VOL. 224 NO. 3 AUGUST 14, 1981
(Biomedical Applications, Vol. 13, No. 3)
THIS ISSUE COMPLETES VOL. 224

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
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Journal of Chromatography, 224 (1981) 355—370
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 892

ANALYSIS OF PROFILES OF CONJUGATED STEROIDS IN URINE BY ION-EXCHANGE SEPARATION AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received February 23rd, 1981)

SUMMARY

A simplified, flexible method for the analysis of metabolic profiles of steroids in urine is described. Solid extraction with Amberlite XAD-2 or Sep-Pak C_{18} cartridges is followed by group fractionation of unconjugated neutral and phenolic steroids, monoglucuronides, monosulphates and disulphates on the lipophilic strong anion exchanger triethylamino-hydroxypropyl Sephadex LH-20 (TEAP-LH-20). Following brief enzymatic hydrolysis or solvolysis the steroids are purified on TEAP-LH-20. O-Methyloxime and trimethylsilyl ether derivatives are prepared and purified by filtration through Lipidex 5000, and are then analyzed by glass capillary column gas—liquid chromatography and gas chromatography—mass spectrometry.

Between 2 and 5 ml of urine are used for a comprehensive analysis. Unconjugated neutral and phenolic steroids are isolated in half a day, corresponding steroids in the conjugate fractions in two days. No fraction containing steroids is discarded, but the analysis can be limited to a selected fraction.

INTRODUCTION

Many methods have been described for analysis of metabolic profiles of steroids in urine [1—8]. In most cases, hydrolysis of conjugated steroids is the first step in the analysis. Since the state of conjugation depends on the structure and metabolic origin of the steroid metabolites, important information about metabolic pathways may be lost by the use of such procedures. A method based on ion-exchange separation of groups of conjugates prior to hydrolysis and analysis by gas—liquid chromatography (GLC) and gas chromatography—mass spectrometry (GC—MS) has been developed in this laboratory [9]. However, this method is time-consuming and less suitable for routine analyses. Various modifications have therefore been tried in order

to simplify and increase the speed of all steps in this procedure. This paper describes a method which is the result of these studies. The steroid conjugates are separated on a strong anion exchanger which yields cleaner fractions. Less time-consuming methods for extractions, hydrolysis and solvolysis are employed. The method has now been in use for about two years.

EXPERIMENTAL

Chemicals and glassware

All solvents were of reagent grade and were redistilled in all-glass apparatus twice before use. Glassware was cleaned in an ultrasonic bath. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Labs., State College, PA, U.S.A.) were redistilled and trimethylsilylimidazole (Supelco, Bellefonte, PA, U.S.A.) was used as supplied. Methoxyamine hydrochloride (Eastman Organic Chemicals, Rochester, NY, U.S.A.) was recrystallized from methanol. Tetrahydrofuran was refluxed and distilled over lithium aluminium hydride. It could be stored for one week. Triethylamine sulphate was prepared as a 0.5 *M* solution in water, pH 7.2.

Helix pomatia digestive juice was from Pharmindustrie (Clichy, France). Immediately before use, 0.3 ml were added to 5 ml of 0.2 *M* sodium acetate buffer, pH 4.5, and the solution was passed through a 80 × 4 mm column of Amberlite XAD-2 at a rate of 0.4 ml min^{-1} [10].

Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) was extensively washed with acid, base, water and solvents and was then stored in ethanol [9]. Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used as supplied. Lipidex 5000 (Packard Instrument, Downers Grove, IL, U.S.A.) was washed with 20% and 50% aqueous ethanol and ethanol at 70°C. It was stored in methanol at 4°C. SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) was converted into the sodium form, washed in the same way as Lipidex 5000 and stored in ethanol at 4°C. Prior to use it was converted into the H^+ form with 0.5 *M* hydrochloric acid, washed with water until neutral, and suspended in 72% methanol. SP-Sephadex was stable in this form at 4°C for a month. Triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20) was prepared as follows [11]. Sephadex LH-20, 50 g, was converted into a chlorohydroxypropyl (23%, w/w) derivative [12]. After washing with 85% aqueous ethanol, ethanol, chloroform and ethanol, the derivative was dried at room temperature and used for the synthesis of TEAP-LH-20 essentially as described for TEAPHA-LH-20 [13]. The gel, about 65 g, was allowed to swell at room temperature in 270 ml 50% aqueous methanol under continuous stirring for 30 min. Sodium hydroxide (6.5 g) in 500 ml of 50% aqueous methanol was then added, followed by 500 ml of triethylamine (50 ml min^{-1}). The temperature was increased to 55°C and the mixture was stirred for 3.5 h. The product was washed on a Buchner funnel with 1 l of the following series of solutions: 50% aqueous ethanol, 0.3 *M* sodium hydroxide in 72% ethanol, water (until neutral) and 72% ethanol. The ion-exchanging capacity was 0.8—1.0 mequiv. g^{-1}. The gel was converted into the Cl^- form with 0.3 *M* hydrochloric acid in 72% ethanol, washed until neutral with water and extracted at 70°C as described for Lipidex. It was then converted into

the acetate form via the OH^- form and stored dry at −20°C. Immediately before use the appropriate amount was washed with 0.3 *M* sodium hydroxide in 72% methanol, water and 72% methanol.

Chromatography columns were 200 × 4 mm with a reservoir of 10 ml. PTFE gauze, 70 μm, and a PTFE tube with stopcock were inserted at the bottom end. Sep-Pak C_{18} cartridges were connected to the glass columns via the PTFE tube. When necessary, appropriate flow-rates were obtained by application of nitrogen (carbon dioxide, see below) pressure.

Steroids

Unlabelled steroids were those used in previous studies [9]. Radioactively labelled steroids were from the Radiochemical Centre (Amersham, Great Britain) and NEN Chemicals (Dreieich, G.F.R.). [6,7-^{3}H]Estrone sulphate and [6,9-^{3}H]estriol-16-glucuronide were kindly donated by Prof. H. Adlercreutz, Helsinki, Finland. The disulphate of 5α-[1,2-^{3}H]androstane-3β,17β-diol was a gift from Dr. U. Rudqvist at this department.

Analytical procedure

A flow scheme of the method is shown in Fig. 1.

Extraction. Urine, 2—5 ml, is diluted with 1—3 ml of water and passed at a flow-rate 0.4 ml min^{-1} through a column of XAD-2 (80 × 4 mm, packed in ethanol and washed with 15 ml of water). The column is washed with

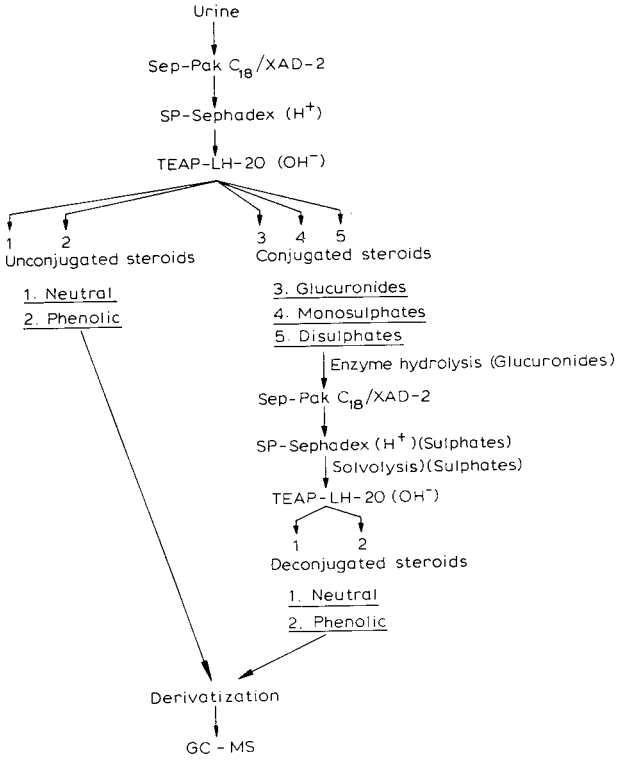

Fig. 1. General flow scheme for analysis of steroids in urine.

3 ml of water, 2 ml of 0.5 *M* triethylamine sulphate and 3 ml of water. Steroids are eluted with 8 ml of methanol.

Following the description of an extraction method based on Sep-Pak C_{18} cartridges [14] this method has been used as a faster alternative to the XAD-2 procedure. The urine is directly filtered through the cartridge (washed with 5 ml each of methanol and water prior to use) followed by 5 ml of water (flow-rate 1 ml min^{-1}). Steroids are eluted with 8 ml of methanol.

Cation exchange. Water, 3 ml, is added to the eluate from the extraction step, and the solution is filtered (flow-rate 0.7 ml min^{-1}) through SP-Sephadex in H^+ form (40 × 4 mm, packed in 72% methanol) followed by a rinse with 3 ml of 72% methanol.

Anion exchange, group separation. The entire effluent from SP-Sephadex is passed through TEAP-LH-20 in OH^- form (40 × 4 mm, packed in 72% methanol, flow-rate 0.5 ml min^{-1}). The effluent is collected together with a 4-ml rinse with 72% methanol. Neutral (uncharged) steroids are present in this fraction. Unconjugated phenolic steroids are then eluted with 8 ml of 72% methanol saturated with carbon dioxide, the flow-rate being maintained by a pressure of carbon dioxide. Monoglucuronides are then eluted with 10 ml of 0.4 *M* formic acid, monosulphates with 10 ml of 0.3 *M* acetic acid—potassium acetate, apparent pH 6.5, and disulphates with 15 ml of potassium acetate—potassium hydroxide, 0.5 *M* in acetate with an apparent pH of 10.0, all solutions being made with 72% methanol.

Glucuronide fraction. The fraction is taken to dryness in vacuo and the residue is dissolved in about 5 ml of the purified enzyme solution. Following incubation for 1 h at 62°C [15] the reaction mixture is extracted with Sep-Pak C_{18} or Amberlite XAD-2 as above, the triethylamine sulphate wash being omitted in the XAD-2 extraction. The methanol eluate is directly passed through a column of TEAP-LH-20 in OH^- form (40 × 4 mm, packed in methanol). The effluent and a rinse with 4 ml of methanol contain the liberated neutral steroids. Phenolic steroids are eluted with 8 ml of methanol saturated with carbon dioxide as above. The fractions are stored in methanol at 4°C until analyzed.

Sulphate fractions. The fractions are concentrated in vacuo to about 2 ml (monosulphates) and 3 ml (disulphates). Water, 5 ml, is added and pH is adjusted to 4.5 with concentrated hydrochloric acid (about 20 µl to the mono- and 200 µl to the disulphate fractions, respectively). If desired, enzymatic hydrolysis may be performed at this stage. However, in the present method, the fractions are extracted with Sep-Pak C_{18} or XAD-2 as described for urine, an additional wash of XAD-2 with 6 ml water being added to remove triethylamine sulphate more completely. Water, 1 ml, is added to the methanol eluate and the solution is filtered and washed through SP-Sephadex as described for the extract of urine. The combined effluent is concentrated in vacuo at 25°C to about 100 µl. Distilled tetrahydrofuran, 5 ml, acidified with 5 µl of 4 *M* aqueous sulphuric acid is added [16]. Following incubation for 1 h at 50°C, 1 ml of methanol is added and the solution is passed (0.5 ml min^{-1}) through TEAP-LH-20 in OH^- form, the column (80 × 4 mm) being packed in methanol and washed with 3 ml of tetrahydrofuran—methanol (5:1, v/v), prior to application of the sample. The column is washed with 6

ml of the latter solvent. The liberated neutral steroids are recovered in the combined effluent. Phenolic steroids are eluted with the same solvent saturated with carbon dioxide (under carbon dioxide pressure). Solvents are removed in vacuo and the fractions are stored in methanol at 4°C until analyzed.

GLC and GC—MS analyses. One microgram of 7-ketocholesterol is added as internal standard to all neutral steroid fractions. O-Methyloxime-trimethylsilyl ether derivatives are then prepared [17] and purified as described [13]. Estrogens are converted into trimethylsilyl ethers after addition of a suitable amount of 5β-cholestan-3α-ol as internal standard [13].

GLC was carried out using a Pye 104 gas chromatograph equipped with a 25 m × 0.3 mm open tubular glass capillary column coated with SE-30 [2]. Nitrogen was used as the carrier gas at an inlet pressure of 50 kPa, giving a flow-rate of about 1 ml min^{-1}. The oven temperature was programmed from 230°C to 265°C at a rate of 1°C min^{-1}.

GC—MS was carried out using a modified LKB 9000 and a glass capillary column (SE-30) connected via a single stage jet separator [13]. Column temperature was programmed from 225°C to 270°C at a rate of 1.2°C min^{-1}. Conditions for repetitive scanning mass spectrometry and computer evaluation were as described previously [18]. The identification of a steroid was based upon the retention time, the complete mass spectrum and partial mass spectra obtained from fragment ion current chromatograms constructed for characteristic ions given by the steroid derivatives. Details have been given in ref. 9.

Relative amounts of steroids were estimated by comparing the GLC peak areas given by the steroids with that of the internal standard. Since a true quantification was not the aim of this part of the study, a mass response factor of 1.0 was used.

RESULTS

The group fractionation of steroids on TEAP-LH-20 was tested with radioactively labelled steroids and steroid conjugates and with extracts of urine from two men given [^{3}H]cortisol and [^{3}H]pregnenolone, respectively. Fig. 2 shows the separation of synthetic steroids added to extracts of urine. A complete and wide separation was achieved of unconjugated neutral and phenolic steroids, glucuronides, monosulphates and disulphates. Table I shows the distribution of radioactivity between different fractions in the separation of metabolites in urine from the two men given the labelled steroids. As expected, cortisol metabolites appeared mainly in the glucuronide fraction while pregnenolone metabolites were excreted mostly as monosulphates. The same pattern was previously obtained using DEAP-LH-20 [9].

The glucuronide fraction was hydrolyzed with Helix pomatia intestinal juice at 62°C for 1 h. This rapid procedure was compared with the method employed in a previous study [9] (48 h, 37°C), using urine from the subject given [^{3}H]cortisol. No significant differences in recovery of radioactive metabolites or GLC profiles were noted. About 85—90% of the radioactivity was recovered as neutral steroids. Less than 5% appeared in the glucuronide fraction. In spite of the good recoveries of [^{3}H]cortisol metabolites, the

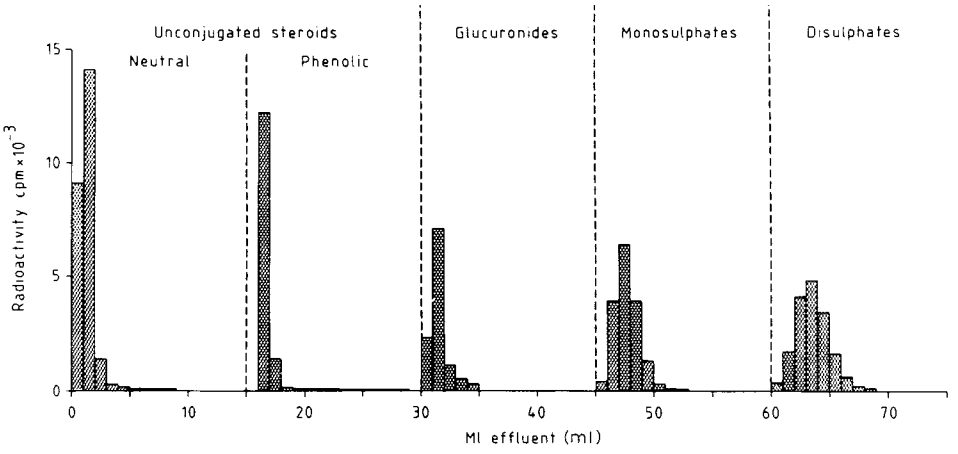

Fig. 2. Separation on a column (40 × 4 mm) of TEAP-LH-20 in OH^- form of radioactive steroids added to an extract of urine. Unconjugated neutral steroids (progesterone and cortisol) were eluted with 72% aqueous methanol, and estrogens (estrone, estradiol and estriol) with the same solvent, saturated with carbon dioxide. Glucuronides (testosterone glucuronide and estriol-16-glucuronide), monosulphates (3β-hydroxy-5-androsten-17-one sulphate) and disulphates (5α-androstane-3β,17β-diol disulphate) were then stepwise eluted with solvents described in Experimental.

TABLE I

PERCENTAGE DISTRIBUTION OF RADIOACTIVE METABOLITES IN URINE FROM TWO MEN GIVEN [^{3}H]CORTISOL AND [^{3}H]PREGNENOLONE, RESPECTIVELY
Six aliquots of each urine sample were extracted and fractionated on TEAP-LH-20.

Steroid fraction	[^{3}H]Cortisol metabolites (% ± S.D.)	[^{3}H]Pregnenolone metabolites (% ± S.D.)
Neutral	10 ± 3	12 ± 2
Glucuronide	86 ± 9	36 ± 2
Monosulphate	4 ± 1	44 ± 3
Disulphate	<1	8 ± 4

precision in the GLC analyses of ring A-reduced metabolites of cortisol and cortisone was poorer than for other steroids (see below).

The sulphate fractions were subjected to rapid solvolysis in tetrahydrofuran—sulphuric acid since enzyme hydrolysis liberated only about 40% of the sulphated metabolites of [^{3}H]pregnenolone. The presence of sulphate ions derived from triethylamine sulphate may contribute to the poor yield.

Fig. 3. GC—MS analyses of steroids in different conjugate fractions isolated from urine of a healthy woman during follicular phase. Fragment ion current chromatograms constructed by the computer are shown for ions characteristic of trimethylsilyl and O-methyloxime-trimethylsilyl derivatives of metabolites of androgens (*m/z* 270—248), progestins (*m/z* 269—253) and corticosteroids (*m/z* 253—431). The principal steroids indicated by the numbers are listed in Table III. A 25-m glass capillary column coated with SE-30 was used, and the oven temperature was programmed at 1.2°C min^{-1} from 225°C to 270°C.

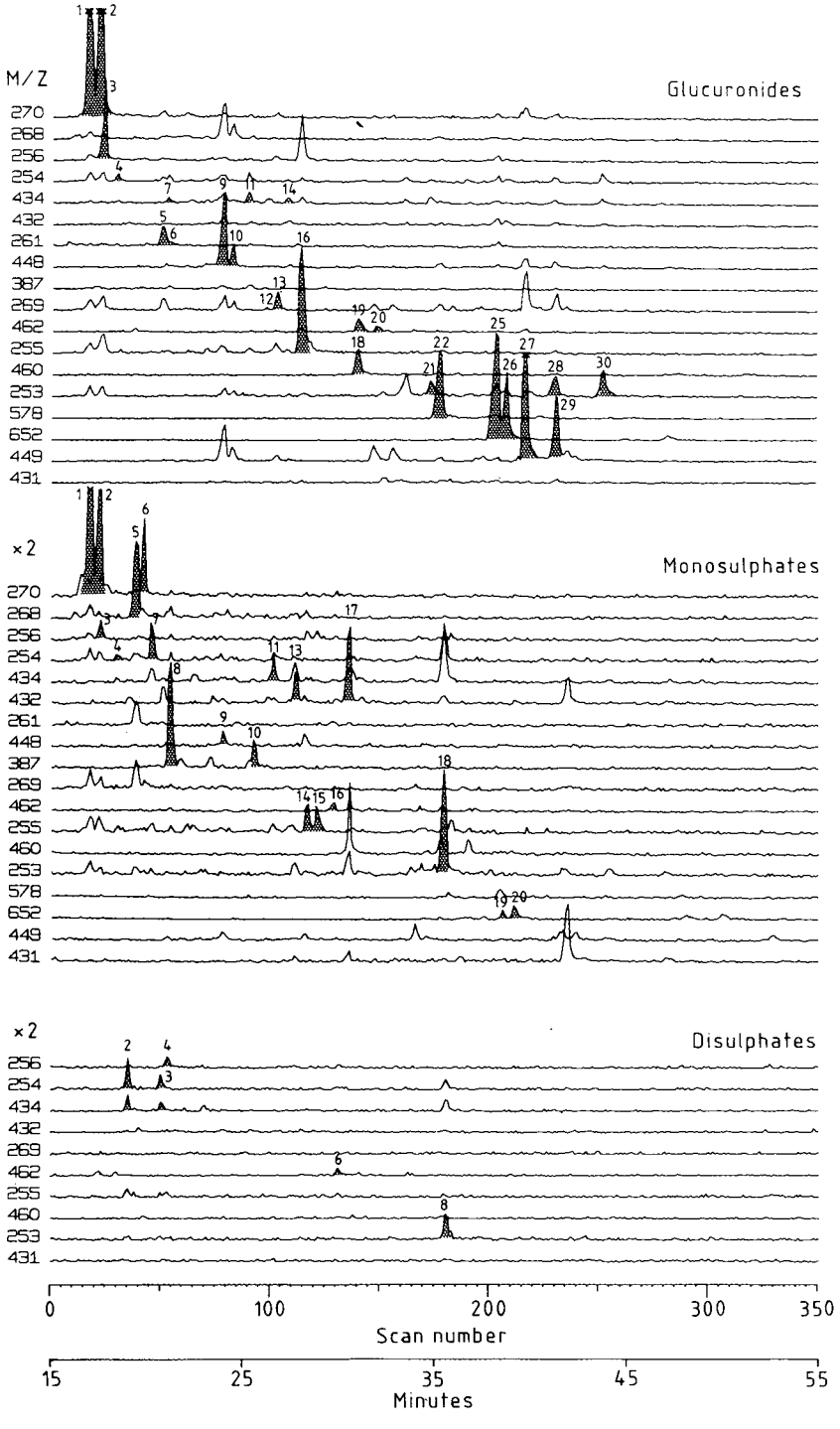
M/Z
Glucuronides
270
268
256
254
434
432
261
448
387
269
462
255
460
253
578
652
449
431
×2
Monosulphates
×2
Disulphates
0
100
200
300
350
Scan number
15
25
35
45
55
Minutes

TABLE II

RECOVERIES THROUGH THE METHOD OF RADIOACTIVELY LABELLED STEROIDS ADDED TO URINE (2—5 ml) AND OF LABELLED STEROID METABOLITES IN URINE AFTER ORAL ADMINISTRATION OF [^{3}H]CORTISOL OR [^{3}H]PREGNENOLONE

Steroid conjugate(s)	Amount added (cpm)	Recovery from columns (%)					
		Extraction and group separation	*n*	Hydrolysis/ solvolysis and purification	*n*	Total*	*n*
[^{3}H]Testosterone glucuronide	7500	94 ± 5	6	79 ± 5	5	76 ± 5	9
[^{3}H]Estriol 16-glucuronide	8000	92 ± 7	2	91 ± 1	2	84 ± 7	2
[^{14}C]3β-Hydroxy-5-androsten-17-one sulphate	8200	100 ± 2	10	94 ± 4	12	94 ± 4	19
[^{3}H]Estrone sulphate	16000	98 ± 3	3	78	1	73 ± 4	3
[^{3}H]Androstane-3β,17β-diol disulphate	7600	79 ± 4	8	88 ± 12	8	73 ± 6	8
[^{3}H]Cortisol metabolites		92 ± 8	6	86 ± 4**	3		
[^{3}H]Pregnenolone metabolites		97 ± 5	6	81 ± 3***	4		

*Filtration of methyloxime-trimethylsilyl ether derivatives through Lipidex 5000 not included
**Glucuronide fraction.
***Monosulphate fraction.

Solvolysis liberated about 80% of these metabolites. Similar results were obtained with ethyl acetate—sulphuric acid (39°C, 16 h), used in a previous study [9].

The recoveries of radiolabelled steroids added to urine or obtained as metabolites of cortisol and pregnenolone are shown in Table II. In most cases the recoveries were better than 90% after extraction on Amberlite XAD-2, filtration through SP-Sephadex and group separation. The 5α-androstane-3β,17β-diol disulphate constituted an exception (see Discussion). Hydrolysis and solvolysis resulted in losses of 6—21% and the recoveries throughout the method varied between 73 and 94%.

The precision of the method was determined by triplicate analyses of urine samples from the follicular (day 4) and luteal (day 23) phases of a healthy woman. The principal steroids in each fraction were identified by GC—MS (Fig. 3) and were quantified from the GLC analyses (Fig. 4). The results are summarized in Table III which also gives the coefficients of variation. It should be pointed out that a mass response factor of 1 was used and that the values in Table III are not true absolute excretion rates.

The method was used to study the changes of the steroid profile during a 24-h period in a healthy woman (day 19 of the cycle). The results are summarized in Table IV. The diurnal variation in the excretion of 11-oxygenated adrenal steroid metabolites is clearly seen. However, if 24-h portions of urine are difficult to obtain, a representative metabolic profile of steroids may be obtained from a single sample, frozen immediately and preferably collected in the morning. Incomplete collection or bacterial conversions during a 24-h

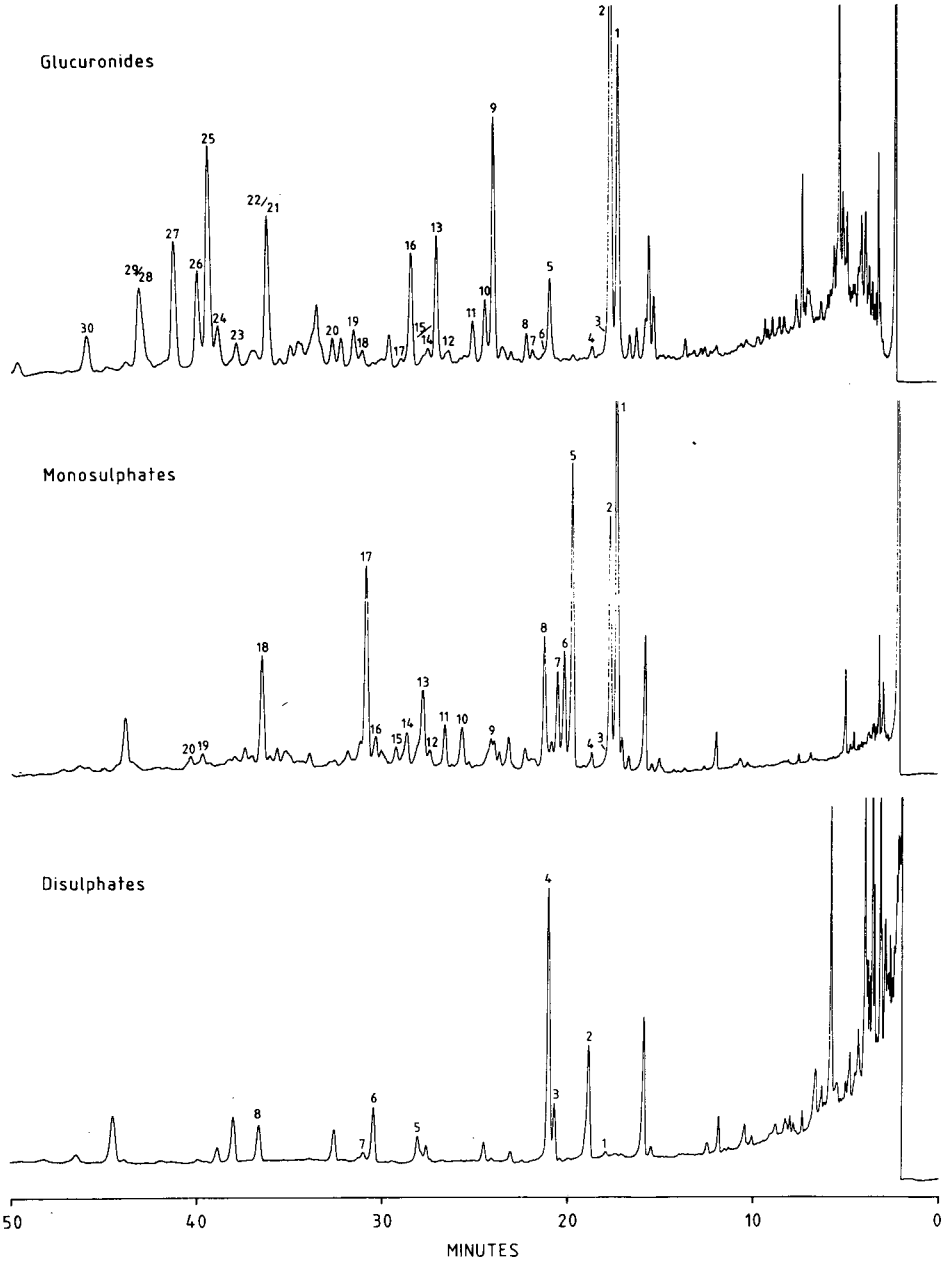

Fig. 4. GLC analyses of steroid profiles in different conjugate fractions isolated from urine of a healthy woman during follicular phase. Aliquots equivalent to 7 μl and 20 μl of urine were injected from the glucuronide and sulphate fractions, respectively. The principal steroids indicated by the numbers are listed in Table III. A 25-m glass capillary column coated with SE-30 was used, and the oven temperature programmed at 1°C min^{-1} from 230°C to 265°C.

