. 5, 1981 Vienna, Austria	**XI International Congress of Clinical Chemistry – IV European Congress of Clinical Chemistry** Contact: Congress Secretariat: Interconvention, P.O. Box 35, A-1095 Vienna, Austria. Tel. (0222) 421352.
Sept. 1–4, 1981 Siofok, Hungary	**3rd Danube Symposium on Chromatography** Contact: Hungarian Chemical Society, H-1368 Budapest, P.O.B. 240, Hungary. Tel. Budapest 427–343. (Further details published in Vol. 189, No. 2).
Sept. 7–10, 1981 Guildford, Great Britain	**4th International Bioanalytical Forum** Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain.
Sept. 9–11, 1981 Chicago, IL, U.S.A.	**2nd International Symposium on Radiopharmacology and Exhibition** Contact: Dr. Lelio G. Colombetti, Pharmacology Department, Loyola University, Stritch School of Medicine, Maywood, IL 60153, U.S.A.
Sept. 20–25, 1981 Philadelphia, PA, U.S.A.	**8th Annual Meeting of the Federation of Analitical Chemistry and Spectroscopy Societies (FACSS)** Contact: Richard J. Knauer, Publicity Chairman, ARMCO INC., P.O. Box 1697, Baltimore, MD 21203, U.S.A.
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749-2623. (Further details published in Vol. 222, No. 2)

Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC '81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 26 20 20.
Oct. 12–15, 1981 Houston, TX, U.S.A.	**"EXPOCHEM '81"** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749–2623.
Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectroscopy** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. J. Albaigés, General Secretary, Plaza de Espana, Barcelona–4, Spain. Tel: 223–31 01.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.
April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umweltforschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.

NEW BOOKS

Sequencing of proteins and peptides, by G. Allen (Laboratory Techniques in Biochemistry and Molecular Biology, edited by T.S. Work and R.H. Burdon, Vol. 9), North-Holland, Amsterdam, New York, Oxford, 1981, XVIII + 327 pp., price Dfl. 61.00, US$ 29.75 (paperback); Dfl. 163.00, US$ 79.50 (hardbound), ISBN 0-444-80254-1 (paperback), 0-444-80275-4 (hardbound). (To be reviewed in *J. Chromatogr., Biomed. Appl.*)

Protides of the biological fluids, Colloquium 28, edited by H. Peeters, Pergamon, Oxford, 1980, 600 pp., price US$ 150.00, £ 65.00 (to be reviewed in *J. Chromatogr., Biomed. Appl.*).

Frontiers in protein chemistry (Proc. Conf. Protein Chemistry, University of Hawaii, Honolulu, HI, July 2–6, 1979), edited by T.-Y. Liu, G. Mamiya and K.T. Yasunobu, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1980, 592 pp., price Dfl. 135.00, US$ 65.75 (outside U.S.A. and Canada) or US$ 60.00 (in U.S.A. and Canada), ISBN 0-444-00443-2.

Catecholamines and stress: Recent advance (proc. 2nd Int. Symp., Czechoslovakia, September 1979), edited by E. Usdin, R. Kvetnansky and I.J. Kopin, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1980, 600 pp., price Dfl. 148.00, US$ 72.25 (outside U.S.A. and Canada) or US$ 65.00 (in U.S.A. and Canada), ISBN 0-444-00402-5.

PUBLICATION SCHEDULE FOR 1981

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

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2							
Chromatographic Reviews							220/1	The publication schedule for further issues will be published later.						
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2							

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

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

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