TABLE III

DAILY EXCRETION OF STEROID CONJUGATES IN URINE FROM A 33-YEAR OLD WOMAN DURING FOLLICULAR AND LUTEAL PHASES

The samples were analyzed in triplicate.

No.	t_R*	RI**	Steroid conjugates***	Excretion (mg per 24 h)§			
				Cycle day 4		Cycle day 23	
				Mean	C.V.§§ (%)	Mean	C.V. (%)
			Glucuronides				
1	0.50	2593	5α-A-3α-ol-17-one	2.47	2	3.10	2
2	0.51	2600	5β-A-3α-ol-17-one	4.06	6	3.41	1
3	0.51	2602	5β-A-3α,17β-ol				
4	0.55	2630	A^5-3β,17α-ol				
5	0.64	2687	5α-A-3α-ol-11,17-one	0.85	12	0.72	2
6	0.65	2693	5β-A-3α-ol-11,17-one				
7	0.65	2693	A-triol				
8	0.68	2703	A-triol				
9	0.76	2753	5α-A-3α,11β-ol-17-one	2.48	12	2.55	2
10	0.78	2760	5β-A-3α,11β-ol-17-one	0.80	17	0.81	10
11	0.80	2770	A-triol				
12	0.90	2815	5α-P-3α,20α-ol				
13	0.91	2821	5β-P-3α,20α-ol	1.39	11	5.25	3
14	0.92	2825	A-triol				
15	0.92	2825	A^{Δ}-triol				
16	0.98	2850	5β-P-3α,17α,20α-ol	1.12	5	1.30	3
17	0.99	2853	5α-P-3α,17α,20α-ol				
18	1.12	2900	P^5-3α,16α,20α-triol				
19	1.14	2908	P-3,16,20-ol				
20	1.21	2930	P-3,16,20-ol				
21	1.42	2990	P^5-3β,17α,20α-ol				
22	1.43	2993	5β-P-3α,17α,21-ol-11,20-one	1.70	25	1.30	10
23	1.52	3018	5β-P-3α,11β,21-ol-20-one	0.23	9	0.24	4
24	1.60	3038	5α-P-3α,11β,21-ol-20-one	0.42	7	0.56	7
25	1.63	3047	5β-P-3α,11β,17α,21-ol-20-one	2.50	25	2.00	25
26	1.66	3053	5α-P-3α,11β,17α,21-ol-20-one	1.10	25	0.90	25
27	1.76	3078	5β-P-3α,17α,20α,21-ol-11-one	1.80	3	1.75	1
28	1.88	3103	5β-P-3α,11β,17α,20β,21-ol	1.22	7	1.26	3
29	1.89	3105	5β-P-3α,17α,20β,21-ol-11-one				
30	2.09	3145	5β-P-3α,11β,17α,20α,21-ol	0.61	7	0.71	1
			Monosulphates				
1	0.50	2593	5α-A-3α-ol-17-one	1.39	1	1.05	4
2	0.51	2600	5β-A-3α-ol-17-one	0.87	5	0.70	6
3	0.51	2602	5β-A-3α,17β-ol				
4	0.55	2630	A^5-3β,17α-ol				
5	0.59	2650	A^5-3β-ol-17-one	0.91	13	0.36	3
6	0.61	2670	5α-A-3β-ol-17-one	0.33	20	0.13	9
7	0.62	2675	A^5-3β,17β-ol	0.34	3	0.16	6
8	0.64	2687	A^5-3β,7α-ol-17-one				
9	0.75	2748	5α-A-3α,11β-ol-17-one				
10	0.83	2788	A^{Δ}-diolone				
11	0.87	2803	A-triol				
12	0.89	2812	5β-P-3α,20α-ol				
13	0.93	2830	A^5-3β,16β,17α-ol	0.34	6	0.30	7

TABLE III (continued)

No.	t_R*	RI**	Steroid conjugates***	Excretion (mg per 24 h)§			
				Cycle day 4		Cycle day 23	
				Mean	C.V.§§ (%)	Mean	C.V. (%)
14	0.98	2850	5β-P-3α,17α,20α-ol	0.12	14	0.10	7
15	1.01	2860	5α-P-3α,17α,20α-ol				
16	1.06	2880	P^5-3β,20α-ol	0.14	11	0.17	20
17	1.09	2890	A^5-3β,16α,17β-ol	0.82	6	1.01	1
18	1.42	2990	P^5-3β,17α,20α-ol	0.32	20	0.18	15
19	1.63	3047	5β-P-3α,11β,17α,21-ol-20-one				
20	1.66	3053	5α-P-3α,11β,17α,21-ol-20-one				
			Disulphates				
1	0.51	2602	5β-A-3α,17β-ol				
2	0.55	2630	A^5-3β,17α-ol	0.31	12	<0.1	
3	0.59	2675	A^5-3β,17β-ol	0.21	15	<0.1	
4	0.63	2680	5α-A-3β,17β-ol	0.91	13	1.20	12
5	0.93	2830	A^5-3β,16β,17α-ol				
6	1.06	2880	P^5-3β,20α-ol	0.15	16	<0.1	
7	1.09	2890	5α-P-3β,20α-ol				
8	1.42	2990	P^5-3β,17α,20α-ol	0.14	5	<0.1	

*Retention time of the trimethylsilyl ether and methyloxime-trimethylsilyl ether derivative relative to that of 5α-cholestane on a glass capillary column, SE-30, 250° C.

**Retention index [19].

***A and P = androstane and pregnane, superscript indicates position of double bond, greek letters denote configuration of hydroxyl groups.

§Values calculated using a mass response factor of 1.00. Absence of a value indicates difficulties in quantitation by GLC due to small amounts or presence of contaminants (see Fig. 3).

§§C.V. = coefficient of variation.

period can probably result in larger variations of the steroid profiles than seen in Table IV.

DISCUSSION

The method for isolation of steroids from urine follows the same general principles as described in previous publications from this laboratory [9, 13, 20]. Thus, a solid extraction method is used followed by purification and group separation on lipophilic ion exchangers. Three column materials are used in this process and have been selected in order to increase simplicity and speed of analysis. In the present form the method permits extraction, separation, hydrolysis and purification of each group of steroids in two days. Unconjugated neutral or phenolic steroids are isolated in a few hours.

Extractions are performed at three stages: as the initial step, for desalting prior to solvolysis, and after enzymatic hydrolysis. Losses of steroid sulphates in extraction with Amberlite XAD-2 have been reported by several workers [21—23]. In our experience, recoveries of steroid sulphates from urine are usually high, whereas large losses occur when the conjugates are extracted

TABLE IV

DAILY EXCRETION OF STEROID CONJUGATES IN URINE FROM A 39-YEAR OLD WOMAN DURING THE LUTEAL PHASE (DAY 19)

Steroid conjugates*	Excretion (mg/24 h)						Mean ± S.D.**	C.V. (%)**	24-h collection
	Calculated from values for urine collected between								
	7.15—9.35	9.35—13.00	13.00—18.35	18.35—22.10	22.10—2.00	2.00—7.15			
Glucuronides									
5α-A-3α-ol-17-one	5.48	4.85	4.26	4.66	4.34	4.07	4.61 ± 0.51	11	4.58
5β-A-3α-ol-17-one	7.17	5.96	5.15	5.26	5.35	5.91	5.80 ± 0.75	13	5.80
5α-A-3α-ol-11,17-one	1.71	1.65	1.55	1.57	1.35	1.13	1.49 ± 0.22	15	1.48
5α-A-3α,11β-ol-17-one	6.38	4.96	3.76	3.03	2.05	3.44	3.94 ± 1.53	39	3.81
5β-A-3α,11β-ol-17-one	1.68	1.54	1.57	1.55	1.24	1.07	1.44 ± 0.23	16	1.44
5β-P-3α,20α-ol	9.13	10.66	8.49	8.31	7.55	7.18	8.55 ± 1.24	15	8.55
5β-P-3α,17α,20α-ol	3.28	3.50	3.31	3.06	2.92	2.52	3.10 ± 0.35	11	3.12
P^5-3β,17α,20α-ol 5β-P-3α,17α,21-ol-11,20-one	1.28	0.77	0.38	1.57	0.59	1.82	1.07 ± 0.57	53	1.09
5β-P-3α,11β,21-ol-20-one	0.43	0.25	0.18	0.40	0.16	0.44	0.31 ± 0.13	42	0.31
5α-P-3α,11β,21-ol-20-one	1.20	0.87	0.55	0.64	0.31	1.02	0.77 ± 0.33	43	0.76
5β-P-3α,11β,17α,21-ol-20-one	3.21	2.28	1.04	2.09	1.18	2.91	2.12 ± 0.88	42	2.08
5α-P-3α,11β,17α,21-ol-20-one	1.57	1.43	0.88	1.57	0.91	1.39	1.29 ± 0.32	25	1.28
5β-P-3α,17α,20α,21-ol-11-one	3.01	3.25	2.28	1.82	1.30	1.83	2.25 ± 0.75	33	2.20
5β-P-3α,11β,17α,20β,21-ol 5β-P-3α,17α,20β,21-ol-11-one	1.77	2.37	1.66	1.62	0.88	1.11	1.57 ± 0.53	34	1.54
5β-P-3α,11β,17α,20α,21-ol	0.93	1.09	0.78	0.76	0.44	0.61	0.77 ± 0.23	30	0.76

Monosulphates									
5α-A-3α-ol-17-one	2.31	1.74	1.93	2.18	1.37	1.53	1.84 ± 0.37	20	1.83
5β-A-3α-ol-17-one	1.70	1.10	1.57	1.83	0.90	1.09	1.37 ± 0.38	28	1.37
A^5-3β-ol-17-one	1.23	1.57	0.88	0.62	0.57	0.78	0.94 ± 0.39	41	0.92
5α-A-3β-ol-17-one	0.27	0.46	0.25	0.29	0.31	0.37	0.33 ± 0.08	24	0.31
A^5-3β,17β-ol	0.14	0.28	0.17	0.17	0.18	0.23	0.20 ± 0.05	25	0.19
A^5-3β,16β,17α-ol	0.64	0.71	0.74	0.48	0.41	0.38	0.56 ± 0.16	29	0.56
A^5-3β,16α,17β-ol	0.96	0.78	1.21	0.80	1.00	0.86	0.94 ± 0.16	17	0.97
P^5-3β,17α,20α-ol	0.09	0.21	0.12	—	—	0.13	0.09	—	0.10
Disulphates									
5β-A-3α,17β-ol	0.09	0.15	0.09	0.17	—	0.13	0.11	—	0.11
A^5-3β,17α-ol	0.07	0.27	0.06	0.30	0.10	0.44	0.21 ± 0.15	71	0.23
5α-A-3β,17β-ol	1.49	1.56	1.18	1.18	1.40	1.37	1.47 ± 0.21	14	1.46
Creatinin (mmol per 24 h)	11.28	12.41	21.10	12.19	11.23	11.11	11.72 ± 0.57	5	11.96

*For abbreviations, see Table III.
**S.D. = standard deviation; C.V. = coefficient of variation.

at low ionic strength. The problem is circumvented if the column is washed with triethylamine sulphate in water prior to elution of steroids with methanol [21]. Triethylamine has been assumed to form ion pairs with the steroid conjugates, thus facilitating elution [21]. However, we have found that the strong adsorption of steroid sulphates is due to ion exchange, Amberlite XAD-2 acting as a weak anion exchanger [24]. The need for a wash with triethylamine sulphate increases the time of extraction, and small amounts of the compound will contaminate the steroid extract. This has an adverse effect on enzyme hydrolysis and solvolysis (see below). Recent studies indicate that sodium sulphate can replace triethylamine sulphate thereby simplifying the procedure [24].

When the present work was in the final stages, extraction of steroids with Sep-Pak C_{18} cartridges was described [14]. Since this method is superior with respect to speed, simplicity and recovery, it has largely replaced the Amberlite XAD-2 extraction steps.

Ion-exchange chromatography

The extract of urine is filtered through SP-Sephadex in the H^+ form. Ion exchange occurs in aqueous methanol and the high capacity permits use of a small column bed. This step may not be necessary for quantitative sorption of steroid conjugates on TEAP-LH-20, as is the case with the weak anion exchanger DEAP-LH-20 (or Lipidex-DEAP). However, it removes substances which affect derivatization and GLC of phenolic steroids. Furthermore, solvolysis does not occur if sulphate fractions extracted with Amberlite XAD-2 are not passed through SP-Sephadex. This is due to the presence of triethylamine sulphate acting as buffer. SP-Sephadex removes triethylamine (provided that water is added to the methanol eluate) and generates sulphuric acid. To avoid decomposition of sulphated steroids, the eluate must not be taken to complete dryness. Filtration through SP-Sephadex may not be needed prior to solvolysis when triethylamine sulphate is omitted, e.g. after extractions with Sep-Pak C_{18}.

The base form of a strong anion exchanger, TEAP-LH-20, is used instead of the acetate form of the weaker DEAP-LH-20 previously employed for group separation of steroid conjugates [9]. The purity of the neutral steroid fraction is higher since weak organic acids are more efficiently sorbed. Furthermore, phenolic steroids may be separately isolated [13]. Glucuronides of neutral steroids and A-ring glucuronides of phenolic steroids can be eluted prior to estrogen glucuronides with a free phenolic hydroxyl group. A similar separation was previously obtained using DEAP-LH-20 [25], and further subfractionation of glucuronides of aromatic steroids has been achieved using DEAE-Sephadex in methanol [26]. However, these methods are more time-consuming. A disadvantage with the present method is that the basic nature of the ion exchanger may affect recoveries of alkali-labile steroids.

TEAP-LH-20 permits ion exchange in a variety of solvents. Thus, the solvolysis mixture can be filtered through the ion exchanger after addition of methanol. This permits direct isolation of neutral and phenolic steroids in two separate fractions simultaneously with the removal of sulphuric acid and acids liberated by the solvolysis. It should be noted that the separations

on TEAP-LH-20 are not achieved in tetrahydrofuran or tetrahydrofuran—water alone.

Hydrolysis of steroid conjugates

In most methods, hydrolysis and solvolysis constitute rate-limiting steps and result in uncontrolled losses of steroids. We have attempted to use as rapid procedures as possible but it is evident that selective losses of steroids may occur, e.g. due to unknown enzyme selectivities and differential solubilities in the solvolysis mixture. Comparison with commonly used slower procedures showed no differences. Some factors affecting reproducibility may be pointed out. The enzyme solution must be purified to remove substances which interfere in the GLC analysis. The method used does not decrease enzyme activity [10]. In the solvolysis, it is important that tetrahydrofuran is freshly distilled and that the flasks are well stoppered. Finally, if mixed conjugates, diglucuronides or unknown types of conjugates are present they will probably escape detection unless additional means of hydrolysis are used. It is an advantage of the method that such compounds can be recovered from the second chromatography on TEAP-LH-20.

Quantitative aspects

The general pattern of steroids obtained with the present method is essentially as expected from previous studies [1—9, 27, 28]. The finding of 5α-androstane-3β,17β-diol as the major steroid in the disulphate fraction seems to be new. This steroid conjugate appears to be particularly sensitive to solvolytic conditions, and is readily lost upon evaporation of acidic solutions. There are other types of steroids which are not quantitatively recovered with the present procedure. Catechol estrogens are partly destroyed on TEAP-LH-20 [29, 30] and the conditions used for formation of O-methyloximes are not suitable for metabolites of aldosterone and 18-hydroxylated corticosteroids [31, 32]. Thus, individual steps in the general method may have to be modified when labile steroids are of particular interest. Depending on the problem to be studied it may also be more suitable to select other types of derivatives for the GLC and GC—MS analyses. In the present study O-methyloxime and trimethylsilyl ether derivatives were selected since they are the most generally applicable ones. Irrespective of the type of derivative selected it is necessary to determine a mass response factor for each steroid to be quantitated. The accuracy of the analyses will then depend mainly on the purity of the fractions isolated, and on the resolving power, dynamic range and sensitivity of the GLC or GC—MS systems.

ACKNOWLEDGEMENTS

The technical assistance of Ms. Monica Bredmyr is gratefully acknowledged. This work was supported by the World Health Organization, the Swedish Medical Research Council (Grant No. 03X-219), Magn. Bergvalls Stiftelse and Karolinska Institutet.

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Journal of Chromatography, 224 (1981) 371—379
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 881

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE SEPARATION AND MEASUREMENT OF DI- AND POLYAMINES AND THEIR DERIVATIVES, AND SPECIFIC PREPARATION OF ISOMERS OF THEIR MONOACETYL DERIVATIVES

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(First received October 27th, 1980; revised manuscript received February 27th, 1981)

SUMMARY

We present a method for separating and quantitating the di- and polyamines and many of their derivatives found in mammalian tissues by high-performance liquid chromatography using a cation-exchange resin with gradient elution. Three different solvent systems are described, each with special advantages. The nitrate anion is used in order to permit increasing the cation concentration without increasing buffering capacity or introducing halide ions, which are corrosive for stainless steel. The specific synthesis of two isomers of N-monoacetylspermidine is described.

INTRODUCTION

Recent observations that N-monoacetylputrescine, two isomeric N-monoacetylated spermidines and the enzymes relevant to their metabolism occur naturally in animal tissues has focused biochemical interest on acetylated derivatives of the polyamines. This paper presents a method suitable for separating these polyamine metabolites in biological samples, and outlines methods for their synthesis.

Several approaches have been used for separating and measuring the polyamines, spermidine and spermine, and their metabolic precursor, putrescine [1]. These include thin-layer chromatography [2], gas—liquid chromatography of derivatives [3—5], radioimmunoassay [3] and high-performance liquid chromatography (HPLC), either by reversed-phase chromatography of fluorescent derivatives such as the dansyl [6, 7] or *o*-phthalaldehyde [8, 9] derivatives or

0378-4347/81/0000—0000/$02.50

by direct chromatography on cation-exchange columns and subsequent derivatization (ninhydrin color [10—12] or fluorescence [13—16]). The most widely used method employs a cationic column with formation of the *o*-phthalaldehyde derivative and measurement by fluorescence. With this approach, amounts as little as 10 pmol can be detected [13].

Polyamines occur naturally in both free and conjugated forms. No method using a chromatographic column with gradient elution without previous derivatization has been proposed for separating and measuring the major simple metabolites such as the monoacetyl derivatives of putrescine, spermidine and spermine, or the partially oxidized derivatives such as γ-aminobutyric acid putreanine, isoputreanine or 2-hydroxyputrescine. Moreover, the methods now in use (with the exception of the derivatized reversed-phase system of Seiler et al. [7]) are based on converted amino acid analyzers and therefore are programmed for stepwise isocratic elution patterns. We have developed a novel system involving a linear gradient which permits separation of most of the major simple metabolic products of the polyamines from one another and from the free amino acids which are usually present in biological samples without precolumn derivatization.

EXPERIMENTAL

Equipment

Solvents are filtered through fritted steel filters and passed through a Gilson Mixograd gradient maker fitted with a C-V General Valve No. 1-17-900 to an Altex 100A double piston pump at 0.8 ml/min and thence through a 2-μm Inline Mobile Phase Filter (Rainin 905-15) to a Rheodyne injection valve (No. 7125) with a 20- or 100-μl injection loop. Samples are applied by the filled loop technique. The flow then passes through a precolumn into a prepacked 250 × 3.5 mm I.D. Partisil PXS 10-25 SCX cation-exchange column (Whatman, Clifton, NJ, U.S.A.) at 50°C. The effluent is led to a T-junction, where it enters at right angles to the flow of the fluorescent reagent. The *o*-phthalaldehyde reagent [17] is pumped at a flow-rate of 0.9 ml/min by an Eldex E-120-S pump through the long limb of the T-connector. The mixed streams then pass through a 10-μl flow cell in a fluorometer (exciting light filtered with a Corning 7-60 filter, peak transmission 330 nm; emitted light filtered through a combination of Corning filters 3289, 5113 and 3-74, peak transmission 455 nm) and the signal from the photomultiplier tube is amplified in a Farand Photometer and recorded on a strip recorder. All connections from the pistons of the pumps to the T-connection are stainless-steel tubing, 1/16 in. O.D. and connections beyond the T-connection are 1/16 in. O.D. PTFE-tubing. It was sometimes advantageous to use isotopic tracers in our biological studies, and on other occasions ^{3}H- or ^{14}C-tracers were added to specimens before analysis in order to verify the location of specific peaks on the chromatogram. When isotopes were used, the effluent from the flow cell could be led directly to a fraction collector, where 1-min samples (1.7 ml fluid volume) were counted after addition of 5 ml of ACS counting fluid (Radiochemical Centre, Amersham, Great Britain).

Reagents

All reagents are analytical grade and are made up in double glass-distilled water, or in redistilled methanol. All aqueous reagents entering the HPLC column are filtered through a 0.3-μm Millipore filter under vacuum to remove dissolved gas and stored at 4°C, when not in use.

The fluorescent reagent is made up from two stock solutions: a potassium borate buffer, 1.0 *M* (pH 10.4), to which has been added 20 ml/l of 5% Brij 35 (Pierce, Rockford, IL, U.S.A.). This reagent can be made up in 4-l lots and stored indefinitely at room temperature. The second stock solution, 2 g *o*-phthalaldehyde in 25 ml methanol (Aldrich No. P 3,940-0, Milwaukee, WI, U.S.A., although the source seems not to affect activity) is stored in a dark bottle in the freezer. Working reagent is prepared by adding to each 100 ml of borate buffer 0.2 ml 14.3 *M* 2-mercaptoethanol and 1.0 ml of the *o*-phthalaldehyde solution. We have found it advantageous to heat this working solution to 55°C for 20 min to remove dissolved gas before using it. The working reagent remains active when kept in a dark bottle at 4°C for several weeks.

The concentrations of the eluting buffers are expressed in terms of their cationic component, i.e., as moles of Na^+. The main system consists of a linear gradient from 0.01 *M* sodium acetate to a mix containing 0.05 *M* sodium acetate in 0.95 *M* sodium nitrate, at a constant pH of 4.60. A second system consisting of a gradient from 0.01 *M* sodium citrate (pH 3.04) to 0.05 *M* sodium citrate in 0.95 *M* sodium nitrate (pH 3.27), is also useful for special separations. When it is necessary to collect fractions for reprocessing under circumstances where removal of the sodium-containing buffer would present a problem, an ammonium formate buffer system may be used, since ammonium formate can be removed by sublimation. In this case, the gradient is from 0.01 to 1.0 *M* ammonium formate at pH 3.40. This system is especially suitable when isotopically labeled compounds are to be detected by their radioactivity, but is not useful for fluorescent measurement since the ammonium ion fluoresces in *o*-phthalaldehyde.

Standards

The following chemical standards were obtained from commercial sources as indicated: putrescine · 2HCl (1,4-diaminobutane · 2HCl; Sigma P-7505, St. Louis, MO, U.S.A.); spermidine · 3HCl (Sigma S-2501); spermine · 4HCl (Aldrich S383-6); γ-glutamylputrescine · HCl (Calbiochem 352491, Los Angeles, CA, U.S.A.); crystalline bovine serum albumin (Sigma A-4503); γ-aminobutyric acid (Calbiochem 1370) and putreanine [N-(4-aminobutyl)-3-aminopropionic acid · 2HCl; Calbiochem 541081]. Monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine were synthesized in our laboratories as described later. A small sample of D,L-2-hydroxyputrescine · 2HCl was kindly supplied by C. Hurwitz, who synthesized it by reduction of 1,4-diaminobutanone [18].

Both ^{14}C- and ^{3}H-radioactive standards were also used, but we found that the purity of the ^{3}H-standards was unreliable. Each batch had to be checked before use, since there was frequently contamination with a front-running radioactive component whose chromatographic mobility remained unchanged after hydrolysis in 8 *N* hydrochloric acid at 110°C for 16 h. It seems likely that some

of this contamination was ^{3}HOH, since part of it could be sublimed away; but the nature of the remainder is uncertain. A possibility is that it represents γ-aminobutyric acid or some other oxidation product. There was negligible contamination with ^{14}C-standards, but their specific radioactivity is so low that they sometimes contributed to the fluorescent measurement when trace amounts were added and the detector set at maximum useful sensitivity. The radioactive materials were obtained from New England Nuclear (Boston, MA, U.S.A.).

Sample preparation

All biological material prepared for application to the column was deproteinized with 5% trichloroacetic acid (TCA). This included samples which had been subjected to proteolysis, since it was essential to remove all traces of protein.

To obtain total polyamines, without concern for the derivatives, samples were hydrolyzed in 8.3 *M* hydrochloric acid at 110°C for 16 h. The hydrochloric acid was then removed in vacuo and the sample reconstituted either in distilled water or in the 0.01 *M* buffer appropriate to the analysis. Acid hydrolysis of biological samples often produced charred residues which were removed by centrifugation prior to lyophilization of the hydrochloric acid. Crystalline bovine serum albumin was prepared in this way to serve as a standard for mixed amino acids.

Chemical syntheses

Monoacetylated diamines. Monoacetyl-1,3-diaminopropane and monoacetyl-1,4-diaminobutane were prepared by the acetic anhydride method described by Tabor et al. [19].

Acetylated spermidines. The nitrile whose reduction yields N^{8}-acetylspermidine was synthesized according to the method of Tabor et al. [19]. After the condensation reaction and subsequent hydrogenation, the catalyst was removed by low-speed centrifugation and washed with water. The pooled sample was concentrated to about 10 ml in vacuo at 40°C. Sulfate ions were removed and the product was converted to the acetate by using the anion-exchange resin Dowex 1 in its acetate form. During concentration of the Dowex 1 eluate, a small amount of white crystalline material appeared, which separated readily, was difficultly soluble in water, had a very high melting point, contained no sulfate and had no demonstrable NMR spectrum. This was filtered off and discarded. The concentrated sample was placed on a 10 × 2.5 cm column of the cation exchanger Dowex 50-X2, 200—400 mesh, in the hydrogen form, and washed with about 50 ml of water. The water eluate was negative to ninhydrin. Elution with 1.5 *N* hydrochloric acid yielded ninhydrin positive material which, after being taken to dryness under reduced pressure at a temperature below 40°C, was repeatedly crystallized from hot absolute ethanol. Low-speed centrifugation was the method of choice for gathering the crystals. A final wash was done with diethyl ether. The yield, based on the nitrile as starting material, was 42%. It was convenient to handle 20 mmol of nitrile.

Tabor and Tabor's method [20] for preparing N^{1}-acetylspermidine was modified as follows: condensation of N-acetyl-1,3-diaminopropane (22 mmol) with 4-bromobutyronitrile (20 mmol) was followed immediately by catalytic hydro-

genation until hydrogen uptake ceased. Sulfate was removed by Dowex 1 as for the N^8-isomer; the preparation was then concentrated and processed with Dowex 50. The elution pattern of the amine was followed by *o*-phthalaldehyde fluorescence. The material eluting with water and with 200 ml of 0.5 *M* hydrochloric acid was discarded. Elution was continued with 1.5 *M* hydrochloric acid. Two hundred ml of eluate were collected, concentrated and recrystallized as for the N^8-isomer. The yield was 19%, based on the nitrile.

Analysis

The identity of the chemicals synthesized was confirmed by elementary analysis, melting point determination, NMR spectrum (Table I) and by hydrolysis to yield the parent compound.

TABLE I

PROOF OF IDENTITY OF SYNTHESIZED COMPOUNDS

Compound*		Elementary analysis (%)**			Melting point corrected (°C)	NMR spectrum*** (1% tetramethylsilane in ^2H_2O)
		C	H	N		
N-Acetyl-1,3-diamino-propane	Observed	39.3	8.7	18.6	160—161	3.27(t,2H,J=6.5 Hz); 3.03(t,2H,J=7.5 Hz); 2.01(s,3H); 1.88(m,2H)
	Theory	39.4	8.6	18.4		
N-Acetyl-1,4-diamino-butane	Observed	43.3	9.3	17.1	138—139	3.22(t,2H,J=6.6Hz); 3.02(t,2H,J=7.5 Hz); 2.00(s,3H); 1.68(m,4H)
	Theory	43.3	9.1	16.8		
N^8-Acetyl-spermidine	Observed	41.0	8.6	17.2	203.5—205	3.42(t,2H); 3.42(t,2H,J=6.6 Hz); 3.13(t,2H,J=7.6 Hz); 3.00(t,2H,J=6.7 Hz); 1.99(s,3H); 1.64(m,6H)
	Theory	41.5	8.9	16.2		
N^1-Acetyl-spermidine	Observed	41.3	9.0	17.3	189—191	3.29(t,2H,J=6.7 Hz); 3.06(m,6H); 2.01(s,3H); 1.90(m,2H); 1.77(m,4H)
	Theory	41.5	8.9	16.2		

*Compounds were prepared as the hydrochloride salts.
**Analysis performed by Baron Consulting Co., Orange, CT, U.S.A.
***NMR spectra were obtained on a Bruker HX-270 instrument.

RESULTS

All of the amino acids are eluted within the first 15 min. During this time, γ-aminobutyric acid and γ-glutamylputrescine also appear. After the gradient has terminated at 40 min, elution is continued isocratically with 1.0 *M* Na^+ if necessary. The retention times and relative fluorescence of the various compounds are shown in Table II. Note that this system does not resolve 2-hydroxyputrescine from N-acetylputrescine, and resolves N^1-acetylspermidine from N^8-acetylspermidine poorly. All other peaks are cleanly resolved.

TABLE II

RETENTION TIMES AND RELATIVE FLUORESCENCE OF POLYAMINES AND RELATED SUBSTANCES

Absolute values of the retention times are individual characteristics of the particular column.

Compound	Acetate buffer			Citrate buffer	
	Retention time (min ± S.D.)	C.V. (%)	Fluorescence vs. putrescine	Retention time (min)	Fluorescence vs. putrescine
γ-Aminobutyric acid	8.1 ± 0.3	3.1	1.48	9.4	1.32
γ-Glutamylputrescine	13.6 ± 1.4	9.9	2.08	15.0 **	1.95
N-Acetylputrescine	16.5 ± 0.3 *	2.0	0.48	15.7	0.49
2-Hydroxyputrescine	16.7 ± 0.1	0.6	0.92	17.7 **	0.76
Putreanine	17.3 ± 0.4	2.0	0.20	17.2 **	0.21
Putrescine	19.2 ± 0.5	2.8	1.00	17.6	1.00
N^8-Acetylspermidine	24.1 ± 0.1	0.4	0.14	21.0 ***	0.09
N^1-Acetylspermidine	25.5 ± 0.8	3.0	0.18	21.4 **	0.10
Spermidine	27.2 ± 0.7	2.6	0.88	21.0	0.61
Spermine	36.4 ± 1.3	3.7	0.38	26.4	0.24
Carnosine	13.3 ± 0.1	0.2	0.36		
Homocarnosine	13.8 ± 0.1	0.1	2.33		
Anserine	16.1 ± 0.7	4.3	0.16		
Cadaverine	21.1 ± 0.1	0.6	1.03		
3,3'-Iminobispropylamine	24.4 ± 1.0	4.0	0.80	18.6	0.76
1,3-Diaminopropane	15.8			16.3	
N-Acetyldiaminopropane	14.1			13.7	
Ammonium ion	10.0			12.7	
Histamine	21.1 ± 1.2	0.8	0.29		
Amino acids	4.5—16			5—13	

*Not resolved by acetate system but resolved by citrate system.
**Not resolved by citrate system, but resolved by acetate system.
***Not resolved by citrate system; partially resolved by acetate system.

The reproducibility of the system is also shown in Table II. Since the peaks are very narrow (Fig. 1) quantitative estimation by measuring peak heights is preferable to attempts to estimate their areas.

The linearity of fluorescence has already been established by others [13] and we could confirm that the fluorescence for any given compound was linear over the range of 20 pmol to 10 nmol. The application of this system to a liver extract is shown in Fig. 2.

A second buffered gradient elution system similar to the sodium acetate system utilized the sodium citrate buffer gradient described previously. This system produced different retention times permitting resolution of N-acetylputrescine from 2-hydroxyputrescine and provided a check on identification of the compounds (Table II).

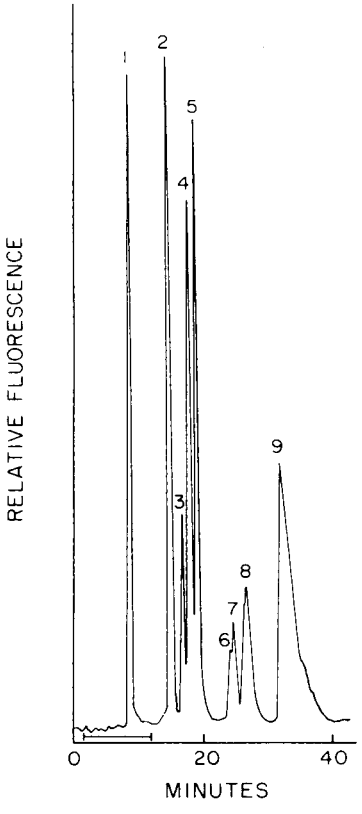

Fig. 1. Chromatogram of mixtures of diamines, polyamines and some of their derivatives, in the acetate system. The compounds are identified as follows: 1, γ-aminobutyric acid; 2, γ-glutamylputrescine; 3, N-acetylputrescine; 4, 2-hydroxyputrescine; 5, putrescine; 6, N^8-acetylspermidine; 7, N^1-acetylspermidine; 8, spermidine; 9, spermine. The location of the amino acids is indicated by the line below the tracing. The vertical line on the left marks the time at which the sample was applied to the column. The total duration of the chromatogram from application of the mixture to the end of the tracing is 39 min. Each peak represents 10 nmol of compound.

DISCUSSION

A linear gradient elution system has advantages over others which have been proposed. It avoids the sudden shifts of baseline which may be seen in making step changes in isocratic systems. The buffers used in this type of system must be fairly acidic in order to maintain complete ionization of the amino groups of the polyamines. The *o*-phthalaldehyde reaction used to generate fluorescence requires a highly basic medium. Sensitivity is lost if the entire increment of cations in the gradient is associated with a buffer anion which results in an altered pH of the reaction mixture. It is therefore advantageous, in the concentrated part of the buffer curve, to provide part of the cations in association with a strong acid such as chloride. However, the use of chloride ion, as recommended by most other workers [9, 11—16], causes corrosion of steel and in-

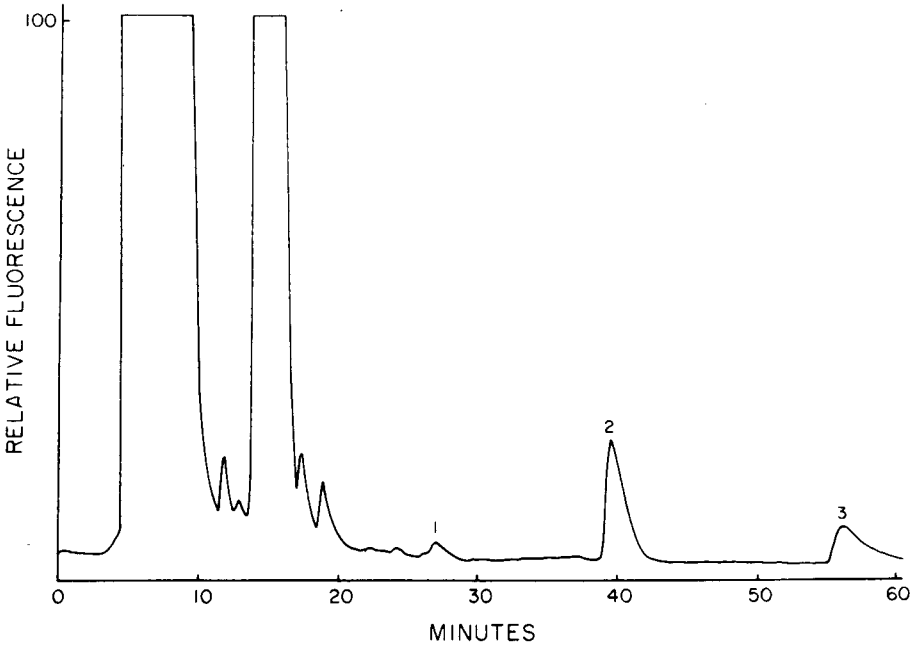

Fig. 2. Chromatogram of an acid extract of the liver of a normal 300-g rat. The tissue was extracted with an equal volume of 0.01 *M* hydrochloric acid. The extract was then precipitated with 10% trichloroacetic acid. The supernatant was hydrolyzed with 8.3 *M* hydrochloric acid at 110°C for 18 h. The hydrochloric acid was then removed in vacuo and the dry residue taken up in distilled water and applied to the column. The peaks were quantitated by standards run before and after the analysis. The tissue levels of putrescine (peak 1), spermidine (peak 2) and spermine (peak 3) (34, 938 and 757 nmol/g wet weight respectively) are in agreement with those recorded in the literature [21]. In addition to the amino acid peaks, several other peaks appear, which have not been identified with certainty.

validates the warranty on such pumps as the Altex which we use. Others have commented on the corrosive effect of chloride-containing buffers, though minimizing the damage it does to the actual valve system [14]. In contrast, the buffers we propose are entirely harmless to the fittings of the HPLC system, since nitrate ion is inert and produces no corrosion. It does not interfere with the assay and is compatible with the compounds under study.

The ability of the system to separate most of the known metabolites of the polyamines from the amino acids present in biological materials as well as from one another without requiring additional steps is a particular advantage in performing metabolic studies. Moreover, because the column can be reconstituted by a wash with the initial buffer for only 20 min, it is suitable for repeated cycling and could easily be automated. In our experience, the columns are suitable for at least 200 cycles, if maintained properly according to the instructions of the manufacturers.

We preferred to use the unique synthesis described here in preparing the monoacetyl derivatives of spermidine because it avoids forming the isomer

which is inevitably present in a synthesis using spermidine as the starting material. Thus, the more cumbersome sequence of synthetic chemical steps was selected in these syntheses because of the level of purity desired in the product.

ACKNOWLEDGEMENTS

We are grateful for the assistance of Dr. John W. Kozarich and Mr. Peter Demou in preparing and interpreting the NMR spectra. Mr. Robert N. Dryer's advice was invaluable in setting up the HPLC system. We thank Drs. H. and C.W. Tabor and Dr. H.G. Williams-Ashman for helpful discussions. Ms. Leslie A. Lande provided valuable technical assistance.

The research was supported by funds from the Veterans Administration.

We wish to acknowledge the support of the NSF Northeast Regional NMR Facility at Yale University which is funded by grant No. CHE-7916210 from the Chemistry Division of the NSF.

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Journal of Chromatography, 224 (1981) 381—388
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 890

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY QUANTITATION OF N-ACETYLNEURAMINIC ACID IN MALIGNANT MELANOMA AND BREAST CARCINOMA

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(First received January 5th, 1981; revised manuscript received March 5th, 1981)

SUMMARY

A rapid isocratic high-performance liquid chromatography (HPLC) method for quantitation of serum N-acetylneuraminic acid (NANA) is described. Separation is achieved on an Aminex HPX-87 cation-exchange resin using a 0.006 *N* sulfuric acid mobile phase. Compared with the more conventional thiobarbituric acid (TBA) method, HPLC is more reliable and has a much improved maximum sensitivity of 0.8 nmol/ml. In a limited study of malignant melanoma patients' sera, HPLC gave slightly higher values for NANA than TBA. In a more detailed study of breast carcinoma patients with measurable tumour burden, HPLC and TBA methods were used on the same sera and compared with concurrent carcinoembryonic antigen (CEA) determinations. HPLC resulted in a clear improvement in discrimination between tumour burden groups compared with either the TBA method or CEA.

INTRODUCTION

We have been interested in the clinical significance of sialoglycoproteins in

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cancer patients' sera. Our studies have shown that serum sialoglycoprotein elevations, as reflected by total serum sialic acid (N-acetylneuraminic acid, NANA), occur in association with a variety of neoplasms and that these elevations are related to tumour burden and prognosis [1—3]. This suggests that total serum NANA can be a valuable clinical tumour marker. In these studies NANA was measured by the thiobarbituric acid (TBA) method [2], essentially as described by Warren [4]. While this method is relatively inexpensive and easy to perform, there may be problems with interfering substances [4], the interassay variation is suboptimal [2], and the basic procedure is relatively time consuming.

The several methods devised for quantitation of sialic acids vary in complexity, sensitivity and specificity. We have examined high-performance liquid chromatography (HPLC) as a method of NANA measurement. In addition to reliability, sensitivity and selectivity, we wished to determine if the relative simplicity of HPLC would lend itself to use as a clinical NANA assay. In this study we describe an HPLC assay for NANA, compare this with the TBA method [2], and illustrate how HPLC may be used for clinical measurement of NANA in cancer patients' sera.

MATERIALS AND METHODS

Serum collection

Whole blood was collected in 10-ml glass vacutainer tubes and the samples allowed to clot at room temperature for 1 h. After centrifugation at 500 *g* for 10 min, the sera were removed, placed in polypropylene tubes and stored at −70°C until used. Prior to assay, the serum samples were allowed to thaw at room temperature. The reliability of this procedure has been previously examined by us [2].

TBA assay

NANA was hydrolyzed from serum sialoglycoproteins by incubation for 1 h at 80°C in a mixture of 0.1 ml test serum with 2 ml 0.1 *N* sulfuric acid. The hydrolysate was then cooled for 5 min in a 20°C water bath. Further analysis was essentially as described by Warren [4], but with the modifications as described by us previously [2]. As previously reported by us, the intra-assay variation (± 2 S.D.) was 1.9% and the interassay variation was 10%.

HPLC

Sialoglycoprotein hydrolysis was performed as for the TBA assay, except that 0.23 μmol/ml N-acetylglucosamine (NAGA) (Sigma, St. Louis, MO, U.S.A.) was incorporated as an internal standard. NANA was analyzed by cation exclusion chromatography on a Model 7000B chromatograph (Micromeritics, Norcross, GA, U.S.A.) using an Aminex HPX-87 strong cation-exchange resin column designed for organic acid analysis, 300 × 7.8 mm at 42°C (Bio-Rad Laboratories, Richmond, CA, U.S.A.). A mobile phase of 0.006 *N* sulfuric acid was used at a flow-rate of 0.65 ml/min and a pressure of $7 \cdot 10^6$ N m^{-2}. The column effluent was monitored by a UV detector at 206 nm and 0.05 absorbance span, using an LKB 2138 Uvicord S detector fitted with an

HPLC micro flow-cell and the photodiode output recorded at a chart speed of 0.33 cm/min. The injection volume for each sample was 50 μl and the total run time per serum sample was 20 min.

NANA content of test samples was calculated from peak height ratios using the NAGA described above as an internal standard and referring to a standard curve using concentrations of NANA (Sigma) from 0.32 to 6.5 μmol/ml in 0.1 *N* sulfuric acid.

Mass spectrometry

Following injection of NANA standard and serum hydrolysate, column effluent was collected at 0.5-min intervals and fractions representing UV absorbance peaks were pooled and lyophilized.

The lyophilized material was placed in a Reacti-Vial (Pierce, Rockford, IL, U.S.A.) and 25 μl of N,O-bis-trimethylsilyl-trifluoroacetamide (Pierce) added. The stoppered tube was then heated in a heating block until the silylation reaction was complete as indicated by total solubilization of the substances [5, 6]. Trimethylsilyl ether-ester derivatives were immediately subjected to high-resolution mass spectrometric analysis on a Finnigan Model 4000 mass spectrometer. The 70-eV electron impact ionization fragmentation patterns were interpreted according to the mass spectra described by Kamerling and Vliegenthart [7].

Stability studies

NANA standards, NAGA standards and serum samples were heated in 0.1 *N* sulfuric acid at 80°C for 1, 5 and 24 h. Standards and serum in distilled water were incubated in parallel for comparison. Additional hydrolyzed serum samples were analyzed immediately and compared with the same hydrolysate analyzed after maintenance at 4°C for 24 and 48 h.

Patients

Forty malignant melanoma patients were selected, representing a broad range of serum NANA concentrations as determined by the TBA method. Fifty-eight breast carcinoma patients were selected for study on the basis of objective measurable tumour burden and assigned to tumour burden groups much as we have previously described [2]. Group I included 20 patients with no evidene of residual neoplasm between 4 and 8 weeks after surgical excision of all known carcinoma; group II, 18 patients, had limited recurrent disease confined to the chest wall and estimated at less than 5 g; and group III consisted of 20 patients known to have more advanced regional or distant metastatic disease estimated at more than 5 g. Breast carcinoma patients had plasma carcinoembryonic antigen (CEA) determined by the Roche method (Roche Laboratories, Nutley, NJ, U.S.A.) on samples obtained at the same time as serum for sialic acid. In addition to specimens from cancer patients, 40 normal control sera were selected from our serum bank for comparison. These were from 40 females with an age range of 19—91 year. All sera were analyzed for sialic acid by both the TBA and HPLC methods as described above. Thirty of these control sera were also available for CEA testing.

RESULTS

HPLC of NANA

The elution of a standard NANA solution from the Aminex HPX-87 column with retention time of 7.8 min is identical to that of the second UV absorbance peak of the serum hydrolysate (Fig. 1). The eluate representing this peak was collected and the trimethylsilyl derivative analyzed by mass spectrometry. Characteristic derivative fragmentions of 668, 624, 478, and 298 daltons identified NANA [7].

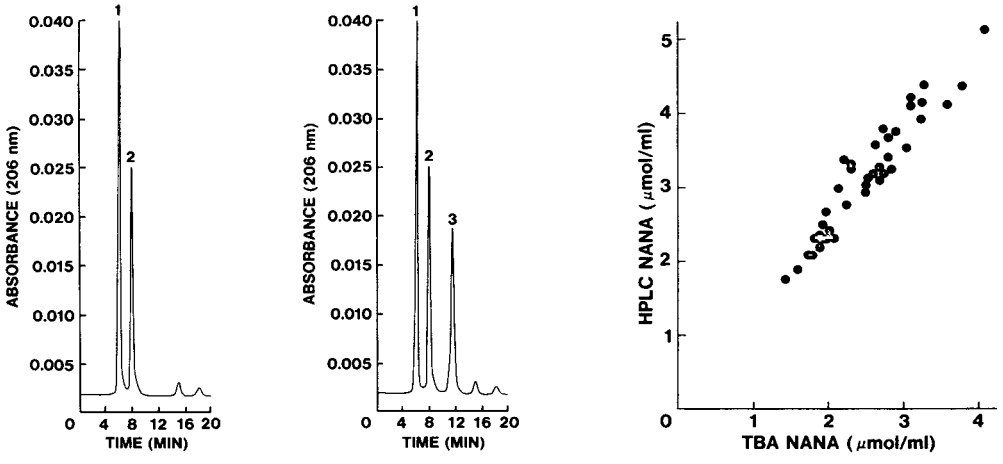

Fig. 1. Chromatogram of serum hydrolysate. Retention time of NANA (peak 2) = 7.8 min. 1 = Acid injection peak.

Fig. 2. Chromatogram of serum hydrolysate with added 0.23 μmol/ml NAGA as internal standard. Retention time of NAGA (peak 3) = 11.36 min. 2 = NANA. 1 = Acid injection peak.

Fig. 3. Linear regression analysis of TBA and HPLC methods for NANA in malignant melanoma patients. Correlation coefficient = 0.98.

The chromatogram of NAGA added to serum hydrolysate is displayed in Fig. 2. NAGA elutes as a sharp peak at 11.36 min, independent of other eluted material. This suggests NAGA could serve as an internal standard provided it remained stable under assay conditions. The effect of heat and acid were examined by HPLC after incubation of serum and standard NANA and NAGA solutions in 0.1 *N* sulfuric acid for 1, 5 and 24 h. The loss for NAGA was acceptable with a reduction of 2.5% in the first hour, decreasing to 1.7% per hour in the final observation period. The reduction in NANA during the first hour was 9.5% in keeping with results of others [8, 9]; decreases in the final observation period were 2.8% per hour. Storage of hydrolysate or standard solutions of NANA or NAGA at 4°C after the standard 1 h hydrolysis at 80°C did not cause any further loss up to 48 h.

NANA concentration was linearly related to peak height, and this relationship was maintained even at low NANA concentrations. The maximum sensitivity for NANA in our system was 0.8 nmol/ml with a signal-to-noise ratio > 3.

On the basis of the above results the HPLC assay detailed in Materials and Methods was used for the analysis of serum samples. Intra-assay variation on 20 serum analyses was 2.1 ± 2% S.D. and the interassay variation on 15 analyses was 5.2%.

Serum studies

HPLC and TBA assays were directly compared in 40 malignant melanoma patients. In each case, both assays were performed on the same serum sample. As shown in Fig. 3, the concordance was very high (correlation coefficient = 0.98). This illustration also suggests HPLC tended to give slightly higher values for NANA than the TBA method. Fig. 4 shows this more clearly and demonstrates that the difference is significantly greater at higher NANA concentrations (F = 16.04 with 2 and 77 degrees of freedom, $p < 0.001$). In this analysis the normal control sera were included to provide NANA values in the lower range.

NANA results for each tumour burden group of the 58 breast carcinoma patients are shown in Fig. 5. Again, generally higher values are apparent for

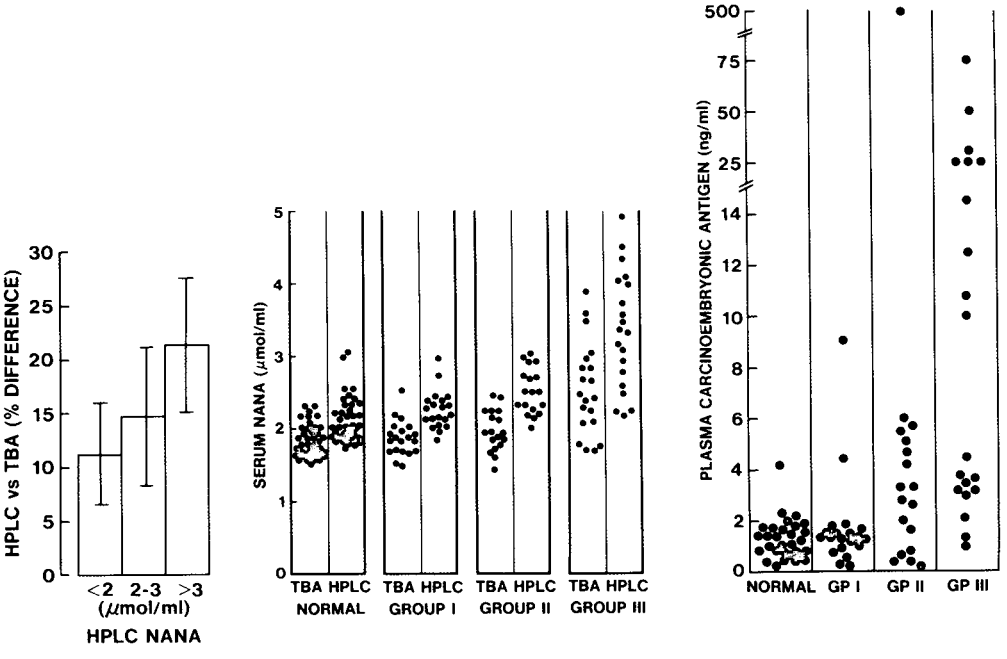

Fig. 4. Relationship of TBA and HPLC methods. Ordinate: percent difference between HPLC and TBA: $\left(\frac{\text{HPLC NANA} - \text{TBA NANA}}{\text{HPLC NANA}}\right) \times 100$. Abscissa: three ranges of HPLC NANA values.

Fig. 5. NANA serum concentrations in normals and breast carcinoma patients determined by both TBA and HPLC on the same sera.

Fig. 6. CEA concentrations concurrently determined on the same breast carcinoma patients represented in Fig. 5. Thirty concurrent normal control plasma were available for CEA testing.

the HPLC method. Results of pairwise statistical analyses by the Mann—Whitney test are displayed in Table I. While there was significant discrimination between groups for HPLC in each case, TBA results did not attain statistical significance for groups I vs. normal, I vs. II, and II vs. normal. Of the remaining three comparisons, confidence levels (p values) were more remarkable for HPLC in each case.

TABLE I

BREAST CARCINOMA GROUP COMPARISONS (MANN—WHITNEY TEST) FOR NANA BY HPLC, NANA BY TBA METHOD, AND CEA

Groups compared	Significance (p)		
	HPLC	TBA	CEA
I vs. normal	0.02	NS*	NS**
I vs. II	0.006	NS	0.02
II vs. III	0.0005	0.002	0.01
II vs. normal	0.00007	NS	0.004**
III vs. I	0.000004	0.0002	0.000009
III vs. normal	0.00000002	0.000005	0.0000001**

*NS, not significant, $p > 0.05$.
**Normal control group included 30 sera.

Concurrent plasma CEA values were available for all breast carcinoma patients and 30 of 40 normal controls (Fig. 6). Pairwise statistical analyses (Table I) appeared marginally more significant for CEA than TBA NANA, but in each case confidence levels were more remarkable for HPLC NANA than CEA.

DISCUSSION

Methods for detection and quantitation of sialic acids have relied principally on colorimetric procedures using reagents such as resorcinol [10], diphenylamine [11], dimethylaminobenzaldehyde [12], and thiobarbituric acid [4, 13]. Most of these procedures are relatively insensitive and lack specificity. Although fluorimetry [8, 14] offers a relatively simple means of improving sensitivity, interfering substances pose a problem [15]. Specificity can be greatly improved through additional preliminary purification steps [15—18], or, to a lesser extent, by development of empirical mathematical formulas to control for interfering substances [4]. Other assays have improved specificity, but more elaborate laboratory procedures impose additional practical limitation. The enzymatic assay of Brunetti et al. [19] requires purified N-acylneuraminic acid aldolase. The ion-exchange method of Svennerholm [17] is time consuming, and both gas—liquid chromatography and mass spectrometry require substrate conversion to volatile derivatives [6, 7, 20, 21].

In contrast to colorimetric methods, HPLC offers the potential of improved sensitivity and selectivity and the advantage of ease of operation compared with the more elaborate procedures.

In our study the HPLC assay was compared directly with the TBA method. In general, HPLC is more easily performed and more reliable. The interassay variation of 5.2% was almost half that of the TBA method [2]. A combination of factors may be responsible for the improved reliability of HPLC analysis: the assay is more direct, NAGA is used as an internal standard, and mathematical manipulations for controlling interfering substances are eliminated. HPLC was 250-fold more sensitive. The lower limit of sensitivity of 0.8 nmol/ml is comparable to more elaborate sensitive chromatographic techniques [22—24]. Fluorimetry can be more sensitive, but there are associated problems with specificity. Although we have not demonstrated absolute specificity for NANA using HPLC, we have not detected any interfering substances, and this is supported by our mass spectrometric studies.

Comparative serum studies have shown general concordance between HPLC and TBA methods (Fig. 3). The source of limited but consistent higher NANA results by HPLC may be related to as yet unidentified effects of serum on the Aminex HPX-87 column during HPLC. The discrepancy between assays is perhaps better explained by serum interference in the TBA assay [25—27]. For example, L-fucose, known to be elevated in breast cancer patients such as ours [28, 29], inhibits color formation in the TBA assay as do many other serum components [30].

Serum NANA elevations have previously been reported in breast carcinoma patients. In a limited study Macbeth and Bekesi [31] reported that NANA elevations in breast carcinoma were confined to advanced disease patients. As part of a larger study Mrochek et al. [23] found a correlation between serial NANA levels and response to treatment in 21 unstaged patients. Hogan-Ryan et al. [32] have recently reported on serum NANA levels in 19 advanced disease patients and 46 patients sampled after primary surgery when there would presumably have been no evidence of residual disease. While significance levels are not reported, those with advanced disease clearly had higher serum NANA concentrations. In a comparison with CEA, the authors felt that NANA was possibly a better tumour marker. Since we studied a different selected population and used a different CEA test, our results are not strictly comparable to those of Hogan-Ryan et al. [32]. However, it is of interest that our results for NANA measured by TBA are in keeping with those reported by others.

Of greater importance, our breast carcinoma study illustrates the potential advantage of HPLC for serum NANA quantitation. Pairwise statistical analysis demonstrated a clear improvement in discrimination among tumour burden groups for HPLC NANA compared with either TBA NANA or CEA. The apparent HPLC sensitivity for relatively small tumour burden is also in contrast to our previous patient studies using the TBA method [2, 33—35]. Since the same sera were used for both HPLC and TBA measurement, the improved discrimination of HPLC in this study is best explained by the superior intra- and interassay reliability.

In our hands the simplicity and reliability of HPLC quantitation of NANA make it a valuable laboratory tool with ready application for clinical use.

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Journal of Chromatography, 224 (1981) 389—397
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 883

QUANTITATION OF INDIVIDUAL TOCOPHEROLS IN PLASMA, PLATELETS, LIPIDS, AND LIVERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received October 8th, 1980; revised manuscript received February 25th, 1981)

SUMMARY

A rapid, sensitive procedure is described for the analysis of the tocopherols (α-, β-, γ-, and δ-) in plasma, platelets, lipids, and liver using high-performance liquid chromatography and fluorometric detection. Excellent recoveries of these tocopherols in plasma were obtained — greater than 90%. Separation and quantitation of the four tocopherols required as little as 0.2 ml plasma.

INTRODUCTION

In order to ascertain vitamin E deficiency syndromes in laboratory animals and the nutritional status of man with respect to this vitamin, investigators should include an assessment of plasma vitamin E levels. In addition, in order to demonstrate or verify the absence of the nutrient in the diet, similar analyses for vitamin E of the dietary preparations should be performed. Investigators have employed serum creatine phosphokinase (ATP:creatine phosphotransferase, EC 2.7.3.2) activity [1—3] or urinary creatine [4] measurements as status parameters; creatinuria, however, may result from other conditions such as choline or potassium deficiencies [5]. In the past, however, tocopherol analyses required laborious, cumbersome techniques, including column or thin-layer chromatography, which often lead to poor recoveries. Most analytical procedures were based primarily on the Emmerie—Engel reaction [6]. Such determinations lack specificity because other substances are often present that will reduce the resultant chromogenic complex and the individual tocopherols

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0378-4347/81/0000—0000/$02.50

also react at varying rates to produce the reduced compound which is measured spectrophotometrically. The former limitation has been partially eliminated by elaborate sample preparation to remove reducing artifacts. Several investigators have also described a thin-layer chromatographic separation of these artifacts from the tocopherols and the use of gas—liquid chromatography for final separation and quantitation of the tocopherols [7, 8]; these procedures are rather lengthy.

Several investigators recently reported high-performance liquid chromatographic (HPLC) techniques that obviate such complicated methodologies for the quantitation of vitamin E. Hatam and Kayden [9] have employed a reversed-phase microparticulate column and fluorometric detection with excitation at 205 nm for the determination of tocopherol content of plasma and cellular components of blood. These investigators did not, however, report the separation of the positional isomers, β- and γ-tocopherols. Taylor et al. [10] measured total tocopherol content of homogenates and subcellular components from rat tissues using fluorometric analysis; these researchers employed 290 nm excitation instead of 215 nm because severe quenching problems reportedly limited the utility of the latter absorption maxima. Abe et al. [11] have also developed an HPLC technique for the determination of α-, β-, γ-, and δ-tocopherols using a spectrofluorometer with excitation at 295 nm; quantitative measurements for these components found in serum [12] and liver [13] were reported. Thompson and Hatina [14] have also reported the quantitation of the tocopherols as well as the tocotrienols in food and animal tissue homogenates employing fluorometric detection in conjunction with HPLC. Bieri et al. [15] have reported an HPLC procedure for simultaneous determination of α-tocopherol and retinol in plasma and the former in erythrocytes, using a reversed-phase microparticulate column and UV absorbance detection; the positional isomers were not separated.

The current investigators sought to verify the universality and reliability of an HPLC—fluorometric technique that was developed in this laboratory [16] for the quantitation of tocopherols found in plasma and casein. Because of the methodological inadequacies of earlier methods, the current investigators were prompted to develop a sensitive, reliable, versatile, and rapid technique. The coupling of fluorometric detection (with saponification) and HPLC provided a unique method for vitamin E analyses in a variety of biological materials.

EXPERIMENTAL

Analytical instrumentation

A Perkin-Elmer Model 1250 liquid chromatograph (Norwalk, CT, U.S.A.) in conjunction with a Fluorichrom filter fluorometer (Varian, Palo Alto, CA, U.S.A.), with a 25-μl cell volume and equipped with a deuterium lamp was employed in this study. Fluorescence was recorded on an Omniscribe B-500 1-mV strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). The analytical column was a microparticulate 5-μm silica gel normal-phase column, Partisil PXS5 (Whatman, Clifton, NJ, U.S.A.), 25 cm × 4.6 mm I.D., preceded by a guard column, 10 cm × 4.6 mm I.D., packed with Corasil II (Waters Assoc., Milford, MA, U.S.A.). The mobile phase was 1.4% isopropanol in hexane

(MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.). Flow-rates approximated 1.2 ml/min. An interference filter was used for excitation of column effluent at 200 nm and band pass filters for emission monitoring at 340 nm.

Four tocopherol standard (α-, β-, γ-, and δ-tocopherols) solutions containing 0.1 mg tocopherol per ml hexane were prepared; equal-volume aliquots of each were mixed together and serially diluted 1:10 with hexane. Standard calibration mixtures were injected every three or four sample injections in order to ensure the constancy of detector response. Sample concentrations were calculated using peak height and standard calibration curves. A typical calibration curve is depicted in Fig. 1. These calibration curves were calculated by linear regression. Excellent linearities were exemplified by correlation coefficients of 0.985, 0.994, 0.993, and 0.992 ($P < 0.001$) for α-, β-, γ-, and δ-tocopherols, respectively.

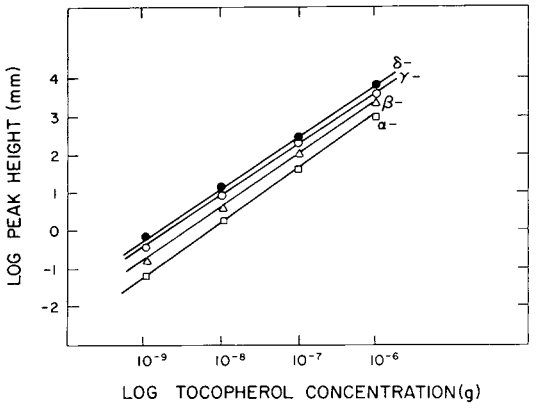

Fig. 1. Calibration curve for tocopherols by HPLC analysis.

Finally in order to ascertain the identity of the four tocopherols, standard solutions of each (approximately 1 mg tocopherol per ml hexane) were prepared for mass spectrometric (MS) analysis on a Hitachi Model RMU-7 electron impact mass spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). An equal-volume mixture of the tocopherol standards was also injected into the chromatograph and the eluate collected in fractions for MS analyses in order to verify the characteristic spectra of each tocopherol. A plasma extract was selected for MS analysis once the investigators observed both β- and γ-tocopherols present by chromatographic retention data. The plasma extract was then reinjected and the eluate collected in fractions which included all four tocopherol components; these fractions were then submitted to MS analysis. The β- and γ-tocopherols are known to be positional isomers which elute closely after one another [17]; the spectra for the β- and γ-tocopherols eluted from the column are shown in Fig. 2. Fragmentation patterns for the two tocopherols were quite similar except for differences in the relative intensity of the molecular ion peak at *m/e* 416 and the fragmentation peak at *m/e* 151. The spectra of all four tocopherols correspond closely to that obtained for each standard and to that reported by De Leenheer et al. [18].

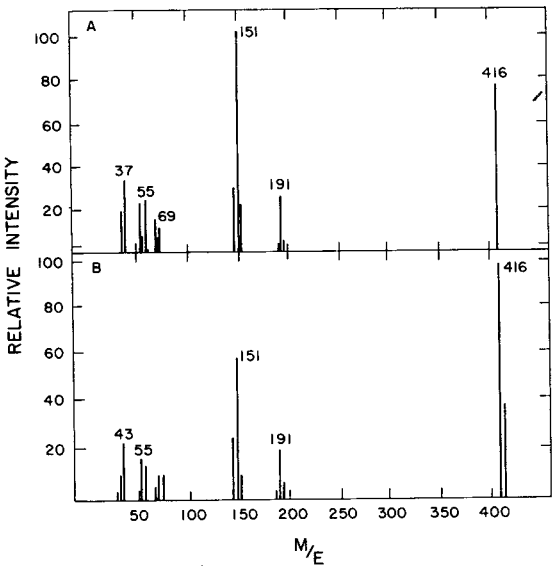

Fig. 2. Mass spectra of plasma HPLC eluates corresponding to (A) γ-tocopherol and (B) β-tocopherol.

Recoveries of the tocopherols were determined by standard additon; mixtures containing both 1 and 5 μg of each of the tocopherols were added to each type of sample matrix at the onset of sample preparation. Recoveries were calculated by comparing "spiked" values to duplicate analyses to which no tocopherols were added.

Plasma determinations

Blood samples (approximately 2 ml) from five male New Zealand white rabbits (Dutchland Laboratory Animals, Denver, PA, U.S.A.) 10 weeks of age were drawn from the central auricular artery using disposable syringes. Blood samples were also obtained from four males, 23—31 years of age. Blood was collected in polypropylene tubes containing 0.2 ml acid—citrate dextrose (ACD) as the anticoagulant. Tubes were stoppered and gently inverted to ensure adequate mixing of anticoagulant. Blood was stored in ice and subsequent analyses were performed in the dark. Blood was centrifuged at 3000 *g* for 10 min at 4°C. Plasma extraction of tocopherols was adapted from the procedures described by De Leenheer et al. [18] and by Abe and Katsui [19], except for the utilization of antioxidants in both aqueous and organic solvents — 3% pyrogallol in absolute ethanol and 0.025% butylated hydroxytoluene (BHT) in *n*-hexane. Following a two-fold hexane extraction, samples were subsequently evaporated under argon and redissolved in 0.2 ml *n*-hexane.

Platelet determinations

Blood, 5 ml, was obtained from rabbits by cardiac puncture or venipuncture from four young Caucasian males. Blood was collected in polypropylene tubes containing 0.6 ml ACD solution. The contents of these tubes were then centrifuged at 100 *g* for 15 min at 21°C to obtain platelet-rich plasma (PRP); following multiple washings of PRP the resultant platelet pellet was resuspended with 1 ml 0.154 *M* sodium chloride solution [20]. An aliquot

(6.6 μl) was then obtained for counting platelets on a Coulter Counter ZBI (Coulter Diagnostics, Hialeah, FL, U.S.A.) [21] and the platelet suspension was adjusted to contain roughly $1 \cdot 10^{10}$ cells.

The remaining resuspended platelet samples were mixed with 1 ml absolute ethanol containing 3% pyrogallol and tubes were allowed to sit for 5 min; 2 ml *n*-hexane containing 0.125% BHT were added to each sample tube. Tubes were then shaken for 10 min followed by centrifugation at 5000 *g* for 5 min at 21°C. The extraction was similar to that described above for plasma.

Liver determinations

Rabbit livers and commercially obtained calf liver, approximately 0.5 g, were homogenized in ice with 2 ml absolute ethanol and 2 ml 1% EDTA, disodium salt. The mixture was then preincubated at 70°C for 5 min. While samples were continuously flushed with argon, 1 ml of 60% aqueous potassium hydroxide solution was added; again contents were mixed vigorously and warmed for an additional 15 min in a 70°C water bath. Tubes were then placed in ice to cool and twice extracted with 6 ml *n*-hexane containing 0.125% BHT. The procedure was performed in a manner similar to those previously described.

Lipid determinations

Aliquots, about 0.5 g, of commercial oils or shortenings were transferred into test tubes; 2 ml of 3% pyrogallol in absolute ethanol were added to the contents of each tube. Contents were then mixed and flushed with argon. Samples were subsequently heated for 15 min in a 70°C water bath. Then 0.5 ml of 60% aqueous potassium hydroxide solution was added to each sample under a continuous stream of argon. Again the contents were heated for an additional 3 min, cooled in ice, and to each tube 2.5 ml deionized water and 3 ml *n*-hexane containing 0.125% BHT were added. Again, extraction was implemented as described earlier.

Dietary preparation determinations

Diets similar to that of Gaman et al. [22] were prepared according to specifications. Aliquots, approximately 1 g and 2.5 g, of pelleted rations were crushed and added to 4 ml absolute ethanol containing 3% pyrogallol in glass stoppered test tubes. Saponification and extraction were conducted in a similar fashion as described previously for liver.

RESULTS AND DISCUSSION

Typical chromatograms of extracts from plasma of a 16-week old rabbit and of an aliquot of control dietary preparation are depicted in Fig. 3. Very few plasma extracts were found to contain all four tocopherols; in fact, only one rabbit in each of two purchases of animals was observed to possess detectable quantities of β-tocopherol. α-Tocopherol is considered to be the most predominant form in plasma, followed by γ-, δ-, and β-tocopherols [23, 24]. In analyses of control diet aliquots, the present investigators observed significant quantities of both α- and γ-tocopherols. α-Tocopherol acetate does not fluoresce [14] but following saponification, quantitation of α-tocopherol was possible. Corn oil in the diet preparation was expected to contribute

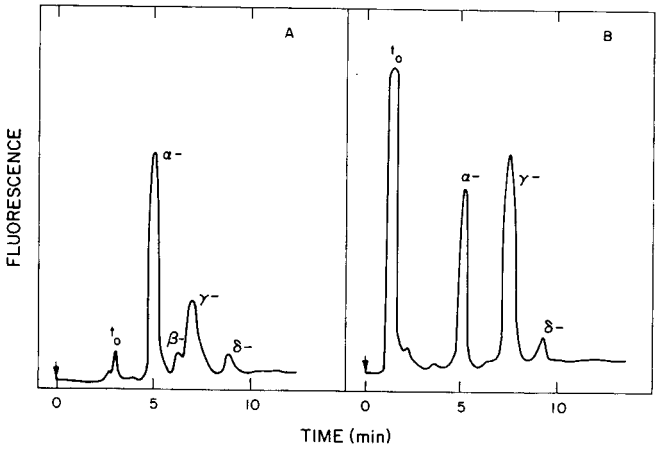

Fig. 3. HPLC separation of tocopherols from extracts of (A) plasma from control rabbit and (B) control diet.

considerable amounts of γ-tocopherol and lesser amounts of α- and δ-tocopherols; analyses of corn oil alone described later confirmed this expectation.

The tocopherol content of several animal tissues are given in Table I. Values for plasma tocopherols in rabbits are in agreement with those of Horn et al. [25] and Diehl and Kistler [26]. Horn et al. [25] utilized a microspectrophotometric technique based upon the Emmerie—Engel reaction for the determination of plasma total tocopherols in New Zealand white male rabbits fed a synthetic diet containing 225 mg DL-α-tocopherol per kg diet; plasma vitamin E levels of control rabbits were reported to be at least 1000 μg/dl. Diehl and Kistler [26] determined serum total tocopherol levels in New

TABLE I

VITAMIN E CONTENT OF SELECTED ANIMAL TISSUES

Values represent means ± S.D. Each analysis was performed in duplicate; the number of samples represents the number of animals used in independent analyses.

Sample	No. of samples	Tocopherol				
		α-	β-	γ-	δ-	Total
Rabbit plasma (mg/dl)	5	0.81 ±0.25	nd*	0.14 ±0.06	nd	0.95 ±0.23
Human plasma (mg/dl)	4	1.05 ±0.22	nd	0.14 ±0.06	0.10 ±0.01	1.25 ±0.21
Human platelets ($\mu g/10^{10}$ cells)	3	4.04 ±0.82	nd	0.96 ±0.06	0.10 ±0.00	5.10 ±0.82
Rabbit liver (μg/g wet wt)	3	20.61 ±4.59	nd	0.74 ±0.06	0.50 ±0.00	21.85 ±4.27
Calf liver (μg/g wet wt)	3	14.23 ±2.67	nd	1.89 ±0.58	nd	16.12 ±3.73

*nd = not detected.

Zealand white rabbits fed a commercial rabbit chow reported to contain 8 mg/kg diet; a similar spectrophotometric procedure was employed and control rabbits exhibited a range of 0.5—1.0 mg tocopherol per dl sera. In contrast, Caravaggi [27] analyzed sera obtained from New Zealand white rabbits by a microspectrophotometric method and observed 0.34 ± 0.43 mg total tocopherol per dl ($\overline{X}$ ± S.D.); the dietary content of vitamin E was not reported. The plasma α-tocopherol level reported by Ishibashi et al. [12] for three rabbits of unknown strain (0.08 ± 0.01 mg/dl, $\overline{X}$ ± S.D.) was nearly 10 times smaller; α-tocopherol is the major component present in plasma. The latter investigators employed an HPLC technique similar to the current researchers with saponification and fluorometric detection; interestingly, human plasma α-tocopherol levels reported in the same study did approximate values reported in the current study.

With regard to platelet determinations, values obtained in the present study for total tocopherols are in agreement with those reported by other investigators. Hatam and Kayden [9], using an HPLC—fluorometric technique, observed 5.10 ± 0.63 μg α-tocopherol per 10^{10} platelets ($\overline{X}$ ± S.D.): in six healthy male subjects, these investigators reported that γ-tocopherol represented 11.4 ± 3.3% ($\overline{X}$ ± S.D.) of the total tocopherols in platelets. Nordøy and Strøm [20], using a spectrophotometric procedure, observed similar levels of total tocopherols in platelets from 12 male subjects.

Liver analyses for vitamin E content should be treated with caution; tocopherol measurements in wet aliquots are often different by 10-fold or greater. Additionally, data may be further subject to question because studies have demonstrated the diminutive effect of hepatic coccidiosis on the vitamin E status of rabbits [26, 28]. Values obtained for control rabbit livers in this study were slightly greater than those reported from non-infected rabbits in the study of Diehl [28]; the latter investigator used New Zealand rabbits of mixed age and sex which were given a commercial chow containing only 8 mg/kg diet. Draper and Csallany [29] observed a greater concentration of α-tocopherol (approximately 30 μg/g fresh tissue) in livers from rabbits; the strain was not reported and animals were of mixed age and sex; these animals were given a low tocopherol diet supplemented with α-tocopheryl acetate at 1 g/kg. Analyses included extraction and column chromatography, followed by a spectrophotometric determination. Factors that may have considerable influence on tissue tocopherol content are age, sex, dietary vitamin E content, and how long the diet was fed. Values for calf livers are similar to those reported in the literature [30, 31].

Since vegetable oils are known to contain significant quantities of the tocopherols, the present investigators analyzed representative oils obtained commercially (Table II). Note the large quantity of β-tocopherol present in wheat germ oil. Data obtained here are in agreement with those previously reported [31, 32]; such agreement exemplifies the degree of reliability and the advantages of such rapid methodology.

Also included in Table II are data obtained for tocopherol content of diets prepared commercially for the present study. Note only minute amounts of δ-tocopherol are present in the control ration. In the deficient preparation only β-tocopherol was detected.

TABLE II

VITAMIN E CONTENT OF SELECTED FOOD MATERIALS

Values represent means ± S.D.

	No. of replicates	Tocopherol (mg/100 g)				
		α-	β-	γ-	δ-	Total
Wheat germ oil, Rexall	5	120.18 ±10.96	71.4 ±4.6	9.42 ±0.17	nd*	201.00 ±10.03
Corn oil, Mazola	4	6.44 ±0.06	nd	68.97 ±3.89	0.92 ±0.03	76.33 ±3.65
Vegetable oil, Crisco	4	7.61 ±0.02	nd	40.23 ±1.72	9.46 ±0.13	57.30 ±1.52
Vegetable shortening, Crisco	4	9.12 ±0.31	nd	66.22 ±2.19	24.45 ±1.15	99.79 ±2.04
Diet, control	4	9.12 ±0.07	nd	3.66 ±0.04	0.31 ±0.01	13.09 ±0.05
Diet, deficient	4	nd	nd	0.00** ±0.00	nd	0.00 ±0.00

*nd = not detected.
**Actual mean was 0.052 μg/100 g.

Recoveries of the 1- and 5-μg spikes in all kinds of biological samples tested ranged from 94—99% for α-, 86—94% for β-, 93—99% for γ-, and 89—92% for δ-tocopherol. Typically the lower per cent recoveries were observed in liver samples.

This HPLC technique appears to be quick and reliable as well as sensitive. The wide applicability of such a method can be justified by the diversity of materials analyzed in this study. Such methodology undoubtedly merits greater consideration for researchers interested in the separation and quantitation of tocopherols.

ACKNOWLEDGEMENTS

The authors thank Hoffmann-La Roche, Nutley, NJ, U.S.A. and Eisai Research Labs., Tokyo, Japan for generously supplying tocopherol standards, Gary Anderson for assistance in rabbit phlebotomies, and Jorge Bedia for mass spectrometric analyses.

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Journal of Chromatography, 224 (1981) 399—405
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 886

COMBINED PAPER AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE STUDY OF PREGNENOLONE AND PROGESTERONE METABOLITES

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(First received December 17th, 1980; revised manuscript received March 11th, 1981)

SUMMARY

A method for the separation and quantitation of steroid metabolites, obtained from enzymatic conversions of tritiated pregnenolone or progesterone, is described. As the first step, paper chromatography is used and the different zones obtained, as monitored by radiochromatogram scanning, are then eluted separately from the paper. The final resolution is achieved by reversed-phase high-performance liquid chromatography in 35% acetonitrile with UV detection at 215 nm. The method has been successfully applied to the study of metabolite patterns obtained from steroid conversion produced by testicular biopsy material under conditions of in vitro incubations.

INTRODUCTION

Methods for the investigation of androgen biosynthesis in the testis have been developed by Steinberger and Ficher [1] and by Tcholakian and Eik-Nes [2], using in vitro incubations of testicular biopsy specimens with a radiolabelled Δ^4-steroid as precursor. After extraction the metabolites were separated by paper and thin-layer chromatography (TLC) and quantified by means of liquid scintillation counting. However, the transfer of the radioactive material from TLC plates requires exact localization of the spots, collection of the layer containing the activity and elution, a very tedious and time consuming procedure. To accomplish adequate separation between different steroids, derivatization is required in certain cases. When [^{3}H] pregnenolone is used as precursor the increased number of possible metabolites requires further TLC steps in order to obtain complete separation. In our opinion, reversed-phase high-performance liquid chromatography (HPLC) as a separation tool should possess definite

0378-4347/81/0000—0000/$02.50

advantages compared to TLC due to its superior resolution, speed and reproducibility. O'Hare et al. [3] in 1976 published a method which combines different gradient elution HPLC systems to separate testicular steroids. A recent report by Cochran and Ewing [4] describes a study on the use of HPLC after Celite column chromatography for the separation of 14 testicular steroids.

In the present paper a systematic investigation of the use of paper chromatography, followed by reversed-phase HPLC for the separation and quantitation of metabolites formed from [^{3}H] pregnenolone and [^{3}H] progesterone via the Δ^5 and Δ^4 pathways, is described in detail. An improved method is proposed for separation and quantitation of the metabolites formed after enzymatic conversions by means of testicular tissue in vitro.

MATERIAL AND METHODS

Biopsy techniques and biopsy material sources

Testicular material was obtained from infertile men by means of regular testicular biopsy. The biopsy specimen was kept cool and taken for enzyme assay within 30 min.

Incubation technique

Teased testicular biopsy specimens (wet weight ca. 50 mg) were incubated in 3 ml of a chemically well defined medium for 3 h in an atmosphere of carbon dioxide—oxygen (5:95) at 37°C under constant shaking and in the presence of 10 μCi of [^{3}H] pregnenolone (3.0 nmol). The medium used consisted of 3.0 ml of Krebs—Ringer bicarbonate buffer (KRBB), pH 7.4, including glucose (11 m*M*), pyruvic acid (3 m*M*), nicotinamide (30 m*M*), NADP (0.4 m*M*), glucose-6-phosphate (3.5 m*M*), NADH (0.4 m*M*) and magnesium chloride (1.5 m*M*) together with ca. 2.0 units/ml of each of the enzymes glucose-6-phosphate dehydrogenase and lactate dehydrogenase necessary for recycling. The reactions were stopped by addition of 0.1 ml 1 *M* hydrochloric acid. To protect the metab-

TABLE I

TRIVIAL NAMES AND ABBREVIATIONS USED

Trivial name	Abbreviation	Chemical name
Androstenediol	A_5	Androst-5-ene-3β,17β-diol
Androstenedione	A_4	Androst-4-ene-3,17-dione
Dehydroepiandrosterone	DHEA	3β-Hydroxyandrost-5-en-17-one
20α-Dihydroprogesterone	20αDH-P_4	20α-Hydroxypregn-4-en-3-one
17β-Estradiol	E_2	Estra-1,3,5(10)-triene-3,17β-diol
Estrone	E_1	3-Hydroxyestra-1,3,5(10)-trien-17-one
17α-Hydroxy-20α-dihydroprogesterone	17α,20α(OH)$_2$-P_4	17α,20α-Dihydroxypregn-4-en-3-one
17α-Hydroxypregnenolone	17αOH-P_5	3β,17α-Dihydroxypregn-5-en-20-one
20α-Dihydropregnenolone	20αDH-P_5	3β, 20α-Dihydroxypregn-5-ene
17α-Hydroxyprogesterone	17αOH-P_4	17α-Hydroxypregn-4-en-3-one
Pregnenolone	P_5	3β-Hydroxypregn-5-en-20-one
Progesterone	P_4	Pregn-4-ene-3,20-dione
Testosterone	T	17β-Hydroxyandrost-4-en-3-one

olites formed, 10 μg of each of the following unlabeled steroids were added: progesterone (P_4), 20α-dihydroprogesterone (20αDH-P_4), 17α-hydroxyprogesterone (17αOH-P_4), androstenedione (A_4), testosterone (T), estrone (E_1), 17β-estradiol (E_2), pregnenolone (P_5), 17α-hydroxypregnenolone (17αOH-P_5), dehydroepiandrosterone (DHEA), androstenediol (A_5) and 20α-dihydropregnenolone (20αDH-P_5) (cf. Table I).

Extraction procedure and paper chromatography

The incubate was repeatedly extracted (8 times) with 25-ml portions of diethyl ether—chloroform (4:1, v/v). The extract, after evaporation under a stream of dry nitrogen, was then chromatographed on a 50-cm paper strip (Whatman no. 1, Clifton, NJ, U.S.A.) in hexane saturated with formamide to the front. The paper strip was impregnated with formamide—methanol (1:2, v/v) beforehand. After evaporation the paper strip was rechromatographed in hexane—benzene—formamide (10:10:1, v/v) to the front as described by Zaffaroni and Burton [5]. The chromatogram was dried overnight at 45°C and then scanned for radioactivity. The zones with radioactive material in the paper strip were then cut out, eluted completely and the eluates evaporated to dryness. Each sample of dried material was then redissolved in 2.00 ml of methanol and an exact volume of each taken to liquid scintillation counting for quantitation of radioactivity. The final separation of the conversion products was achieved using reversed-phase HPLC.

Thin-layer chromatography

The TLC separations were performed on glass plates (20 × 20 cm) coated with a 0.25-mm layer of silica gel G F_{254} (Merck, Darmstadt, G.F.R.) using four different solvent systems as developing media: A, chloroform—acetone (4:1, v/v); B, chloroform—acetone (5:1, v/v); C, chloroform—acetone—ethyl acetate (3:1:1, v/v); D, toluene—ethyl acetate (4:1, v/v). To accomplish separation of T from 17αOH-P_4 derivatization of T to T-acetate was necessary [6].

Reversed-phase HPLC

Instrumentation. The chromatograph was constructed from a Constametric III constant-flow solvent pump, a Rheodyne Model 7125 injection valve provided with a 20-μl loop, a Lichroma 150 × 4.6 mm stainless-steel column, slurry-packed in our laboratory with 5-μm Nucleosil C18, a Schoeffel Model 770 variable-wavelength UV detector and a Linear Model 264 potentiometric recorder.

Chromatographic conditions. All separations were performed under isocratic conditions with acetonitrile—water mixtures as the mobile phase. The flow-rate was 2.0 ml/min.

Measurement of radioactivity

The paper chromatograms were scanned with a Packard Model 7200 radiochromatogram scanner and the TLC plates with a Berthold thin-layer scanner Model LB 2723.

Liquid scintillation counting was performed with a Packard Model 2450 Tri-Carb spectrometer. The scintillation medium was composed of Permablend III

(Packard, Downers Grove, IL, U.S.A.) dissolved in toluene (5.5 g/l) and the counting efficiency for tritium on single isotope analysis was 50%.

Chemicals

Radioactive steroids. Δ^5-[7(n)-^{3}H] Pregnenolone and [1,2(n)-^{3}H]-progesterone were obtained from the Radiochemical Centre (RCC) (Amersham, Great Britain) or from New England Nuclear (NEN) (Boston, MA, U.S.A.). 17α-Hydroxy[7(n)-^{3}H]-pregnenolone and dehydro-[7(n)-^{3}H]-epiandrosterone were from RCC and androst-5-ene-3β,17β-diol[1,2(n)-^{3}H], estrone-[2,4,6,7(n)-^{3}H], estradiol-[2,4,6,7(n)-^{3}H] and estriol-[2,4,6,7(n)-^{3}H] were from NEN.

Unlabeled steroids. These compounds were obtained from different commercial suppliers.

Solvents. All solvents were of pro analysi quality. The acetonitrile of HPLC grade S was obtained from Rathburn Chemicals (Walkerburn, Great Britain).

RESULTS AND DISCUSSION

Separation efficiency

The complete separation scheme is outlined in Fig. 1. As shown, all paper zones could be taken directly to HPLC after elution. However, under the conditions used, optimal column performance was always a prerequisite for complete resolution of A_4 from DHEA (paper zone VI).

Chromatographic retention data for the various steroids obtained in the final steps are summarized in Table II. The TLC systems used supplemented with derivatization of T permit separation of the Δ^4-metabolites. However, the Δ^4- and Δ^5-metabolites in paper zone VI can not be adequately separated with the TLC systems used as seen in Table II. The HPLC system permits adequate separation of T and 17αOH-P_4 without previous acetylation.

The column efficiency as monitored by simultaneous peak tracing by UV and by liquid scintillation counting is shown in Fig. 2. At the detector wavelength 215 nm the Δ^4- as well as the Δ^5-metabolites could be visualized, thus permitting an exact localization of the peaks as required for eluate collection.

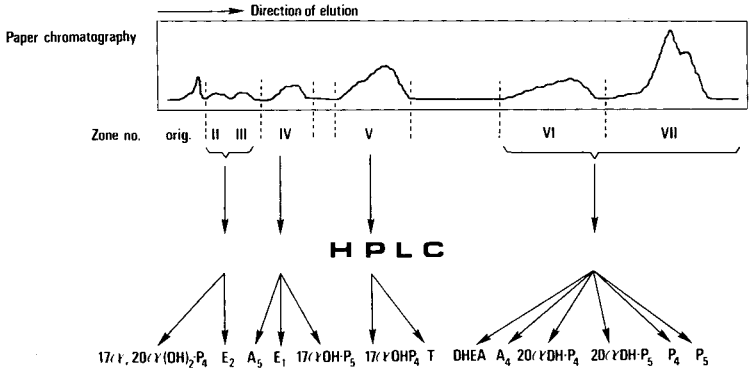

Fig. 1. The complete separation scheme, permitting the determination of 13 identified testicular steroid metabolites.

TABLE II

CHROMATOGRAPHIC RETENTION DATA

Solvent systems: HPLC, 35% acetonitrile; TLC, as indicated, full details in Materials and Methods.

Zone No.	Steroid	HPLC	TLC	
		Capacity factor, k'	R_F	Solvent system
II–III	17α,20α(OH)$_2$-P$_4$	13.5	0.20	B
	E$_2$	16.1	0.38	
IV	A$_5$	19.3	0.37	C
	17αOH-P$_5$	22.8	0.46	
	E$_1$	18.1	0.69	
V	T	19.5	0.35 (acetate)	D
	17αOH-P$_4$	22.3	0.14	
VI	A$_4$	22.3	0.60	A
	DHEA	24.8	0.46	
	20αDH-P$_4$	57.8	0.44	
	20αDH-P$_5$	62.8	0.45	
VII	P$_4$	83.3	0.64	A
	P$_5$	96.0	0.48	

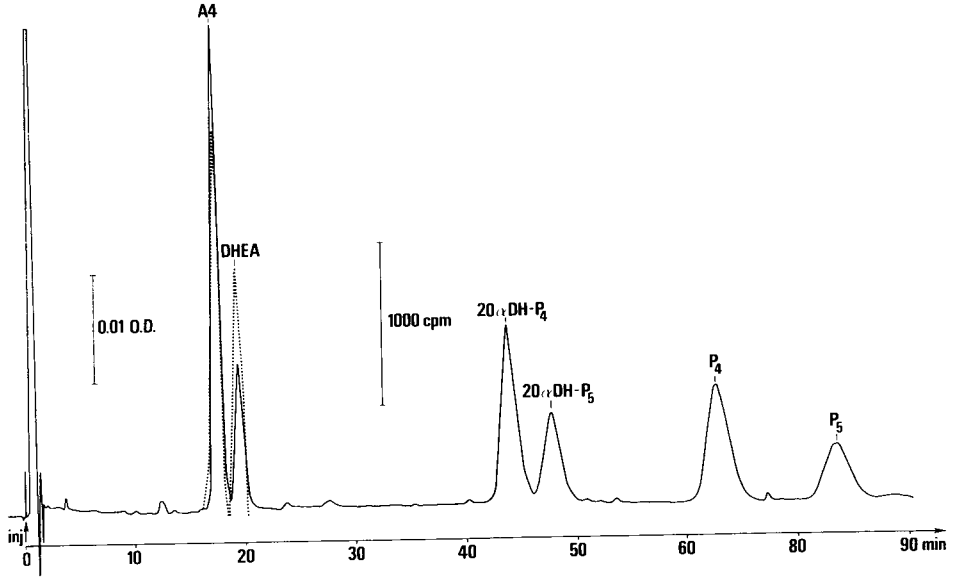

Fig. 2. Illustration of column efficiency and chromatographic resolution. The solid and dashed lines refer to monitoring of UV absorption at 215 nm and to liquid scintillation counting, respectively. For the latter case fractions were collected at intervals of 10 sec.

Extremely purified solvents are absolutely necessary at this wavelength to avoid UV absorbance by impurities.

Reproducibility and methods comparison

The reproducibilities obtained on incubation, extraction, paper chromatography and TLC separation have been reported previously [6].

Several experiments were performed to compare the results of steroid quantitation via TLC and HPLC, respectively, of the zones after the initial paper chromatography. Thus, the paper zones from a given biopsy incubation experiment were eluted and the eluate from each zone divided into two identical parts, one for TLC and the other for HPLC analysis.

Table III shows the results of such an experiment, which demonstrate clearly the satisfactory agreement between the two methods. However, the slightly larger amounts of unidentified radioactive material obtained by HPLC should be consistent with the higher separation efficiency of this method.

Repeated separations of a steroid mixture obtained from paper zones VI and VII gave the results shown in Table IV. ^{14}C-Labelled A_4 was used in order to demonstrate its complete chromatographic resolution from DHEA (Table IV and Fig. 2).

TABLE III

COMPARISON OF RESULTS USING TLC AND HPLC AS FINAL QUANTITATION STEPS

Data obtained from a testicular biopsy incubation with P_5 as the precursor.

Zone No.	Steroid	Radioactivity (%) obtained according to	
		TLC	HPLC
II + III	17α,20α(OH)$_2$-P_4	5.4	4.7
	ui*	4.1	4.5
IV	—	—	—
V	T	4.2**	2.1
	17αOH-P_4	16.2	19.4
	ui	0.5	2.9
VI	A_4	1.3	0.9
	DHEA	—	0.2
	20αDH-P_4	6.0	5.0
	20αDH-P_5	—	0.2
	ui	1.0	—
VII	P_4	38.8	34.8
	P_5	1.4	0.4
	ui	6.0	9.5
Recovery of activity (%)		84.9***	84.6***

*ui denotes unidentified compounds.

**Derivatized to acetate.

***The remaining activity is completely located at the origin (zone I) and at the solvent front in the paper chromatogram.

TABLE IV

ILLUSTRATION OF THE REPRODUCIBILITY ON THREE SUBSEQUENT HPLC SEPARATIONS OF STEROIDS FROM PAPER ZONES VI AND VII (FIVE ^{3}H-LABELLED Δ^{5}- AND ONE ^{14}C-LABELLED Δ^{4}-SPECIES)

Results are presented as percent recovered radioactivity of each steroid related to the total amount of radioactivity injected on the column.

Run No.	1	2	3	Mean	Range
[^{3}H]DHEA	18.7	18.1	17.6	18.1	17.6—18.7
[^{3}H]20αDH-P_4	13.7	13.3	12.8	13.3	12.8—13.7
[^{3}H]20αDH-P_5	18.9	17.9	17.2	18.0	17.2—18.9
[^{3}H]P_4	18.3	20.2	21.8	20.1	18.3—21.8
[^{3}H]P_5	24.5	26.6	25.3	25.5	24.5—26.6
[^{3}H]ui*	5.9	3.9	5.3	5.0	3.9—5.9
	100	100	100	100	
[^{14}C]A_4	99.7	100.5	102.9	101.0	99.7—102.9

*ui denotes unidentified compounds.

CONCLUSIONS

Compared to TLC, HPLC methods are generally more adequate for quantitative work. Retention parameters can be obtained with high precision, and measurements on the eluate can be performed without destroying the chromatographic system as is the case on elution from TLC plates. The continuous operation of HPLC systems represents a definite advantage, especially if clinical demands of sample processing capacity are taken into consideration.

ACKNOWLEDGEMENT

This project has been supported by the Swedish Medical Research Council (Project No. 02020).

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Journal of Chromatography, 224 (1981) 407—413
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 899

ELECTROPHORESIS WITH TWO BUFFERS IN ONE DIMENSION IN THE ANALYSIS OF GLYCOSAMINOGLYCANS ON CELLULOSE ACETATE STRIPS

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(Received January 20th, 1981)

SUMMARY

We have systematically examined the electrophoretic behavior of hyaluronic acid, desulfated chondroitin, heparan sulfate, and chondroitin sulfate with two different buffers under varying conditions of buffer concentration, electrophoresis time, and voltage. An anodal effect was observed with barium acetate buffer in which the glycosaminoglycans behaved as if they became positively charged as they neared the anode, whereas with monovalent buffers the migration was nearly linear with time. Such differences in behavior permitted us to develop a two-buffer monodirectional electrophoresis technique which offers some of the advantages of resolution seen with two-dimensional methods, yet retains the advantages of band comparison and quantitative analysis characteristic of one-dimensional electrophoresis.

INTRODUCTION

Despite the widespread use of electrophoresis on cellulose acetate as an analytical tool for glycosaminoglycans (GAGs) its usefulness in separating complex mixtures of these polyanions has been limited by the similarity in migration of several of the more common species. Solutions to this problem have been sought by modifying buffer systems or support media [1—4], by comparing two separate electrophoretic analyses under different buffer conditions [5, 6], or by combining the two analyses into a single two-dimensional electrophoresis [7, 8]. Cappelletti et al. [9] recently used the differential solubilities of GAGs in ethanol during electrophoresis to permit the simultaneous separation of at least eight species. Their technique appears to offer the greatest resolution of published methods, but the use of an ice-containing chamber in which the strips are submerged in chilled decane during electrophoresis may be too restrictive for many applications.

We have attempted to combine these various methods in a technique which

would be convenient, reproducible, and permit the clean separation of the four major GAGs of embryonic tissue: hyaluronic acid, heparan sulfate, chondroitin sulfate and unsulfated chondroitin. Unsulfated chondroitin is of increasing interest in studies of GAG biosynthesis [10—12]; yet there is no published method for demonstrating it electrophoretically. The usual method of analysis of unsulfated chondroitin involves enzymatic degradation [13]. Our technique is analogous to two-dimensional electrophoresis in that it makes use of the properties of two buffers, but it is run in one dimension through the sequential applications of the buffers. This produces bands, rather than the diffuse and irregularly shaped spots obtained by two-dimensional techniques, and it also offers the convenience and analytical advantages of comparing several samples and standards on a single strip. In this paper we briefly present experiments which provide the rationale for this approach, and include some observations on a significant [14] anodal effect that should be of theoretical importance in the design of electrophoretic analyses.

MATERIALS AND METHODS

Reagents

NIH standards of heparan sulfate (HS), chondroitin-4-sulfate (C-4S), and chondroitin-6-sulfate (C-6S) were generous gifts of J.A. Cifonelli (University of Chicago). The other GAGs were from Sigma (St. Louis, MO, U.S.A.). Desulfated chondroitin (C-0S) was prepared from Sigma C-4S with acidic methanol [15]. Sepraphore III cellulose acetate strips (2.5 × 17 cm and 5.7 × 14.4 cm) and the electrophoresis chamber were from Gelman (Ann Arbor, MI, U.S.A.). Titan III cellulose acetate plates (6.0 × 7.6 cm) from Helena Laboratories (Beaumont, TX, U.S.A.) were tested successfully but not used for the work reported here.

Electrophoresis

Standard conditions are described; any variations are indicated in the text. The buffers were 0.05 *M* barium acetate adjusted to pH 5.8 with glacial acetic acid [1, 16] and 0.05 *M* formic acid adjusted to pH 3.0 with pyridine [17, 18]. Alcian blue staining solution was prepared by dissolving the dye (0.1%, w/v) in a washing solution, which consisted of 0.025 *M* sodium acetate (pH 5.8) and 0.05 *M* magnesium chloride diluted with an equal volume of ethanol [16].

(1) Dry cellulose acetate strips were marked with ink 2.5 cm from one end to indicate the future site of sample application and were then hydrated for at least 10 min in a one-tenth dilution of the formate—pyridine buffer.

(2) A buffer interface was prepared in order to stack the sample in a narrow band, by a technique based on that of Cappelletti et al. [9]. The hydrated strip was blotted on filter paper and immersed in full-strength (0.05 *M*) formate—pyridine buffer from the future anodal end of the strip up to about 1 cm in front of the site of sample application. Three rapid in and out immersions were sufficient to exchange the buffer. The strip was blotted again and placed in the electrophoresis tray.

(3) The samples (1—2 μl containing 0.1—5 μg of GAG) were applied im-

mediately on small chips of cellulose acetate strip, and the first electrophoresis was performed for 30 min at room temperature at 200 V (20 V/cm). Full-strength buffer was at the anode, but the one-tenth dilution was at the cathode to help maintain the interface during sample application.

(4) After electrophoresis the strips were immersed in 95% ethanol for 5 min to precipitate the GAGs and transferred to a solution of the second buffer (0.05 *M* barium acetate) and ethanol (1:3, v/v), for another 5 min.

(5) The strips were blotted, and the alcohol was allowed to evaporate until speckled dry areas began to appear (usually within 1 min). They were immediately blotted again between two filter papers which had been saturated with 0.05 *M* barium acetate but which had been pressed with a rubber roller to leave them only slightly damp. This last step completed the transfer into the second buffer. Direct immersion into the second buffer without prior precipitation was avoided in quantitative studies to prevent loss of sample.

(6) The second electrophoresis was carried out for 20 min at 200 V with 0.05 *M* barium acetate buffer at both the anode and cathode.

(7) The strips were immersed for 5 min in 95% ethanol and stained with Alcian blue for 30 min with continuous gentle agitation. They were then blotted and destained in three 10-min baths of washing solution with agitation [16].

RESULTS

Comparison of electrophoresis conditions

Two buffers which are widely used to separate GAGs are barium acetate [1, 5, 16] and formate—pyridine [5, 6, 17, 18]. Accordingly, we compared the migration of HA and C-6S with these buffers under different conditions (Fig. 1). With increasing molarity of either barium acetate (pH 5.8) or formate—pyridine (pH 3.0), the bands migrated closer together (Fig. 1a), but the

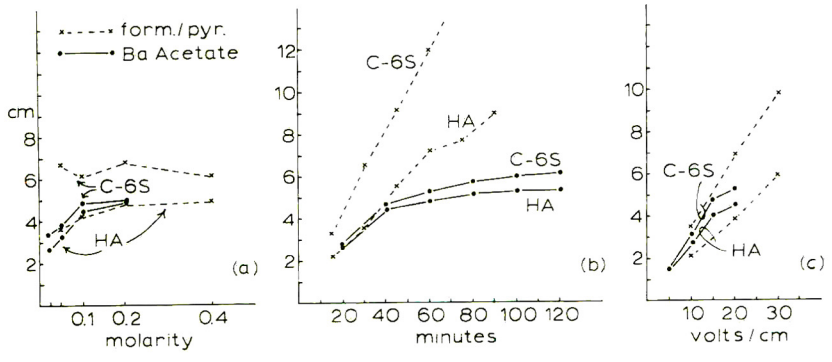

Fig. 1. Effects of varying buffer concentration, electrophoresis time, and voltage. Standard HA and C-6S (1 μg in 1 μl) were applied separately to cellulose acetate strips and electrophoresed under varying conditions. The GAGs were stained with Alcian blue and recorded for measurement by xerography. (a) Migration distance vs. buffer concentration at 20 V/cm for 30 min in formate—pyridine (pH 3.0) or 60 min in barium acetate (pH 5.8). (b) Migration distance vs. time at 20 V/cm in 0.05 *M* buffers. The C-6S in formate—pyridine buffer moved off the end of the strip at 12.5 cm. (c) Migration distance vs. voltage in 0.05 *M* buffers for 30 min in formate—pyridine and 60 min in barium acetate.

bands were also less diffuse at higher molarities. As the buffer molarities approached zero the GAGs were the major charged species and showed strong mutual repulsion with consequent band spreading. The migration of GAGs in formate–pyridine was essentially linear with time (Fig. 1b) or voltage (Fig. 1c), but that in barium acetate was clearly non-linear. All conditions which increased the distance between the two GAGs also promoted band spreading. Optimum conditions were selected as those producing the minimum separation needed to cut apart the strip into distinct bands for further analysis.

Anodal effect

The strong non-linearity seen with the barium acetate buffer could be attributed to an anodal effect; it did not appear to be a function of the parameters varied in Fig. 1, but was dependent upon the distance to the anode. The divergence from linearity was seen at between 4 and 5 cm under all conditions in Fig. 1. The non-linearity was even more striking when electrophoresis was started at the anode (Fig. 2). In this experiment samples were applied simultaneously to the anodal and cathodal ends and to the center of a strip. Those in sodium acetate behaved nearly the same regardless of where they were placed (Fig. 2a), whereas those in barium acetate all migrated toward

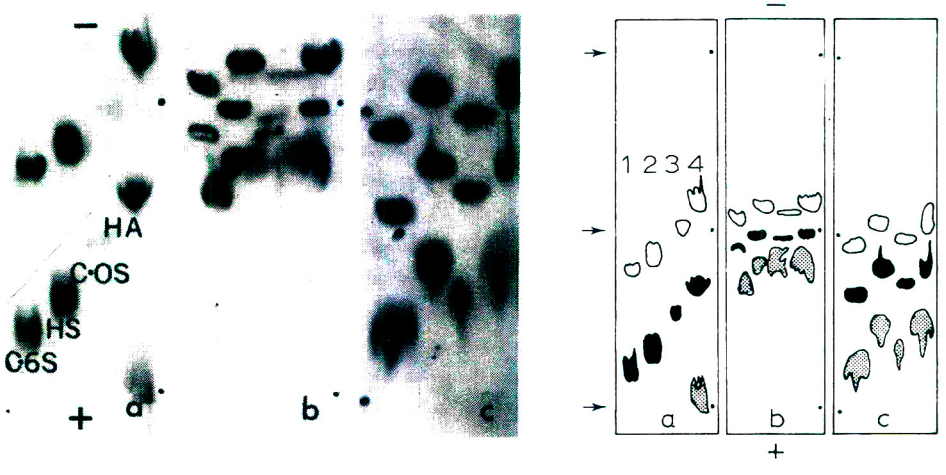

Fig. 2. The anodal effects with barium acetate buffer. Standard HA (4), C-0S (3), HS (2), and C-6S (1) (1 μg in 1 μl) were spotted at three origins along each strip; one was in the middle and the other two were 3 mm from the support bar at each end of the tray (10 cm between the support bars). Only the middle and anodal origins (ink dots) are shown here to save space. Because the buffer wetted the support bars by capillarity the end samples were effectively applied 3 mm from the cathode or anode, respectively. Buffers for hydrating the strips and at the two poles were (a) 0.1 *M* sodium acetate, (b) 0.1 *M* barium acetate, or (c) 0.05 *M* barium acetate. Electrophoresis was at 20 V/cm for 30 min (0.1 *M* buffers) or 40 min (0.05 *M* buffer). All three groups of four GAGs migrated toward the center of the strip (upper dot) in barium acetate (b and c). In sodium acetate (a) they all migrated toward the anode; those originating at the anode migrated off the strip. The same four GAGs (indicated in Fig. 2a) were used in the same order for all three strips. The line drawing is a reduced size tracing of the photographs, showing the entire strip for orientation. The samples spotted at each origin (arrows) are identified by shading: cathodal (outline), middle (solid), and anodal (stippled).

the center of the strip, the migration being more rapid in 0.1 *M* buffer (Fig. 2b) than in 0.05 *M* buffer (Fig. 2c). The anodal effect was only seen with divalent cationic buffers; with sodium acetate (Fig. 2a) or with formate—pyridine (Fig. 1b) migration was more nearly linear, and the samples simply moved off the end of the strip into the buffer.

The anodal effect was observed recently by Noordegraaf [14] when he spotted HA and C-6S at the anode in 0.1—0.3 *M* calcium acetate (pH 7.2 or 3.2). He interpreted the non-linearity of migration as an electrofocusing effect, but he did not provide evidence for the requisite stable ionic or pH gradient. The phenomenon may more simply be explained as electroendosmosis of the GAG—cation complexes, which no longer bear a negative charge.

A two-buffer system

Four common embryonic GAGs (HA, C-0S, HS, and C-6S, or C-4S), did not all separate cleanly in either formate—pyridine or barium acetate buffer alone; HS and C-6S or C-4S comigrated in the former (Fig. 3a, b), and C-0S and C-6S comigrated in the latter (Fig. 2 and Fig. 3c, d). In sodium acetate buffer (Fig. 2a) C-6S and HS showed an overlap that prevented quantitative separation, similar to their behavior in formate—pyridine buffer. However, with the sequential application of buffers, using conditions based on the behavior of GAGs in each buffer, all four GAGs could be separated on a single strip (Fig. 3 e—h). As long as the electrophoresis conditions were carefully

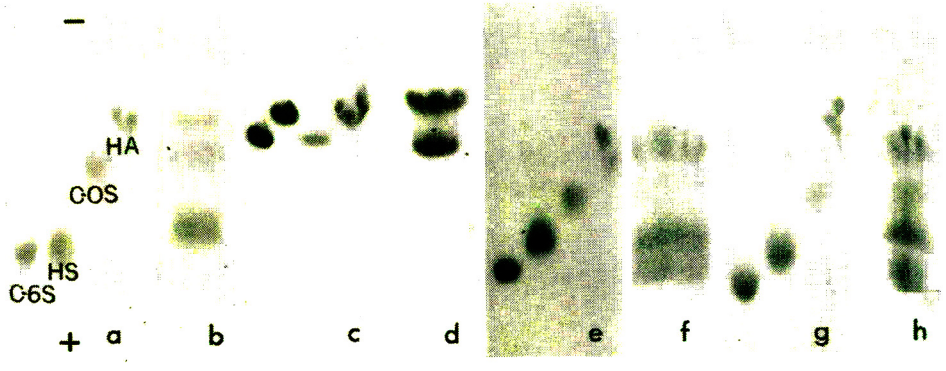

Fig. 3. Comparison of single and double buffers. Standard HA, C-0S, HS, and C-6S were applied either alone (1 μg in 1 μl) or as a mixture (0.5 μg of each in 2 μl) and electrophoresed at 20 V/cm using a 0.005 *M*—0.05 *M* buffer interface. The four single standards were applied to full-width (2.5 cm) strips, and the mixtures were applied to strips cut in half lengthwise. The position of the origin was beyond the top of these photographs and is omitted to save space. (a, b) 0.05 *M* Formate—pyridine buffer (pH 3.0) at 20 V/cm for 40 min. (c, d) 0.05 *M* Barium acetate buffer (pH 5.8) at 20 V/cm for 90 min. (e, f) Two buffers, with electrophoresis first in 0.05 *M* formate—pyridine buffer at 20 V/cm for 30 min. The strips were immersed in 95% ethanol for 5 min, 75% ethanol in 0.05 *M* barium acetate buffer for 5 min, and blotted. After a brief drying, they were blotted on filter papers dampened with 0.05 *M* barium acetate and immediately electrophoresed in this buffer for 20 min at 20 V/cm. (g, h) Two buffers, with electrophoresis first in 0.05 *M* barium acetate for 45 min and then in 0.05 *M* formate—pyridine for 30 min. The alcohol washes and blotting were as for Fig. 3e, f except that formate-pyridine buffer was used for the diluent with the 75% ethanol and for blotting.

controlled, nearly the same results could be obtained regardless of which buffer was used first. Electrophoresis using formate—pyridine as the first buffer was preferred, however, because the GAGs maintained their same relative positions during the second electrophoresis in barium acetate. If barium acetate was used first, HS had to catch up with and migrate through C-0S and HA in the formate—pyridine (compare Fig. 3a and c). This tended to produce band tailing (Fig. 3h).

DISCUSSION

We have shown that differences in the migration of GAGs in different buffers can be utilized on a single strip to attain a separation of closely related GAGs that is superior to that with most other one-dimensional techniques. The technique of Cappelletti et al. [9] may offer a better resolution of the more highly sulfated GAGs, but in our hands it offered no advantage for the four major GAGs of embryonic tissue tested here to offset the greater complexity in protocol. Theoretically, two-dimensional techniques should offer the best resolution, but for the GAGs so far tested this way (e.g., refs. 7, 8, 19) they offer no advantage over the present technique or that of Cappelletti et al. [9] and they do not have the advantage of a precise comparison of sample migration.

A problem that is inherent in the present two-buffer system, as well as in two-dimensional electrophoresis, is the requirement to switch buffers completely, but at the same time to prevent loss of sample [7]. We have attempted to solve the problem by precipitating the GAGs on the strip with alcohol, changing buffers in the presence of alcohol, evaporating some of the alcohol, and finally allowing fresh buffer to be absorbed from damp filter paper. When there was inadequate infiltration by the second buffer, the bands tended to diffuse to varying degrees, but this did not appear to interfere with the relative mobilities of the GAGs. We have experimented with transferring the strips directly, without blotting or any manipulation, to allow the buffers to change electrophoretically. This occasionally gave excellent separation, but the results were inconsistent because of uneven buffer replacement, which resulted in U-shaped bands. GAGs also were precipitated on the strips with 1% cetylpyridinium chloride and washed in varying concentrations of the second buffer, but we were unable to dissociate the complex reproducibly without loss of sample. This technique, however, could be of value when electrophoretically separating GAGs with widely differing precipitation characteristics or when separating GAGs from contaminants and, therefore, deserves further investigation. Another technique was to do the first run in a volatile buffer (e.g., formate—pyridine), allow the strips to air-dry, and then immerse them in the second buffer directly [19]. This procedure gave excellent band separation, but the staining intensity was significantly reduced and was not reduced the same for all GAGs, making this procedure of qualitative value only.

The anodal effect, first reported by Noordegraaf [14], needs further investigation. By selecting divalent cations with different binding affinities for either carboxyl or sulfate groups it may be possible to enhance the separation

of certain GAGs as they approach the anode. Often, electrophoresis of GAGs in barium acetate buffer is reported to be carried out for several hours [1, 5, 7, 16, 19], and it seems likely in these instances that an anodal effect must be acting to alter the relative migration of specific GAGs. For example, by cutting a strip lengthwise and staining half of it after about 45 min while continuing electrophoresis on the other half for 90 min we have observed that HA will continue migration toward the anode after C-6S has begun to migrate back toward the cathode.

We conclude that the discontinuous electrophoresis of GAG in one dimension can enhance resolution over standard one-dimension techniques employing a single buffer. In addition, the technique should be easily modifiable by appropriate selection of buffers to permit the separation of closely related proteins or other macromolecules in other electrophoresis systems.

ACKNOWLEDGEMENT

This research was supported by grants to J.E.M. from the National Institutes of Health, HD14071, and the National Science Foundation, PCM7725412.

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Journal of Chromatography, 224 (1981) 415—422
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 900

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF ETHAMBUTOL IN HUMAN PLASMA*

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(Received January 20th, 1981)

SUMMARY

A quantitative gas chromatographic—mass spectrometric assay has been developed for the determination of ethambutol (EMB) in human plasma. Plasma samples were taken from a patient after oral administration of EMB (with proven tuberculosis infection). Deuterated EMB and a non-deuterated analogue of EMB were synthesized and used as internal standards in this procedure; both gave excellent agreement in the analysis. The derivatizing agent used was trifluoroacetic anhydride (TFAA) and quantitative derivatization was complete in one hour, forming EMB-$(TFA)_4$. Selective ion monitoring was utilized to monitor the gas chromatographic effluent. Ions were generated by electron impact at 70 eV. The limit of detection was 36 ng EMB per ml plasma. This method is compared with the electron-capture gas chromatographic procedure of Lee and Benet.

INTRODUCTION

Tuberculosis (TB) is a prevalent disease in the U.S.A. with a reported incidence in 1979 of 12.6 per 100,000 population (27,669 newly diagnosed cases in one year) [1]. It is estimated that there are more than 80,000 patients with TB at any one time. Ethambutol (EMB), (+)-2,2′-(ethylenediimino)-di-1-butanol, is an effective drug used in the treatment of TB. It is usually given in combination with other anti-TB drugs for it suppresses the growth of resistant tubercle bacilli which develop after exposure to these drugs. Approximately 75% of the human type strains of *Mycobacterium tuberculosis* are sensitive to 1 μg/ml of EMB and resistance to EMB develops slowly and with difficulty in vitro [2, 3].

A number of chromatographic methods have been developed for the anal-

*A preliminary report was presented at the combined SE—SW regional ACS Meeting, New Orleans, LA, December 1980.

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ysis of EMB. Richard et al. [4] reported a flame ionization gas chromatographic method. The drug was derivatized forming a trimethylsilyl (TMS) derivative; however, this procedure was found not to be sensitive enough for measurement of EMB in concentrations found in human plasma after therapeutic doses. Lee and Benet [5] developed an electron-capture gas chromatographic (ECGC) method which could be used effectively for measurement of small quantities of EMB in human plasma and urine. The derivatizing agent used was trifluoroacetic anhydride (TFAA) and the internal standard for quantitation was (+)-2,2′-(ethylenediimino)-di-1-propanol (MEMB). Blair et al. [6] developed a chemical ionization gas chromatographic—mass spectrometric (CI-GC—MS) method using deuterated EMB as internal standard; derivatization with TMS was carried out by the procedure of Richard et al. [4].

In this paper is described the development of a new GC—MS procedure for the analysis of EMB in human plasma. Electron impact (EI) ionization was used at 70 eV and the derivatizing agent was TFAA. [$^2H_4$]EMB and MEMB were synthesized and used as internal standards in this procedure. Methane CI was also studied to see if greater sensitivity could be obtained over EI ionization. A comparison of sensitivities and reproducibilities is made between this GC—MS procedure and the ECGC method of Lee and Benet [5].

EXPERIMENTAL

Materials

Nanograde chloroform and pyridine were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Trifluoroacetic anhydride was obtained from Pierce (Rockford, IL, U.S.A.). Ethambutol was obtained from Lederle Labs. (Pearl River, NY, U.S.A.). (+)-2-Amino-1-butanol, (+)-2-amino-1-propanol and 1,2-dibromoethane were obtained from Aldrich (Milwaukee, WI, U.S.A.). 1,2-Dibromo-[$^2H_4$]ethane was obtained from Merck & Co. (Rahway, NJ, U.S.A.).

Synthesis of internal standards

The procedure of Blair et al. [6] was not found to be workable in our laboratory for the synthesis of [$^2H_4$]EMB. Therefore, the original procedure of Wilkinson et al. [7] was used for the synthesis of [$^2H_4$]EMB and MEMB with slight modification. (+)-2,2′-(Ethylene-[$^2H_4$]diimino)-di-1-butanol was synthesized by heating a stirred solution of 4.5 g (0.050 mole) of (+)-2-amino-1-butanol with 0.95 g (0.005 mole) of 1,2-dibromo-[$^2H_4$]ethane under reflux for 25 min at 100—115°C. The reaction mixture was allowed to cool (30—40°C) before the addition of 0.65 g potassium hydroxide in 5.0 ml hot *n*-propanol (which precipitated potassium bromide). After cooling in a dry ice bath, the reaction mixture was filtered to remove potassium bromide. The filtrate was concentrated (under reduced pressure), and the residue, an orange oil, was dissolved in 5.0 ml of acetone—*n*-propanol (1:1, v/v), and cooled again (in a dry ice bath) and filtered to effectively remove all potassium bromide. The filtrate was diluted with 4.0 ml of 7.8 *N* ethanolic hydrochloric acid. The solution was concentrated under reduced pressure and the residue (a red gum) was dissolved in a minimum amount of cold absolute ethanol.

If precipitation of white crystals did not occur, then cold acetone was added to affect precipitation. The mixture was cooled in a dry ice bath for 30 min before filtration and the solid residue was washed with cold acetone. The material was recystallized twice from hot ethanol or benzene which yielded 0.56 g of white crystals (45% yield) of m.p. 189—190°C.

MEMB was synthesized by the procedure described above: a mixture of 3.75 g of (+)-2-amino-1-propanol (0.050 mole) and 0.95 g (0.005 mole) of 1,2-dibromoethane was heated under reflux for 1 h. The product, 70 mg (8% yield) of m.p. 170—171°C was isolated as above.

Instrumentation

MS was performed with a Finnigan 4000 GC—MS quadrupole mass analyzer spectrometer with a Model 6000 automated data system. GC was accomplished on a 1.8 m × 2 mm I.D. glass column of 3% OV-17 on Gas-Chrom Q (100—200 mesh). The column temperature was maintained at 160°C (helium flow-rate of 20 ml/min), injection port at 190°C, jet separator at 200°C and the ion source at 250°C. The ionization potential was 70 eV. When MEMB was used as the internal standard for quantitation of EMB, the ions monitored were *m/e* 280 (MEMB) and *m/e* 307 (EMB). For quantitation of EMB with [$^2H_4$]EMB as internal standard, the ions monitored were *m/e* 307 (EMB) and *m/e* 310 ([$^2H_4$]EMB). In experiments using CI, methane was used as the reagent gas.

ECGC was performed on a Varian 3700 gas chromatograph with ^{63}Ni detector with a Varian CDS-111 microprocessor. Samples were analyzed on a 1.8 m × 2 mm I.D. glass column with 3% SE-30 on Gas Chrom Q (100—120 mesh). The column temperature was maintained at 170°C (nitrogen flow-rate of 30 ml/min), injection port at 190°C and detector temperature at 250°C. A 10% retention index window was imposed on EMB and MEMB. Only MEMB could be used as the internal standard for this procedure.

Sample collection and preparation

Informed consent was obtained from a patient who exhibited proven infection with tubercle bacilli but in otherwise good clinical status. The patient was not taking other medications at the time of the study. A heparin lock was placed in a superficial arm vein for a 24-h sampling period. EMB at a dose of 800 mg (as tablets and 300 mg isoniazid) was given daily and 3 ml of blood were withdrawn at 0, 1, 2, 4, 6, 12, 24 h after dosing. Plasma was obtained by centrifugation for 10 min at 500 *g*. The extraction procedure was similar to that of Lee and Benet [5]. Plasma (200 μl) was diluted with 800 μl of water containing 3.0 μg of MEMB for ECGC analysis or 3.0 μg [$^2H_4$]EMB or MEMB for GC—MS analysis. After addition of 10 ml chloroform and 1 ml of 4 *N* sodium hydroxide, the solution was shaken for 10 min and centrifuged for 10 min at 500 *g*. The organic layer (8 ml) was transferred into a clean conical centrifuge tube (15 ml) and evaporated under a stream of air at room temperature. The residue was dissolved in 100 μl of benzene—pyridine (7:1, v/v) and 25 μl of TFAA were added. Following derivatization at 4°C for 1 h, the reaction mixture was washed with 400 μl of 0.1 *N* hydrochloric acid to remove excess TFAA (shaking for 2 min and centrifuga-

tion for 10 min at 500 *g*). The sample tubes were kept refrigerated until analyzed. All samples were analyzed (by ECGC or GC—MS) within 4 h after completion of derivatization.

RESULTS AND DISCUSSION

The EI mass spectra of EMB, MEMB, and [$^2H_4$]EMB are presented in Figs. 1—3, respectively. The ions monitored at 70 eV were *m/e* 280 (MEMB), *m/e* 307 (EMB), and *m/e* 310 ([$^2H_4$]EMB). The M^+ ions were not observable by EI. The relative abundances of *m/e* 280, 307, and 310 were 100%, 35.3%, and 29.8%, respectively. These compounds did not have any interfering contribution to the above selected ions from the presence of the other two compounds. The signal intensities of the above mentioned ions were 50% less at 30 eV than at 70 eV.

To test if greater sensitivity could be achieved, spectra of pure standards of each compound were obtained using CI with methane as reagent gas. The relative abundances of the $(MH)^+$ ions of MEMB (*m/e* 561), EMB (*m/e* 589), and [$^2H_4$]EMB (*m/e* 593) were 0.12%, 1.02%, and 2.87%, respectively. The relative abundances of ions *m/e* 280, 307, and 310 were 80.0%, 48.6%, and 37.5%, respectively. It was found that the CI signal intensities at 70 eV of these ions (*m/e* 280, 307, and 310) were three times less than the EI signal intensities at 70 eV. Since there were no other characteristic ions of sufficient

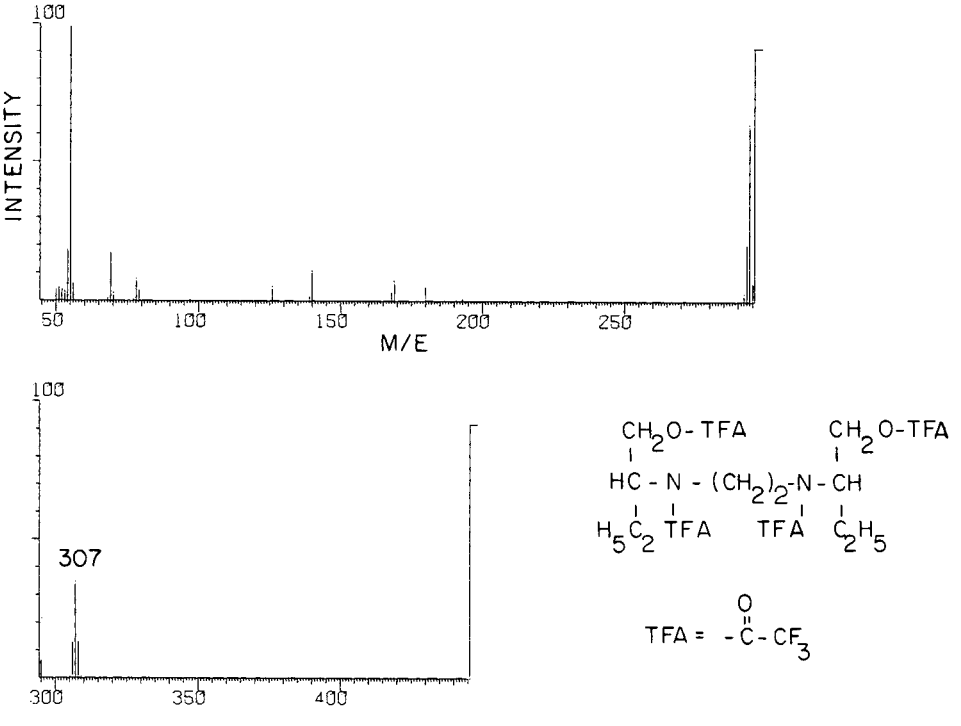

Fig. 1. EI mass spectrum of EMB-$(TFA)_4$.

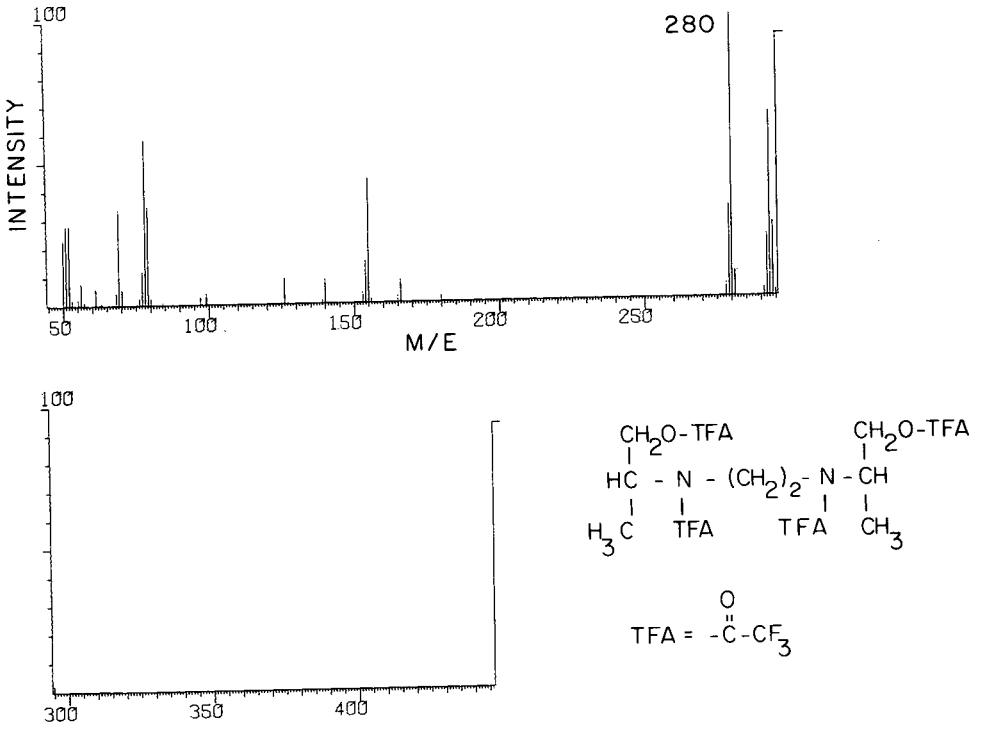

Fig. 2. EI mass spectrum of MEMB-$(TFA)_4$.

intensity generated by Cl that could be employed for quantitative analysis, EI at 70 eV was used for measurement.

The minimum detectable amount (with a 2:1 signal-to-noise ratio) of pure EMB was 1.5 ng (*m/e* 307) by GC—MS. The high sensitivity of the present method enables quantitative analysis (2:1 signal-to-noise ratio) with a detection limit of 10 ng EMB per ml plasma.

Calibration curves were constructed in which known amounts of EMB (0—10 μg) were added to water or plasma containing a fixed amount of [$^2H_4$]-EMB (3 μg) or MEMB (3 μg) and carried through the extraction and derivatization procedure. Samples containing EMB—[$^2H_4$]EMB or EMB—MEMB were analyzed by GC—MS while those containing only EMB—MEMB were run by ECGC. The calibration curves were constructed by a least-squares fit of area ratio versus weight ratio of EMB to internal standard used and the slopes, intercepts and correlation coefficients are given in Table I. As can be seen by the data in Table I, excellent agreement is found in the standard curves regardless of the method chosen or internal standard used.

In Fig. 4 is presented a mass fragmentogram of EMB (with [$^2H_4$]EMB as internal standard) extracted from human plasma following oral dosing. The chromatographic retention time of 0.80 min of the isolated EMB was identical to that of its deuterated analogue; MEMB had a chromatographic retention time of 0.70 min (3% OV-17 column). In Fig. 5 is presented an

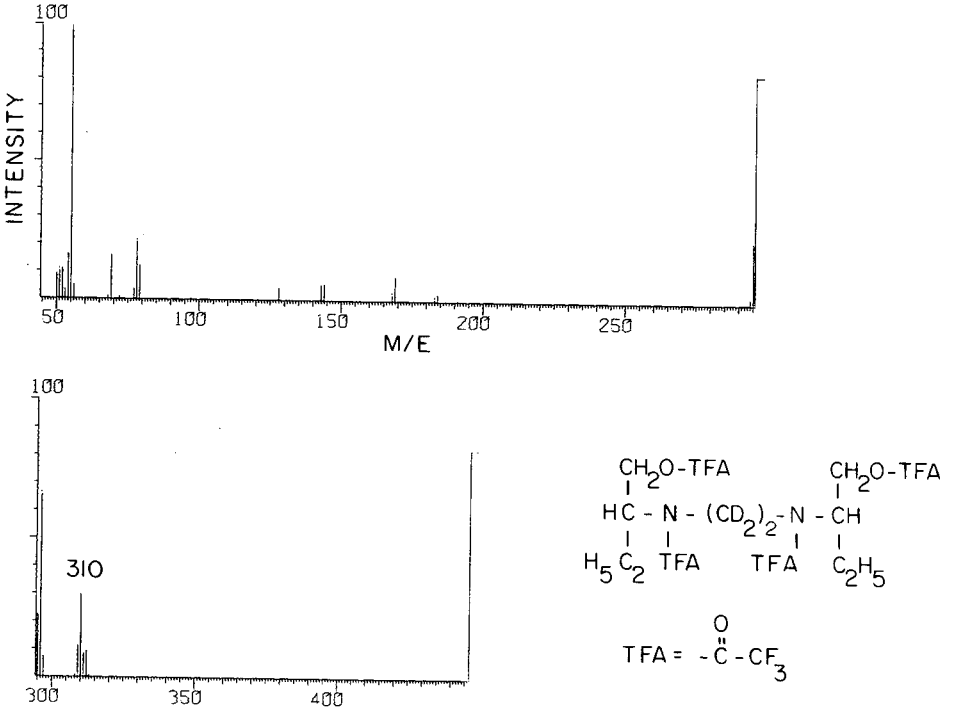

Fig. 3. EI mass spectrum of [$^2H_4$]EMB-(TFA)$_4$.

TABLE I

STANDARD CURVE DATA FOR ETHAMBUTOL BY GC—MS AND ECGS

Internal standard	GC—MS			ECGC		
	Slope	Intercept with *y*-axis	Corr. coeff.	Slope	Intercept with *y*-axis	Corr. coeff.
MEMB	1.140*	—0.009	0.999	1.140**	—0.002	0.999
[$^2H_4$]EMB	1.145***	—0.005	0.999	—	—	—

*Area ratio *m/e* 307/280.
**Area ratio EMB/MEMB.
***Area ratio *m/e* 307/310.

ECGC chromatogram of EMB and MEMB; retention times for the two compounds were 2.73 and 4.31 min, respectively (3% SE-30 column). The total time required for ECGC analysis is 7 min whereas the run time required for GC—MS is 2.5 min. The GC—MS selective ion monitoring is relatively free of interfering peaks.

In Table II are given the levels of EMB after an oral dose of 800 mg EMB

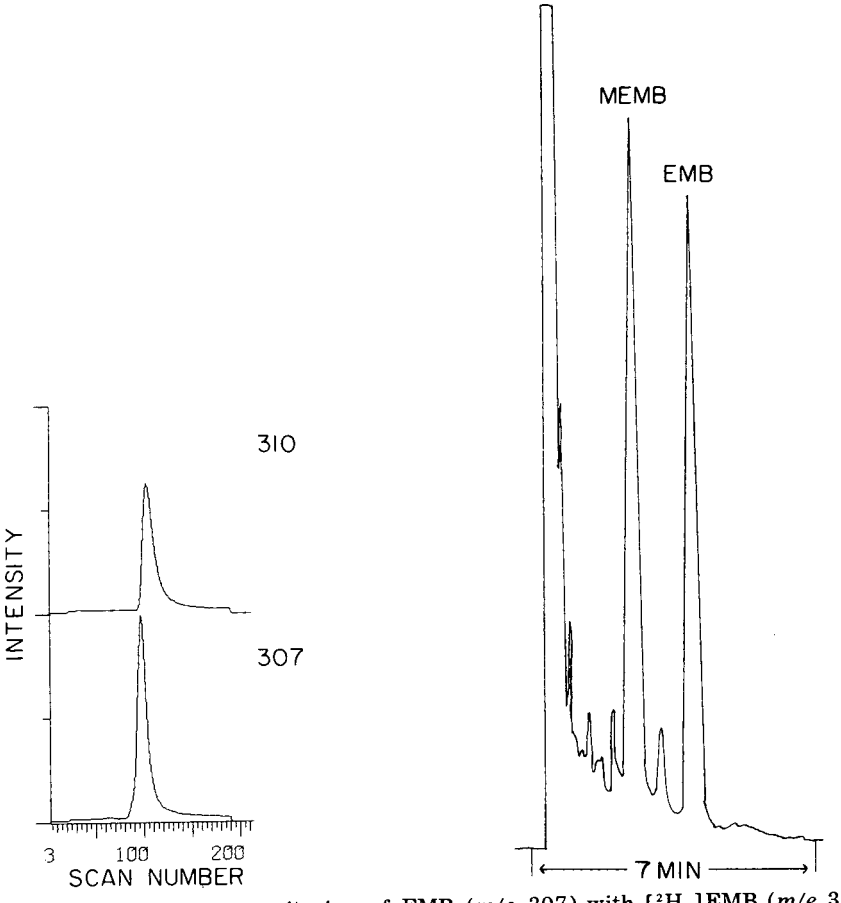

Fig. 4. Selected ion monitoring of EMB (*m/e* 307) with [$^2H_4$]EMB (*m/e* 310) as internal standard from plasma sample.

Fig. 5. ECGC chromatogram of EMB with MEMB as internal standard.

TABLE II

LEVELS OF ETHAMBUTOL IN HUMAN PLASMA DETERMINED BY THE GC—MS AND ECGC PROCEDURES

Levels expressed as μg EMB per ml plasma (average of three determinations) ± standard deviations. Either [$^2H_4$]EMB or MEMB was used as internal standard.

Time* (h)	GC—MS		ECGC
	[$^2H_4$]EMB	MEMB	MEMB
0	0.30 ± 0.10	0.33 ± 0.07	0.31 ± 0.11
1	2.66 ± 0.27	2.58 ± 0.20	2.78 ± 0.29
2	3.36 ± 0.39	3.66 ± 0.44	3.71 ± 0.40
4	1.68 ± 0.30	1.71 ± 0.29	1.81 ± 0.35
6	1.15 ± 0.31	1.17 ± 0.38	1.19 ± 0.28
12	0.80 ± 0.20	0.80 ± 0.19	0.86 ± 0.17
24	0.48 ± 0.09	0.42 ± 0.07	0 52 ± 0.12

*Time after an oral dose of 800 mg EMB and 300 mg isoniazid.

(in the presence of a 300-mg dose of isoniazid). The concentration (μg/ml) in plasma of EMB was determined by GC—MS with either [$^2H_4$]EMB or MEMB as internal standards. These data are compared with values obtained by ECGC using MEMB as internal standard. As can be seen by the data, good agreement was obtained by either method regardless of the internal standard used for quantitation for GC—MS.

CONCLUSION

The GC—MS procedure presented in this paper is useful for the determination of EMB in human plasma. EI at 70 eV was used and the derivatizing agent was TFAA. The total time required per analysis was 2.5 min for GC—MS. [$^2H_4$]EMB and MEMB were used as internal standards for GC—MS and gave comparable results. This GC—MS procedure was compared with the ECGC method of Lee and Benet [5] for measuring EMB in plasma following oral dosing with the drug and both methods gave similar results. The GC—MS procedure was more rapid and specific than the ECGC procedure used.

ACKNOWLEDGEMENTS

Partial support for this project was provided by Sigma Xi, the Scientific Research Society by a Grant-in-Aid for Research (M.R.H.) and NIH Grant AG-01928. Also, we are grateful to Mr. Frank VanMiddlesworth for help in the synthesis of the internal standards used in this study.

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Journal of Chromatography, 224 (1981) 423—429
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 891

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF PLASMA 5-FLUOROURACIL AFTER ADMINISTRATION OF 1-HEXYLCARBAMOYL-5-FLUOROURACIL TO DOGS AND HUMANS

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(First received January 13th, 1981; revised manuscript received March 9th, 1981)

SUMMARY

A simple, sensitive and specific method for determining 5-fluorouracil (5-FU) in plasma after the administration of 1-hexylcarbamoyl-5-fluorouracil (HCFU) was developed using gas chromatography—mass spectrometry. Thymine was used as the internal standard. After removal of interfering substances with chloroform, diethyl ether and Amberlite XAD-2 resin, 5-FU and thymine were extracted with 16% *n*-propanol in diethyl ether and methylated with trimethylanilinium hydroxide. Fragment ions at *m/e* 158 and 154, the molecular ion of the dimethyl derivatives of 5-FU and thymine, respectively, were used to monitor 5-FU and thymine. The sensitivity of the method is 10 ng/ml, which is sufficient to determine the 5-FU levels in plasma after the administration of therapeutic doses of HCFU to patients.

INTRODUCTION

1-Hexylcarbamoyl-5-fluorouracil (HCFU), synthesized as a masked form of 5-fluorouracil (5-FU) [1], has been found to be more active than 5-FU against experimental solid and ascites tumours in mice [2]. The anti-tumour activity of HCFU is thought to be due to 5-FU, one of its metabolites. Previously, microbiological [3] and high-performance liquid chromatographic methods [4] have been reported for the determination of plasma 5-FU after the oral administration of HCFU.

Although the microbiological method is highly sensitive (10 ng/ml in plasma) and facilitates the measurement of plasma 5-FU after the administration of therapeutic doses of HCFU, in practice it is inconvenient for analysing large numbers of samples because it involves lengthy procedures. Further, if patients simultaneously receive antibacterial agents, the antibiotics may interfere with the assay.

On the other hand, although the high-performance liquid chromatographic

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method is highly selective and simple, its sensitivity is only about 100 ng/ml, which is not sufficient for determining low plasma 5-FU levels.

Therefore, a simple, specific and sensitive method was needed to deal with large numbers of samples. We describe here such a method, which utilizes gas chromatography—mass spectrometry for the determination of plasma 5-FU after the administration of HCFU.

EXPERIMENTAL

Chemicals

5-FU, HCFU, 1-(5-carboxypentylcarbamoyl)-5-fluorouracil (CPEFU) and 1-(5-carboxypropylcarbamoyl)-5-fluorouracil (CPRFU) were kindly supplied by Mitsui Pharmaceutical (Tokyo, Japan). Thymine and 0.2 *M* trimethylanilinium hydroxide in methanol (Methelute®) were obtained from Kohjin (Tokyo, Japan) and Pierce (Rockford, IL, U.S.A.), respectively. The test organism for microbiological assay was *Staphylococcus aureus* (ATCC 6538P); the medium used was Mueller Hinton medium (Eiken, Tokyo, Japan). Other reagents were commercial products and were of analytical-reagent grade.

Gas chromatography—mass spectrometry

A JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) and a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) were used. Separations were carried out on a glass column (1.8 m × 1.8 mm I.D.) packed with 3% OV-17 on Chromosorb W (80—100 mesh). The column temperature was maintained isothermally at 185°C for approximately 4 min, then increased at 32°C/min to 260°C, and held there isothermally for 2 min. The injector, separator and ion source temperatures were 300, 250 and 200°C, respectively. The flow-rate of the carrier gas (helium) was 30 ml/min. The ionization potential and total current were 70 eV and 300 μA, respectively. The multiplier voltage supply was set at 1.6—2.2 kV.

Extraction procedure

To each plasma sample (0.5 ml), thymine (1.5 μg) was added as the internal standard (I.S.). After adding 0.1 *M* hydrochloric acid (2.5 ml), the plasma sample was extracted with chloroform (4 ml). The aqueous layer was then shaken with Amberlite XAD-2 resin (0.5 ml) for 1 h. After removal of XAD-2 resin, the aqueous layer was saturated with sodium hydrogen carbonate and extracted with an equal volume of diethyl ether. A 2-ml volume of the aqueous layer was added to 30 ml of 16% *n*-propanol in diethyl ether and shaken for 20 min. The organic layer was collected and evaporated in a water-bath at 45°C. Residual *n*-propanol was evaporated to dryness under reduced pressure at 40°C. Methelute (30 μl) was added to the residue immediately before measurement; 3-μl aliquots were injected into the column for gas chromatography—mass spectrometry and the fragment ions at *m/e* 158 and 154 were used to monitor 5-FU and thymine, respectively.

Calibration graph

The calibration graph was prepared by subjecting human plasma samples, to which known amounts of 5-FU had been added, to the above procedures. The peak height ratio of 5-FU to the I.S. was plotted against 5-FU concentration.

Extraction recoveries

Control plasma samples (0.5 ml) containing 5-FU (150 ng) were carried through the above procedure without adding I.S. Thymine (1.5 μg) was added to the 16% *n*-propanol—diethyl ether extracts and the solutions were evaporated to dryness. The subsequent procedures were as described above. Recoveries were calculated by comparing the peak height ratios with those obtained when 5-FU and I.S. were processed without the extraction procedure.

Recoveries of 5-FU from the mixtures of 5-FU, HCFU, CPEFU and CPRFU were obtained as follows: (1) 5-FU (150 ng) and I.S. (1.5 μg) were added to control plasma samples (0.5 ml) and carried through the above procedures; (2) the same procedures were followed with control plasma samples (0.5 ml) containing 5-FU (150 ng), I.S. (1.5 μg), HCFU, CPEFU and CPRFU (500 ng each). The percentage recovery of 5-FU was calculated as 100 (peak height ratio obtained with procedure 1/peak height ratio obtained with procedure 2).

Microbiological method

The microbiological method of Watanabe et al. [3] was used.

Animal studies

After overnight fasting, three male beagle dogs were given a 100-mg tablet of HCFU. Blood samples (10 ml) were obtained from the antecubital vein with heparinized syringes, immediately cooled with ice and, within 15 min of sampling, centrifuged for 15 min at 1000 *g* in a refrigerated centrifuge (4°C). Plasma samples were collected, 1 *N* hydrochloric acid (0.2 ml) was added to each sample and they were stored at −20°C until taken for assay.

Human studies

We were requested to measure the plasma 5-FU concentrations in patients who had been administered HCFU by clinicians; these samples were used for human studies.

RESULTS AND DISCUSSION

HCFU is rapidly and extensively metabolized in laboratory animals and humans. Its major metabolites are 5-FU, CPEFU, CPRFU, α-fluoro-β-guanidopropionic acid, α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine [5]. The potent anti-tumour activity of HCFU is thought to be due to the metabolite 5-FU, and in order to determine the optimal dose for safety and efficacy the 5-FU plasma concentration after oral administration of HCFU was previously determined by microbiological assay [3] and high-performance liquid chromatography [4, 6]. However, as neither of these methods was

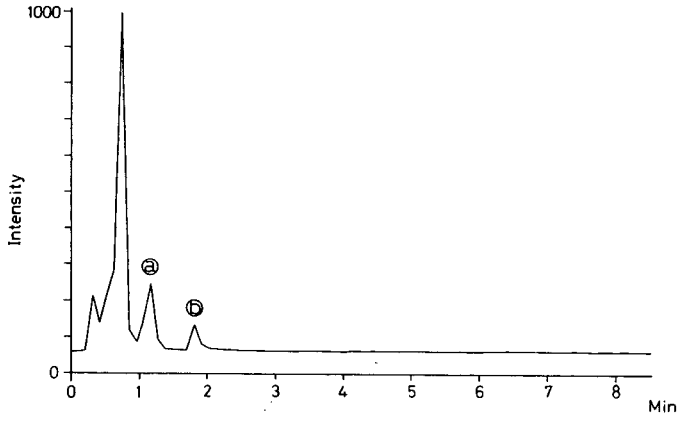

Fig. 1. Gas chromatogram obtained by total ion current detection of HCFU in the presence of trimethylanilinium hydroxide.

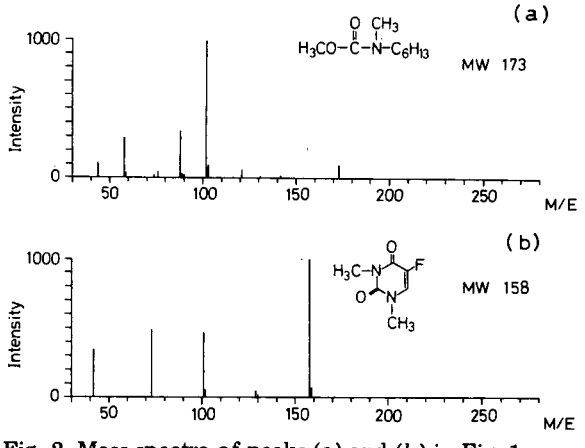

Fig. 2. Mass spectra of peaks (a) and (b) in Fig. 1.

completely satisfactory, we developed a simple, specific and sensitive gas chromatographic—mas spectrometric method for processing large numbers of samples.

Several gas chromatographic—mass spectrometric methods have been reported for determining plasma 5-FU after the administration of 5-FU [7—10] and 1,3-bis(tetrahydro-2-furanyl)-5-fluorouracil [11, 12]. In these methods, 5-FU was converted into its methyl, butyl or silyl derivative, which was subjected to gas chromatography—mass spectrometry. In the present study, trimethylanilinium hydroxide was used as the reagent for methylation [7].

HCFU, CPEFU or CPRFU, injected directly with trimethylanilinium hydroxide in methanol into the column, decomposed into two products, resulting in two peaks. The total ion chromatogram and mass spectra of the products from HCFU are shown in Figs. 1 and 2, and the compounds were identified as N-methoxycarbonyl-N-methylhexylamine (Fig. 2a) and dimethyl derivatives of 5-FU (Fig. 2b) from the high-resolution mass spectrometric data. CPEFU and CPRFU were also decomposed to dimethyl derivatives of

5-FU and 6-(N-methoxycarbonyl-N-methyl)aminohexanoic acid or 4-(N-methoxycarbonyl-N-methyl)amino-*n*-butanoic acid, respectively. These results indicate that the degradation product, 5-FU, interfered in the assay of plasma 5-FU. Further, HCFU, CPEFU and CPRFU are labile at neutral and alkaline pH and hydrolyse easily to 5-FU [3].

The previous methods [7—12] were not suitable for determining 5-FU in plasma after the administration of HCFU, because the physico-chemical properties of HCFU and its metabolites were unsuitable. These difficulties were overcome by removing the HCFU, CPEFU and CPRFU at acidic pH before gas chromatography. HCFU was completely removed and CPEFU and CPRFU were partly removed by extraction with chloroform. The remaining CPEFU and CPRFU in the aqueous layer were removed by shaking with Amberlite XAD-2 resin [3].

Endogenous interfering peaks, which appeared within 3 min on the gas chromatogram, could not be removed by the above extraction procedures, and were removed by extraction with an equal volume of diethyl ether [7].

To confirm the specificity of the assay, the recovery of 5-FU (300 ng/ml) from plasma to which HCFU, CPEFU and CPRFU (1 μg/ml of each) had been added, was determined. The recovery was 98.4 ± 2.6% (mean ± standard error, n = 6), demonstrating that these compounds were completely removed by this procedure.

Thymine was used as an internal standard. The molecular ions at *m/e* 158 and 154 were used to monitor the dimethyl derivatives of 5-FU and thymine, respectively. Chromatograms obtained from human plasma are shown in

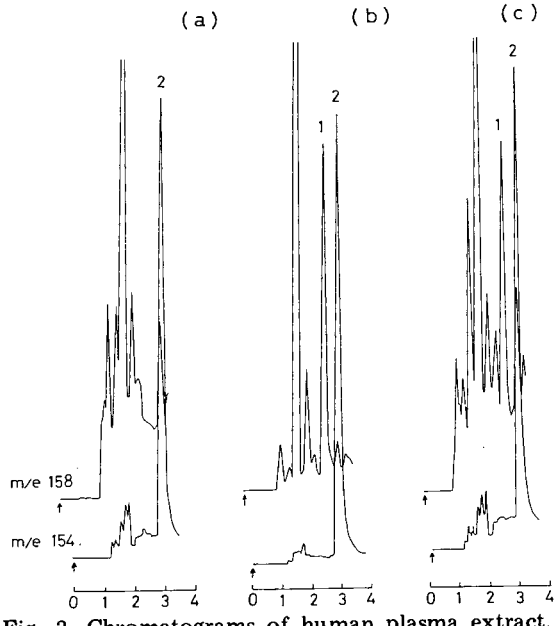

Fig. 3. Chromatograms of human plasma extract. (a) Control plasma containing 3 μg/ml of I.S.; (b) calibration standard with 500 ng/ml of 5-FU in plasma; (c) 8-h plasma sample after the oral administration of 600 mg of HCFU to a cancer patient. Peaks: 1 = 5-FU; 2 = thymine (I.S.).

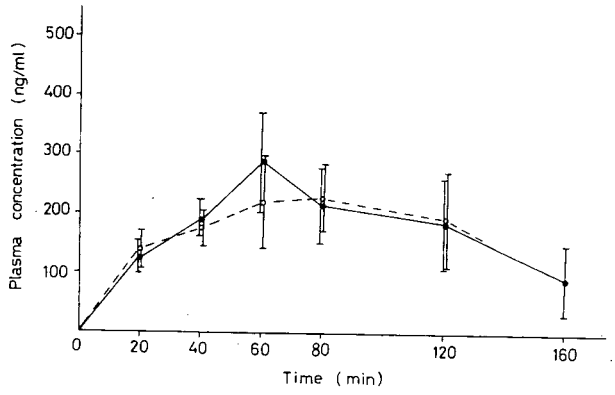

Fig. 4. Plasma 5-FU concentration after the oral administration of 100 mg of HCFU to dogs. o— — — o, Microbiological method; •———•, gas chromatographic—mass spectrometric method.

Fig. 3. The retention times of the dimethyl derivatives of 5-FU and thymine were 2.7 and 3.0 min, respectively. No endogenous peak disturbing the determination of 5-FU was found in the control plasma. About 4 min after sample injection, the column temperature was increased to 260°C and held there for 2 min. As endogenous substances derived from plasma were eluted within these times, this procedure shortened the time required for measurement. About 11 min were needed for measuring each sample. The calibration graph for 5-FU showed good linearity in the range 10—500 ng/ml. The sensitivity of the method was 10 ng/ml, which is sufficient for determining the plasma concentration of 5-FU after a therapeutic dose of HCFU. The extraction recovery of 5-FU added to plasma at a concentration of 300 ng/ml was 94.1 ± 2.1% (mean ± standard error, $n = 6$).

The existence of two new metabolites of HCFU, 1-(5'-oxohexylcarbamoyl)-

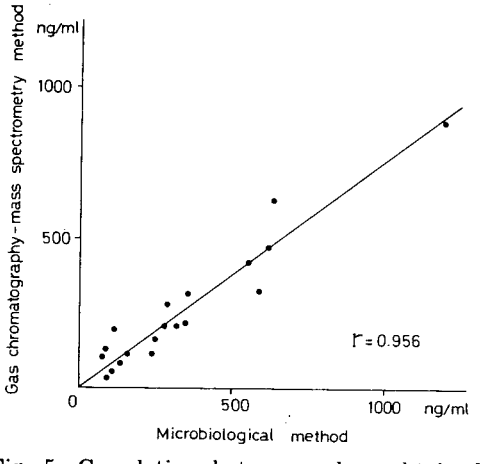

Fig. 5. Correlation between values obtained by the microbiological and the gas chromatographic—mass spectrometric method on plasma samples of cancer patients administered 200—600 mg of HCFU.

5-fluorouracil and 1-(5'-hydroxyhexylcarbamoyl)-5-fluorouracil [6], was reported while our study was in progress. Judging from their chemical constitution, these metabolites may be removed in our procedure, although we have not yet determined whether they interfere in the assay.

Plasma 5-FU levels after the oral administration of HCFU to dogs and humans were determined by our method and the results were compared with those obtained by the microbiological method. Fig. 4 shows the plasma concentration versus time curves obtained after the oral administration of 100 mg of HCFU to dogs. The values obtained by the two methods were in good agreement. Fig. 5 shows the correlation between the values obtained by the two methods after the oral administration of HCFU (200—600 mg) to cancer patients. The correlation coefficient was 0.956.

Because of its simplicity and high specificity, the proposed method should be useful for assaying large numbers of samples. Chemotherapeutic agents appear not to interfere and, owing to the high sensitivity, plasma 5-FU levels as low as 10 ng/ml can be determined using a small amount of sample.

ACKNOWLEDGEMENTS

The authors thank Dr. A. Tachibana and Mrs. Y. Noshiro (Yamanouchi Pharmaceutical Co., Ltd.) for the microbiological assays.

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Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 896

DETERMINATION OF ISONIAZID AND ITS HYDRAZINO METABOLITES, ACETYLISONIAZID, ACETYLHYDRAZINE, AND DIACETYLHYDRAZINE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received October 27th, 1980; revised manuscript received March 20th, 1981)

SUMMARY

A gas chromatographic—mass spectrometric assay for isoniazid and its hydrazino metabolites in human plasma was developed. The trimethylsilyl derivatives of diacetylhydrazine and acetylisoniazid and of the benzaldehyde hydrazones of acetylhydrazine and isoniazid were separated on a 1% OV-17 column and quantitated by single ion monitoring using a LKB 9000 mass spectrometer. Deuterated analogues served as internal standards. The method is well suited for the determination of the hepatotoxic hydrazino metabolites of isoniazid in human plasma following an oral therapeutic dose of isoniazid.

INTRODUCTION

Isoniazid is widely used for the treatment and prophylaxis of tuberculosis. Unfortunately, isoniazid therapy is not without risk. Up to 2.3% of isoniazid recipients will develop clinically overt hepatitis, which has a mortality of about 10% [1]. Older patients, alcoholics and patients on concomitant drug therapy such as rifampin appear to be at a higher risk [2, 3]. Animal studies suggest that it is the metabolic activation of the isoniazid metabolite acetylhydrazine, and possibly diacetylhydrazine, that causes the isoniazid-induced liver injury [1]. If similar mechanisms are responsible for isoniazid hepatotoxicity in man, a kinetic analysis of the hydrazino metabolites of isoniazid might explain why the patient populations mentioned above are more susceptible to the toxic effects of isoniazid. Unfortunately, such an analysis has not been possible with current assays. We have, therefore, developed a specific and sensitive gas chro-

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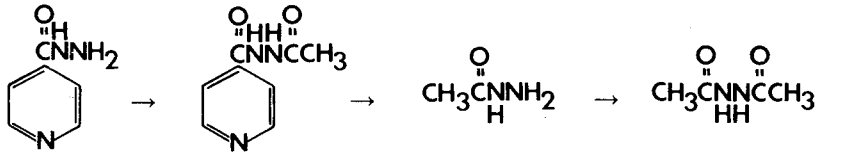

Fig. 1. Structures of isoniazid and its hydrazino metabolites.

matographic—mass spectrometric (GC—MS) method for the simultaneous measurement of isoniazid, acetylisoniazid, acetylhydrazine and diacetylhydrazine in human plasma (Fig. 1).

MATERIALS AND METHODS

Synthesis of internal standards

d_6-1,2-Diacetylhydrazine was synthesized by refluxing 6.7 mmole of hydrazine hydrate (Fisher Scientific, Pittsburgh, PA, U.S.A., as an 85% solution) with 20 mmole d_6-acetic anhydride (Merck, Sharp and Dohme Isotopes, Rahway, NJ, U.S.A.) for 90 min. After distillation of the volatile substances in vacuo, the residue was recrystallized from methanol—diethyl ether (40% yield, m.p. 139—139.5°C). Homogeneity was confirmed by thin-layer chromatography on silica gel with *n*-butanol—ethanol—0.4 *N* ammonium hydroxide (4:1:1), $R_F = 0.56$. d_3-Acetylhydrazine was synthesized according to Nelson et al. [4].

d_3-Acetylisoniazid was synthesized according to Fox and Gibas [5] using d_3-acetic anhydride (Merck, Sharp and Dohme Isotopes) and tetrahydrofuran instead of acetic anhydride and glacial acetic acid.

Isoniazid d_6-benzaldehyde was synthesized by mixing equimolar amounts of isoniazid with d_6-benzaldehyde (Merck, Sharp and Dohme Isotopes) in methanol at 35°C. The hydrazone was crystallized twice, first from methanol and then from ethyl acetate.

Reference compounds

Isoniazid, diacetylhydrazine, acetylhydrazine and benzaldehyde were obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetylisoniazid, [^{14}C] acetylisoniazid and [^{14}C] acetylhydrazine were synthesized as described previously [4, 6]. [^{3}H] Diacetylhydrazine was prepared like the deuterated analogue using [^{3}H]-acetic anhydride (New England Nuclear, Boston, MA, U.S.A.). [^{3}H] Isoniazid was purchased from Amersham Corporation, Arlington Heights, IL, U.S.A.

Sample preparation

For the determination of the concentrations of hydrazino metabolites in human plasma following an oral dose of 300 mg of isoniazid, venous blood was drawn into heparinized tubes. The plasma was immediately separated and stored at −20°C until the time of the assay which took place within five days. For the generation of standard curves between 0.02 and 2 μg of acetylhydra-

zine, diacetylhydrazine and acetylisoniazid and 0.5—15 μg of isoniazid were added to pooled, citrated plasma.

Each of the internal standards (20 μl of 0.1 mg/ml in methanol) was added to 1 ml of plasma, except for isoniazid d_6-benzaldehyde. The plasma was then extracted with 10 ml of methylene chloride to remove lipids interfering with the GC—MS assay. After centrifugation, the aqueous layer was added to 10 μl of benzaldehyde containing 10 μg isoniazid d_6-benzaldehyde. Under frequent shaking, the solution was reacted for 20 min at room temperature to form the benzaldehyde hydrazones of acetylhydrazine and isoniazid. Thirty milliliters of ethyl acetate were then added. While mixing the sample on a vortex mixer, 2 g of anhydrous sodium sulfate powder were slowly added. The ethyl acetate was decanted and evaporated under a stream of nitrogen in a water bath of 40°C. After transfer of the sample into Reactivials, 30 μl of BSTFA (both from Pierce, Rockford, IL, U.S.A.) were added, and the sample was reacted for 1 h at 80°C. A 1—5 μl aliquot was then injected onto the column.

GC—MS assay

A LKB 9000 gas chromatograph—mass spectrometer equipped with a LKB 9020 peak matcher was used. The trimethylsilyl derivatives were separated on 1% OV-17 in a glass column, 1.8 m × 2 mm I.D., using helium, 30 ml/min, as the carrier gas and a temperature program which ran from 90—270°C at 10°C/min. The MS analysis was performed under the following conditions: electron energy 20 eV, accelerating voltage 3.5 kV, electron multiplier 2400 V. A full scan spectrum was obtained of each compound and each internal standard in individual runs to confirm its identity and to determine the major ion closest to the molecular ion for single ion monitoring. For the simultaneous measurement of the metabolites and the corresponding deuterated internal standard, the accelerating voltage was increased periodically and the mass of the selected ion of the metabolite and the mass of the ions of the internal standards 3 or 6 mass units, respectively, greater than the metabolite ion were monitored alternatively. In order to correct for slight drifts of the mass marker during a day's run, the sweep generator modulated the magnetic field with an amplitude chosen to cover ± 0.2 mass units on both sides of the monitored ion.

For quantitation, the peak height ratio of the selected ions of each metabolite and its deuterated analogue were compared with a computer fitted standard curve which was run each day.

Recovery studies were performed with radioactively labeled analogues that were carried through the extraction and derivatization procedures. To assess the yield of hydrazone formation plasma samples spiked with [^{3}H]isoniazid were chromatographed after derivatization and extraction on Avicel F (Analtech, Newark, DE, U.S.A.) using the solvent system described above. Bands containing isoniazid (R_F 0.45) and isoniazid—benzaldehyde—hydrazone (R_F 0.85) were scraped off, eluted with methanol and counted by liquid scintillation spectrometry.

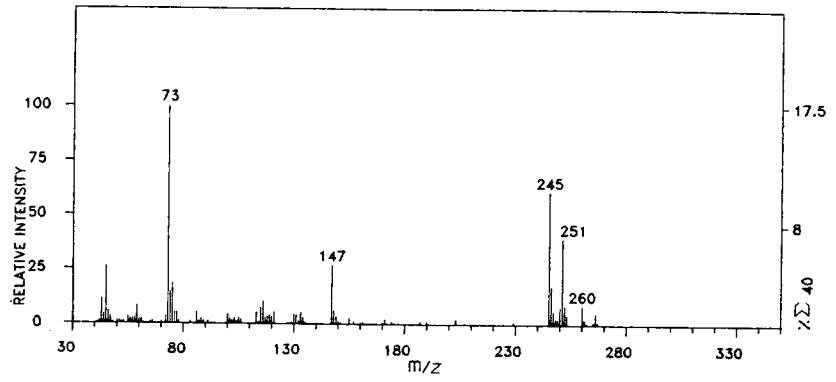

Fig. 2. Mass spectrum of the trimethylsilyl derivatives of diacetylhydrazine and the corresponding internal standard, d_6-diacetylhydrazine. For conditions see text. The ions at *m/z* 245 and 251 were monitored for quantification.

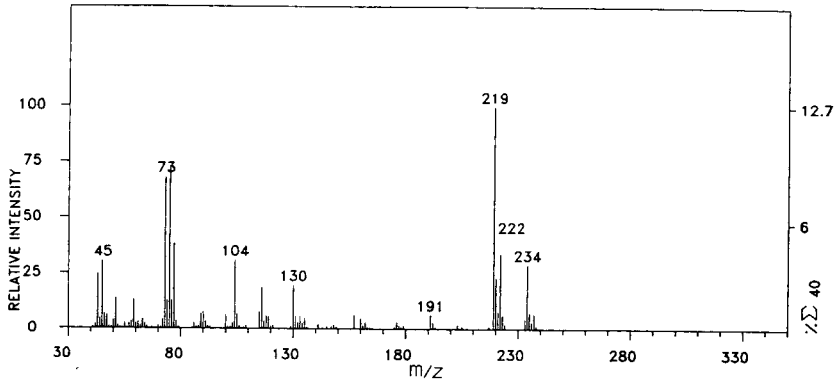

Fig. 3. Mass spectrum of the trimethylsilyl derivative of acetylhydrazine benzaldehyde hydrazone and the corresponding internal standard d_3-acetylhydrazine. The ions at *m/z* 219 and 222 were monitored for quantification.

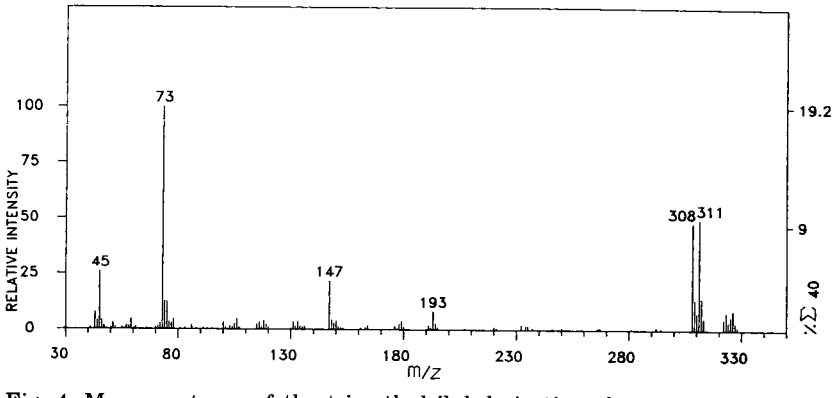

Fig. 4. Mass spectrum of the trimethylsilyl derivative of acetylisoniazid and the corresponding internal standard d_3-acetylisoniazid. The ions at *m/z* 308 and 311 were monitored for quantification.

RESULTS

Full scan spectra of the measured compounds and the corresponding internal standards are shown in Figs. 2—5. The spectra of synthetic standards and of isoniazid metabolites formed in man were identical. For the quantitative analysis by single ion monitoring the most abundant ion, M^+-15, was chosen (Fig. 6). Each of the internal standards appears shortly before the corresponding metabolite. This isotope effect results in a characteristic peak configuration for each

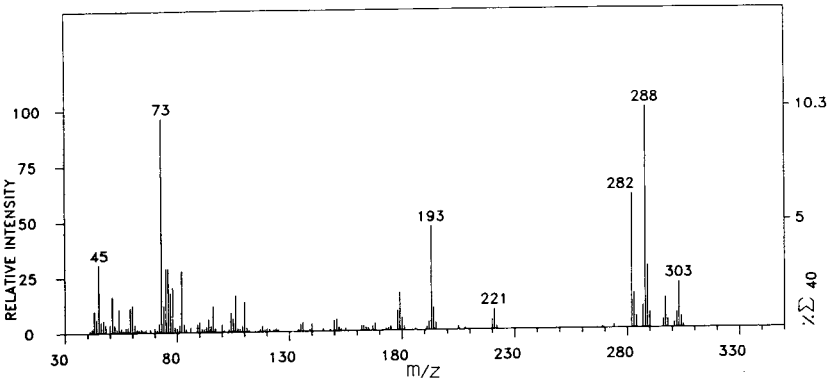

Fig. 5. Mass spectrum of the trimethylsilyl derivative of isoniazid benzaldehyde hydrazone and the corresponding deuterated internal standard. The ions at *m/z* 282 and 288 were monitored for quantification.

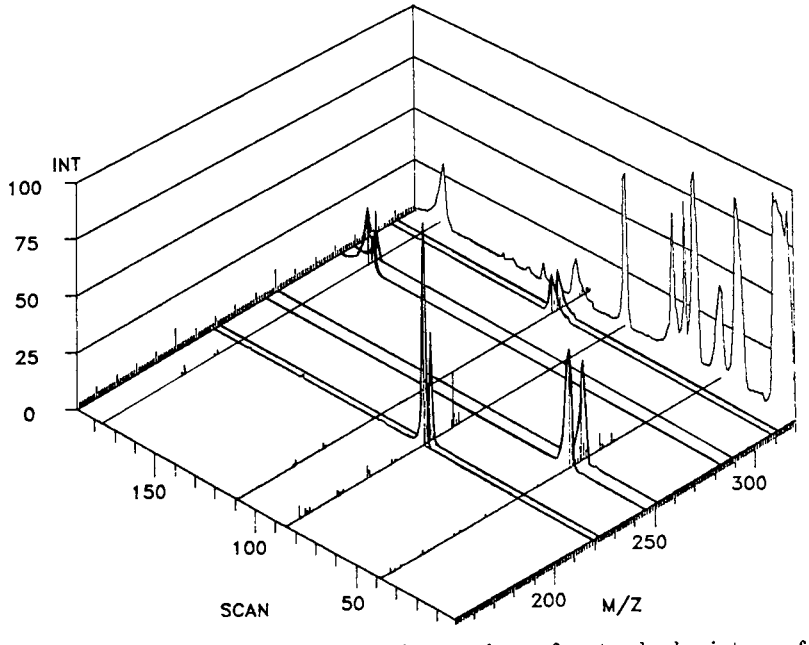

Fig. 6. Total ion current and single ion tracings of a standard mixture of diacetylhydrazine (*m/z* 245), acetylhydrazine (*m/z* 219), acetylisoniazid (*m/z* 308) and isoniazid (*m/z* 282) and their deuterated internal standards. For GC—MS conditions see text.

metabolite which, together with the retention time, allows a ready identification of each peak of interest. The recoveries of the sample preparation and the precision of the method are shown in Table I. Thin-layer chromatography of plasma extracts demonstrated that an average of 90.3% of the isoniazid is derivatized to the benzaldehyde hydrazone. The peak ratios of the metabolites and their corresponding internal standards are proportional over the concentration range encountered in plasma of slow and rapid acetylators following an oral therapeutic dose of 300 mg isoniazid (Fig. 7).

TABLE I

RECOVERY OF SAMPLE PREPARATION AND PRECISION OF METHOD

Recovery was measured with radioactively labeled compounds as described in Materials and methods.

	Recovery (%)	Coefficient of variation (%) of three determinations at indicated concentrations				
		0.05 μg/ml	0.1 μg/ml	0.5 μg/ml	1 μg/ml	5 μg/ml
Isoniazid	76	—	11	8	5	4
Acetylisoniazid	71	—	9	5	6	—
Acetylhydrazine	70	8	11	6	3	—
Diacetylhydrazine	64	9	5	6	4	—

Following the addition of either α-ketoglutarate acetylhydrazone or pyruvate acetylhydrazone to pooled plasma, no free acetylhydrazine was found under the chosen conditions indicating that the assay does not measure hydrazones of acetylhydrazine circulating in plasma.

DISCUSSION

Based on the extensive animal studies from this laboratory, the toxicologically important metabolites of isoniazid are acetylhydrazine and diacetylhydrazine [1]. A number of methods has been proposed to measure these metabolites. Earlier colorimetric and fluorometric assays of uncertain specificity have been used to quantitate the urinary levels of these metabolites but are insufficiently sensitive to quantitate plasma concentrations [7]. More recently published GC methods for acetylhydrazine and diacetylhydrazine, although more specific, are also not sensitive enough to measure these metabolites in plasma [8, 9]. Our highly specific GC—MS assay, on the other hand, is clearly sensitive enough to accurately determine acetylhydrazine and diacetylhydrazine plasma concentrations in the range of 0.01—2 μg/ml following an oral therapeutic dose of isoniazid. Our assay is least sensitive for the parent compound isoniazid itself, mainly due to the long and slow temperature program which is required to analyze the more volatile metabolites. Under the described chromatographic conditions, however, the method is sensitive enough to accurately determine the disappearance rate of isoniazid which is present in high enough concentrations for the first few hours after its ingestion. The sensitivity of the assay for

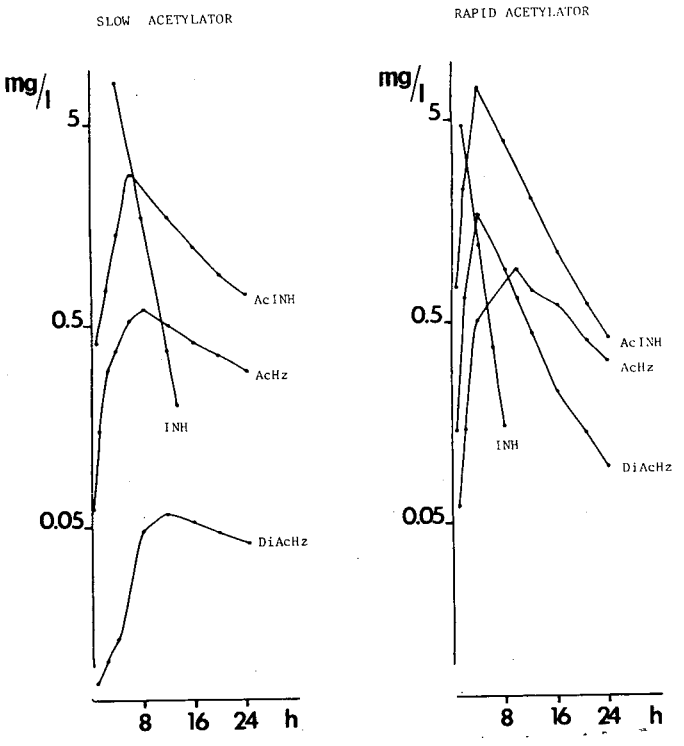

Fig. 7. Concentrations of isoniazid (INH), acetylisoniazid (AcINH), acetylhydrazine (AcHz), and diacetylhydrazine (DiAcHz) in the plasma of a patient with slow acetylator phenotype and one with rapid acetylator phenotype following the oral administration of 300 mg isoniazid.

isoniazid can be markedly increased if the temperature program of the GC separation is started at 200°C. Under these conditions, however, acetylhydrazine and diacetylhydrazine appear in the solvent front and can not be measured.

After acid hydrolysis of urinary hydrazones, the urinary excretion of metabolites can be followed with the identical procedure. Thus, the assay allows a complete kinetic analysis of the toxicologically important metabolites of isoniazid and may help elucidate factors determining the variable susceptibility of patients to isoniazid liver injury.

ACKNOWLEDGEMENTS

This work was supported by Grant Nos. NIGMS-GM-26611 and NIGMS-GM-13901. B.H. Lauterburg was the recipient of the Pharmaceutical Manufacturers Association Foundation Faculty Development Award in Clinical Pharmacology and J.R. Mitchell the Burroughs Wellcome Scholar in Clinical Pharmacology.

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Journal of Chromatography, 224 (1981) 439—448
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 895

SIMULTANEOUS MICRODETERMINATION OF THEOPHYLLINE, CAFFEINE AND PHENOBARBITAL IN BLOOD COLLECTED ON PAPER

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(First received January 9th, 1981; revised manuscript received March 18th, 1981)

SUMMARY

A sensitive and selective gas chromatographic method has been developed for the simultaneous determination of theophylline, caffeine and phenobarbital. Blood collection is performed by dropping 30 μl of blood onto a disc of a special paper. Vinbarbital is used for quantitation by the internal standard method. The chromatographic separation is performed on a 3% OV-17 column, after pentylation of the methylxanthine and internal standard, and the compounds are detected with a nitrogen-sensitive detector.

The sensitivity of the method allows the monitoring of theophylline therapy in premature newborns by the differential determination of caffeine and theophylline. The sampling method does not affect the accuracy and precision and is very suitable for the collection of small blood samples.

INTRODUCTION

The methylxanthines theophylline and caffeine are widely used for the prevention and treatment of apnea in the premature newborn. The metabolism

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and pharmacokinetic parameters of these drugs show important differences in premature newborns in comparison with those in older children or adults [1—6]. It has been shown that theophylline is converted into caffeine in premature newborns [7—10] and this original metabolic pathway has been demonstrated by use of molecules labelled with stable isotopes [11]. Caffeine has nearly the same pharmacological activity as theophylline, so it is necessary to measure both drug levels during theophylline therapy. Some neonatologists prefer to use caffeine instead of theophylline to treat apneoic attacks in the premature newborn [3]. As theophylline is a metabolite of caffeine, it is also important to measure both caffeine and theophylline plasma levels after caffeine administration. Therefore, in the drug monitoring of apneas in premature newborns, it is of utmost importance to measure the total methylxanthines plasma levels in order to ensure the efficacy of the treatment and to prevent toxicity.

The analytical method has to be able to separate theophylline from caffeine because the elimination rate constants of these two drugs are different in premature newborns. The mean half-life of theophylline is 30 h [9, 12] and that of caffeine is about 100 h [13]. Moreover, phenobarbital is often given at birth to prevent neonatal jaundice [14]. The half-life of this drug is very long in the newborn (200 h) [15], so it may be important to measure phenobarbital plasma levels simultaneously.

The lower and upper limits of the therapeutic range of plasma theophylline or caffeine concentrations are dictated by efficacy and toxicity. Available data [6] suggest that the range for theophylline is probably about 7—15 $\mu g \cdot ml^{-1}$. Plasma levels effective for the control of apnoeic spells can be lower, ranging between 3 and 5 $\mu g \cdot ml^{-1}$ [16, 17]. Plasma concentrations of caffeine as low as 3—4 $\mu g \cdot ml^{-1}$ can abolish apnea and regularize breathing patterns. Generally, therapeutic caffeine plasma levels range between 3 and 12 $\mu g \cdot ml^{-1}$ [18].

As many departments of neonatology do not have a laboratory available for assaying these drugs rapidly, we propose here a simultaneous method for the microdetermination of caffeine, theophylline and phenobarbital from blood collected on a special paper. This makes very easy the collection of blood by heel pricks from babies in incubators. Moreover, samples can be easily transported to a remote laboratory by letter mail.

EXPERIMENTAL

Materials

Theophylline, theobromine, caffeine and phenobarbital were purchased from Sigma (St. Louis, MO, U.S.A.). Tetramethylammonium hydroxide, N,N-dimethylacetamide, iodopentane, isopropanol, ethyl acetate and chloroform (analytical-reagent grade) were purchased from Merck (Darmstadt, G.F.R.) and used without further purification.

Stock 100 $\mu g \cdot ml^{-1}$ solutions of theophylline, caffeine, theobromine and phenobarbital in ethanol were prepared. The internal standard was vinbarbital, a barbiturate not often used in therapeutics. All stock solutions were stored at 4°C and used within 1 month. Working solutions (10 $\mu g \cdot ml^{-1}$) were prepared each day by dilution of the stock solution with ethanol. The extraction solvent

was chloroform—isopropanol (95:5). For extractions, blood was buffered with acetate buffer (pH 5.2) to improve the extraction efficiency.

A Schleicher & Schüll (Dassel, G.F.R.) Type 2992 paper was used for blood collection; 30 μl of blood were dropped into an 11 mm diameter circle printed on the paper and dried.

Extraction

A disc of 10 mm diameter containing the whole blood spot was stamped out, transferred into a 20-ml screw-capped centrifuge tube and rinsed with 300 μl of acetate buffer (pH 5.2) for 5 min. Then 20 μl of vinbarbital standard solution were added and thoroughly mixed, 1.5 ml of the extraction solvent were added and the blood was extracted for 1 min on a vortex mixer. After centrifugation, an aliquot of the organic phase was transferred into another centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C.

Derivatization of the N—H group was performed according to the reaction described by Greeley [19]. A 50-μl volume of N,N-dimethylacetamide and 20 μl of tetramethylammonium hydroxide solution (0.1 *M*) were added to the dry residue and shaked vigorously for 10 sec. Then 10 μl of iodopentane were added to the solution, which was shaken and allowed to stand at room temperature for 10 min. After centrifugation (2 min at 2000 *g*) the organic phase was transferred into a third centrifuge tube and evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 50 μl of ethyl acetate and 1—3 μl were injected into the column for chromatographic analysis.

Standardisation

Various blood samples spiked with concentrations of caffeine, theophylline and phenobarbital ranging between 0.5 and 20 $\mu g \cdot ml^{-1}$ were prepared. These blood samples were dropped on to the paper and the spots dried, then 10 mm diameter discs, corresponding to 30 μl of blood, were stamped out of the blood spots and treated as described above.

Calibration graphs for each drug were obtained by plotting peak area ratio (peak area of drug / peak area of vinbarbital) against drug concentration.

Chromatographic separation

The gas chromatograph used was a Hewlett-Packard Model 5710A with a thermionic nitrogen—phosphorus-sensitive detector. The column used was a silanized glass tube (2 m × 1.8 mm I.D.) coated with 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh). Separations were performed isothermally at 230°C (injector and detector temperature 250°C). Nitrogen was used as the carrier gas at a flow-rate of 30 $ml \cdot min^{-1}$. The output signal was integrated and the results were calculated using a Hewlett-Packard 3385A electronic integrator.

RESULTS

Chromatograms

A typical chromatogram of a standard mixture containing caffeine, theo-

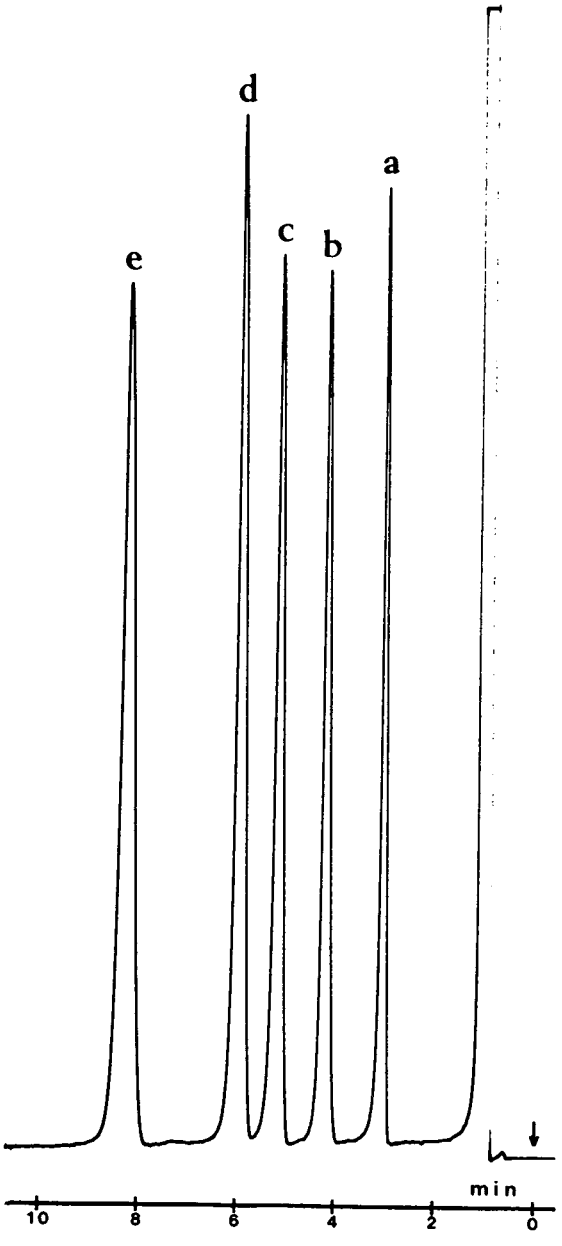

Fig. 1. Typical chromatogram of a standard mixture containing (a) caffeine, (b) vinbarbital, (c) theophylline, (d) paraxanthine and (e) phenobarbital.

phylline, paraxanthine and phenobarbital is shown in Fig. 1. Absolute and relative retention times of methylxanthines and various barbiturates are given in Table I.

Fig. 2 shows chromatograms of extracts of (A) a blood sample spiked with drugs and (B) the blood of a premature newborn treated with theophylline and

TABLE I

RETENTION TIMES OF METHYLXANTHINES AND BARBITURATES

Compound	Absolute retention time	Relative retention time
Caffeine	1.80	0.71
Allobarbital	1.80	0.71
Aprobarbital	1.92	0.76
Butalbital	1.92	0.76
Butobarbital	1.97	0.78
Amobarbital	2.10	0.83
Mebubarbital	2.35	0.93
Pentobarbital	2.35	0.93
Hexobarbital	2.40	0.95
Secobarbital	2.50	0.99
Vinbarbital	2.53	1.00
Theophylline	3.33	1.31
Paraxanthine	3.64	1.44
Theobromine	3.67	1.46
Brallobarbital	3.74	1.48
Mephobarbital	4.40	1.74
Phenobarbital	5.31	2.10
3-Methylxanthine	6.15	2.43
1-Methylxanthine	7.06	2.49
7-Methylxanthine	7.34	2.90

phenobarbital. Blood samples were collected on paper discs. Fig. 3 shows the chromatogram of a blank. The 3% OV-101 column is also suitable for the chromatographic separation of methylxanthines and barbiturates. On such a column the retention time of vinbarbital is greater than that of theophylline but the resolution is still very good.

Linearity

Concentrations were measured under the conditions described above, using blood samples spiked with concentrations of 0.5—20 $\mu g \cdot ml^{-1}$ of each drug and collected on paper. Standardization was performed using the internal standard method. A linear regression analysis of the three graphs (peak area ratio versus concentration) indicated an almost linear fit of the data:

caffeine: slope = 0.102; intercept = 0.022; $r = 0.998$;
theophylline: slope = 0.168; intercept = 0.020; $r = 0.999$;
phenobarbital: slope = 0.089; intercept = −0.002; $r = 0.999$.

Accuracy and repeatability

The accuracy and repeatability of the method in the therapeutic range were studied by measuring concentrations of blood samples spiked with 2, 5, 10 and 15 $\mu g \cdot ml^{-1}$ of each drug. Measurements were performed immediately on the whole blood, then 24 h and 8 days after collecting the same blood on paper and keeping the samples at room temperature. Five samples were assayed corresponding to each drug concentration. The results are given in Tables II—IV.

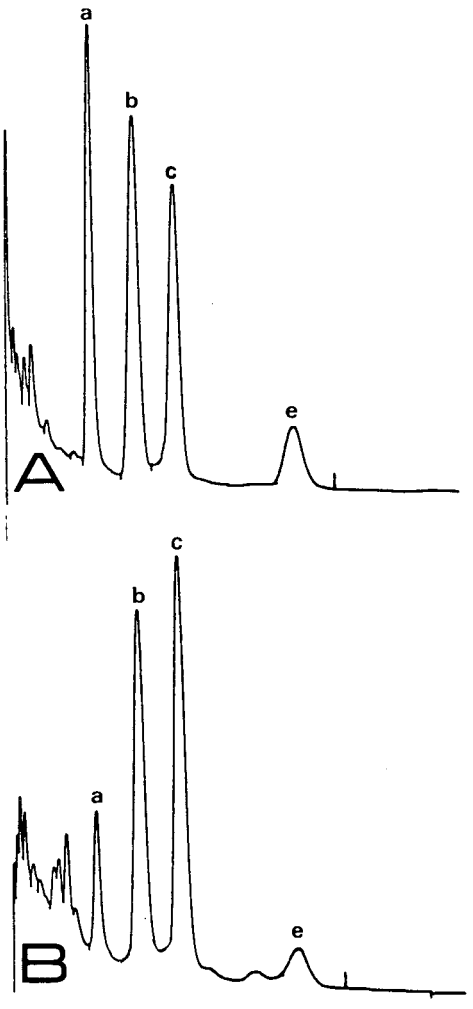

Fig. 2. (A) Chromatogram of an extract of blood collected on paper and spiked with (a) caffeine, (b) vinbarbital, (c) theophylline and (e) phenobarbital (10 $\mu g \cdot ml^{-1}$ of each). (B) Chromatogram of an extract of the blood of a premature newborn treated with theophylline and phenobarbital (collection on paper). Caffeine, 3.45 $\mu g \cdot ml^{-1}$; theophylline, 14.6 $\mu g \cdot ml^{-1}$; phenobarbital, 3.01 $\mu g \cdot ml^{-1}$.

Correlations between added and measured concentrations obtained from whole blood gave the following results:

caffeine: slope = 1.02 ± 0.0096; intercept = −0.063; r = 0.9991;
theophylline: slope = 1.01 ± 0.011; intercept = 0.078; r = 0.9987;
phenobarbital: slope = 1.02 ± 0.0106; intercept = −0.023; r = 0.9989.

The correlations between drug concentrations measured in whole blood and paper discs 24 h and 8 days after blood collection are given in Table V.

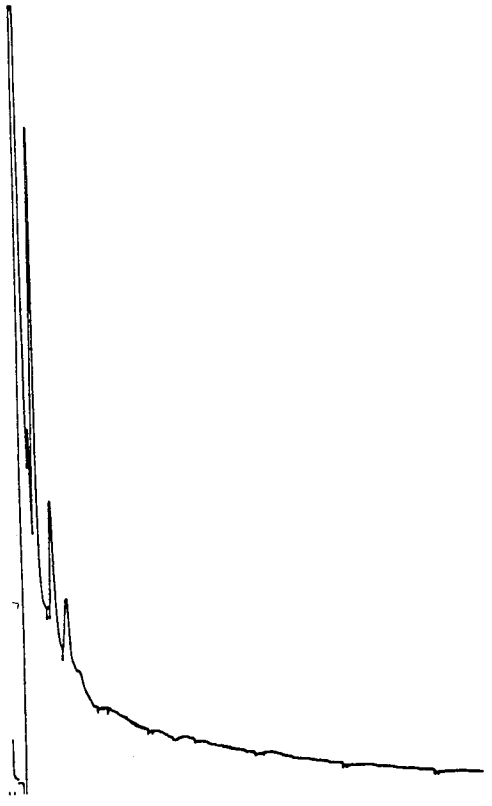

Fig. 3. Chromatogram of a blank.

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OF BLOOD COLLECTED IN TUBES ($n = 5$)

Compound	Added (μg/ml)	Measured (μg/ml) ($\bar{m} \pm \sigma$)	Relative error (%)	Coefficient of variation (%)
Theophylline	15	15.17 ± 0.27	1.13	1.78
	10	10.20 ± 0.34	2.00	3.33
	5	5.24 ± 0.18	4.8	3.44
	2	1.98 ± 0.06	1.0	3.03
Caffeine	15	15.46 ± 0.25	3.07	1.62
	10	10.06 ± 0.25	0.60	2.49
	5	5.09 ± 0.11	1.80	2.16
	2	2.05 ± 0.10	2.50	4.76
Phenobarbital	15	15.23 ± 0.17	1.53	1.12
	10	10.43 ± 0.26	4.30	2.49
	5	5.16 ± 0.11	3.00	2.13
	2	1.91 ± 0.13	4.50	6.7

TABLE III

ACCURACY AND PRECISION OF THE ASSAY 24 HOURS AFTER COLLECTION OF BLOOD ON PAPER ($n = 5$)

Compound	Added (μg/ml)	Measured (μg/ml) ($\bar{m} \pm \sigma$)	Relative error (%)	Coefficient of variation (%)
Theophylline	15	15.19 ± 0.48	1.26	3.15
	10	10.23 ± 0.68	2.30	6.64
	5	5.16 ± 0.27	3.21	5.20
	2	2.04 ± 0.09	2.00	4.41
Caffeine	15	15.27 ± 0.42	1.50	2.75
	10	10.16 ± 0.74	1.60	7.20
	5	4.84 ± 0.36	3.20	7.40
	2	2.06 ± 0.13	3	6.30
Phenobarbital	15	15.25 ± 0.65	1.67	4.20
	10	9.77 ± 0.77	2.30	7.60
	5	5.19 ± 0.25	3.80	4.80
	2	2.08 ± 0.14	4	6.72

TABLE IV

ACCURACY AND PRECISION OF THE ASSAY 8 DAYS AFTER COLLECTION OF BLOOD ON PAPER ($n = 5$)

Compound	Added (μg/ml)	Measured (μg/ml) ($\bar{m} \pm \sigma$)	Relative error (%)	Coefficient of variation (%)
Theophylline	15	15.41 ± 1.1	2.63	7.1
	10	10.25 ± 0.83	2.50	8.09
	5	5.17 ± 0.35	3.40	6.76
	2	2.04 ± 0.12	2.00	5.8
Caffeine	15	15.61 ± 1.30	4.06	8.30
	10	9.65 ± 0.85	3.50	8.80
	5	5.13 ± 0.40	2.60	7.70
	2	2.04 ± 0.14	2.00	6.80
Phenobarbital	15	14.60 ± 1.0	2.61	6.80
	10	10.21 ± 0.81	2.10	7.90
	5	5.11 ± 0.37	2.20	7.24
	2	2.07 ± 0.15	3.50	7.23

CONCLUSION

Blood levels of xanthines used to prevent apneas of premature newborns must be monitored with care because of the low therapeutic index of these drugs. This monitoring must be performed easily on very small blood samples by a specific and sensitive method. The choice of the internal standard is very important, especially for measurements of theophylline levels. Theophylline is

TABLE V

CORRELATIONS BETWEEN RESULTS FOR WHOLE BLOOD AND BLOOD ON PAPER DISCS

Correlations: A, whole blood versus paper discs 24 h after blood collection; B, whole blood versus paper discs 8 days after blood collection.

Correlation	Drug	Slope ± S.D.	Intercept	r
A	Theophylline	1.0009 ± 0.007	0.0022	0.9999
	Caffeine	0.9952 ± 0.019	−0.043	0.9996
	Phenobarbital	0.9757 ± 0.041	0.082	0.9981
B	Theophylline	1.016 ± 0.012	−0.078	0.9988
	Caffeine	1.001 ± 0.029	−0.070	0.9991
	Phenobarbital	0.943 ± 0.007	0.27	0.9993

methylated at the N_7 position by premature newborns to give caffeine. Caffeine can be present in the blood of babies in the first few days of life from placental transfer and afterwards as a result of breast feeding, so caffeine must not be used as an internal standard. Moreover, gas—liquid chromatographic methods involving methylation are not suitable because of the transformation of theophylline into caffeine. The method is also suitable for caffeine therapeutic monitoring. This methylxanthine is also widely used in the prevention and treatment of apneas of premature newborns [4, 18, 20]. Moreover, theophylline is one of the numerous metabolites of caffeine. Hence vinbarbital is a good internal standard; this barbiturate is not used in paediatric therapeutics, is quantitatively extracted (98%) and gives an N,N-dipentyl derivative that is easily chromatographed with a retention time between those of caffeine and theophylline.

The accuracy of the method is good when the results obtained for whole blood collected in tubes are compared with those for added drug concentrations. The accuracy and precision of the results obtained from blood collected on paper discs are also very good.

The accuracy remains nearly the same 24 h and 8 days after collection. The coefficient of variation increases from a mean of 3% for blood collected in tubes to 7% for blood collected on paper discs and assayed 8 days after collection for each of the three drugs.

Blood can be collected very easily on paper discs from premature babies in incubators. Hence it is possible to collect the very small amount of blood (30 μl) necessary for the drug assay and this technique is very practical for both collecting and sending the blood samples to a remote laboratory.

The method described has been used successfully for more than 1 year in pharmacokinetic studies and drug monitoring in premature newborns treated in various hospitals.

The sensitivity, specificity, accuracy and sampling technique make the proposed method very suitable for the complete therapeutic monitoring of the treatment of apneas of premature newborns.

ACKNOWLEDGEMENTS

The authors are very grateful to Miss M. Gros for secretarial assistance.

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Journal of Chromatography, 224 (1981) 449—455
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 884

DETERMINATION OF BARBITURATES AND SOME NEUTRAL DRUGS IN SERUM USING QUARTZ GLASS CAPILLARY GAS CHROMATOGRAPHY

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(First received December 15th, 1980; revised manuscript received February 27th, 1981)

SUMMARY

Rapid methods for the glass capillary gas chromatographic determination of barbiturates and some neutral drugs are described. The analysis of barbiturates was performed using a nitrogen—phosphorus selective detector (NPD). The barbiturates were recovered from serum using charcoal adsorption followed by extraction with methylene chloride. The drugs were then alkylated by means of the Claisen carbonate method. Neutral drugs were extracted simultaneously with the barbiturates. The neutral drugs were determined underivatized with a flame ionization detector. In the underivatized form the barbiturates were not stable on the quartz column used. The selectivity of derivatization combined with an NPD was used to determine the barbiturates in the presence of neutral drugs with the aid of retention data.

INTRODUCTION

Barbiturate analysis by glass capillary gas chromatography (GC/GC) has been described by Garle and Petters [1]. An extractive alkylation procedure was used to recover barbiturates and glutethimide selectively from other types of drugs. A sensitive GC/GC determination of barbiturates was reported by Dünges et al. [2], who claimed that barbiturates could be identified using the Claisen carbonate method to prepare various derivatives of the same compound. A rapid procedure for the extraction of drugs from serum was studied by Meola [3]. Barbiturates and other drugs were adsorbed on activated charcoal, from which they were extracted with a solvent mixture. After evaporation of the organic phase, the residue was dissolved in a small volume of ethyl acetate and the solution was injected directly into a gas chromatograph equipped with packed columns. This method is very simple.

Preliminary studies on underivatized barbiturates using commercially available deactivated quartz glass capillary columns showed serious adsorption/

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catalysis on the glass surface, resulting in poor sensitivity. Sandra et al. [4] separated underivatized barbiturates successfully on a persilylated glass capillary column coated with OV-1. However, free barbiturates give low responses but alkylated barbiturates give high responses if a nitrogen-sensitive detector was used. Therefore, the derivatization method proposed by Dünges et al. [2] was combined with quartz GC/GC and the nitrogen—phosphorus selective detector (NPD) was applied.

Serum samples were investigated using a minor modification of the rapid extraction procedure of Meola [3] followed by the alkylation procedure reported by Dünges et al. [2].

EXPERIMENTAL

Apparatus

One Packard 427 gas chromatograph with an NPD and one Packard 427 gas chromatograph with a flame ionization detector (FID), both equipped with split injectors, were used. The gas chromatographs were connected to Sigma 10 Chromatography Data Station (Perkin-Elmer, Norwalk, CT, U.S.A.). Fused silica capillary columns (25 m × 0.20—0.21 mm I.D.), deactivated with Carbowax 20M, and with SP-2100 as the stationary phase, part number 19091-60025 (Hewlett-Packard, Avondale, PA, U.S.A.), were applied. The GC/GC conditions were as follows: injection port temperature, 230°C; oven temperature, programmed from 110 to 230°C at 10°C/min and then kept isothermally at 230°C for 5 min. Nitrogen was employed both as a carrier gas at a flow-rate of 0.8 ml/min and as the make-up gas at a flow-rate of 10 ml/min. The splitting ratio was 1:10.

Reagents

The reagents used were of analytical-reagent grade, unless specified otherwise: acetone (Merck, Darmstadt, G.F.R.); activated charcoal (Riedel-de Haën, Hannover, G.F.R.); methylene chloride (Baker, Phillipsburg, NJ, U.S.A.) (HPLC grade); ethyl iodide (iodoethane) (BDH, Poole, Great Britain); potassium carbonate, anhydrous.

Serum standards, barbiturates

To drug-free serum were added the barbiturates metharbital, barbital, aprobarbital, butalbital, amobarbital, pentobarbital, vinbarbital, secobarbital, hexobarbital, phenobarbital, cyclobarbital and heptabarbital, all in the range 5—40 μg/ml.

Serum standard, neutral drugs

To drug-free serum were added hexapropymate (30 μg/ml), methyprylon (10 μg/ml), persedon (10 μg/ml), phenazone (5 μg/ml), methaqualone (10 μg/ml) and meprobamate (10 μg/ml).

Internal standard solution for the determination of barbiturates

A 10-mg amount of allobarbital was dissolved in 10 ml of ethanol.

Extractant

Methylene chloride containing 7.5 μg/ml of eicosane ($C_{20}H_{42}$) (eicosane was used as an internal standard for the determination of underivatized neutral drugs) was employed.

Procedure

To 500 μl of serum were added approximately 10 mg of activated charcoal with the aid of a calibrated spatula, then 10 μl of the allobarbital internal standard solution were added (if underivatized neutral drugs were of prime interest, addition of this solution was omitted) and the mixture was vortexed for 30 sec. After centrifugation for 2 min at 2500 *g* the aqueous phase was aspirated off and discarded. The charcoal precipitate was evenly distributed in the bottom of the extraction tube by vortexing. The drugs were then extracted with 300 μl of methylene chloride containing eicosane by vortexing for 1 min. After centrifugation for 2 min at 2500 *g* the organic phase was transferred into a conical mini-vial (volume approximately 400 μl) and evaporated to dryness using a gentle stream of air.

Screening of neutral drugs, GC/GC—FID

The residue was dissolved in 10 μl of acetone and 1 μl of the extract was injected into a gas chromatograph equipped with an FID.

Alkylated barbiturates, GC/GC—NPD

An additional 30 μl of acetone, 10 μl of ethyl iodide and a few grains of potassium carbonate were added to the mini-vial, which was sealed, vortexed and placed in an oven at 80°C for 30 min. Then 0.5 μl of the extract was injected into the gas chromatograph equipped with an NPD.

RESULTS

Fig. 1 (left chromatogram) shows the chromatographic separation of thirteen barbiturates, including the internal standard allobarbital, using the NPD. Retention times were very stable (± 1.2 sec). No major interfering peaks of endogenic origin were found in the serum extracts. Fig. 1 (right chromatogram) shows a serum blank. The GC/GC calibration graphs (peak area ratios to that of the internal standard, allobarbital, versus barbiturate concentration) were linear in the range 5—40 μg/ml for all of the barbiturates studied. All of the correlation coefficients were greater than 0.98. The detection limits were approximately 1 μg/ml or better, except for that of phenobarbital (2 μg/ml). Table I gives the precision data obtained by replicate analyses of sera to which all of the barbiturates had been added (data for three levels are given). Retention times are given in Table II.

As pointed out, many other drugs are co-extracted with the barbiturates. Some of the drugs may be analysed by GC/GC and detected underivatized with the FID. Fig. 2 (upper chromatogram) shows results for neutral drugs analysed in this way. Eicosane was used as the internal standard for the FID procedure. Eicosane was not detected by the NPD and thus did not interfere with the determination of barbiturates. Fig. 2 (lower chromatogram)

shows a serum blank obtained with the FID procedure. The GC/GC calibration graphs were linear in the ranges shown in Table III. The precision data were comparable to those obtained in the analysis of barbiturates (see Table I).

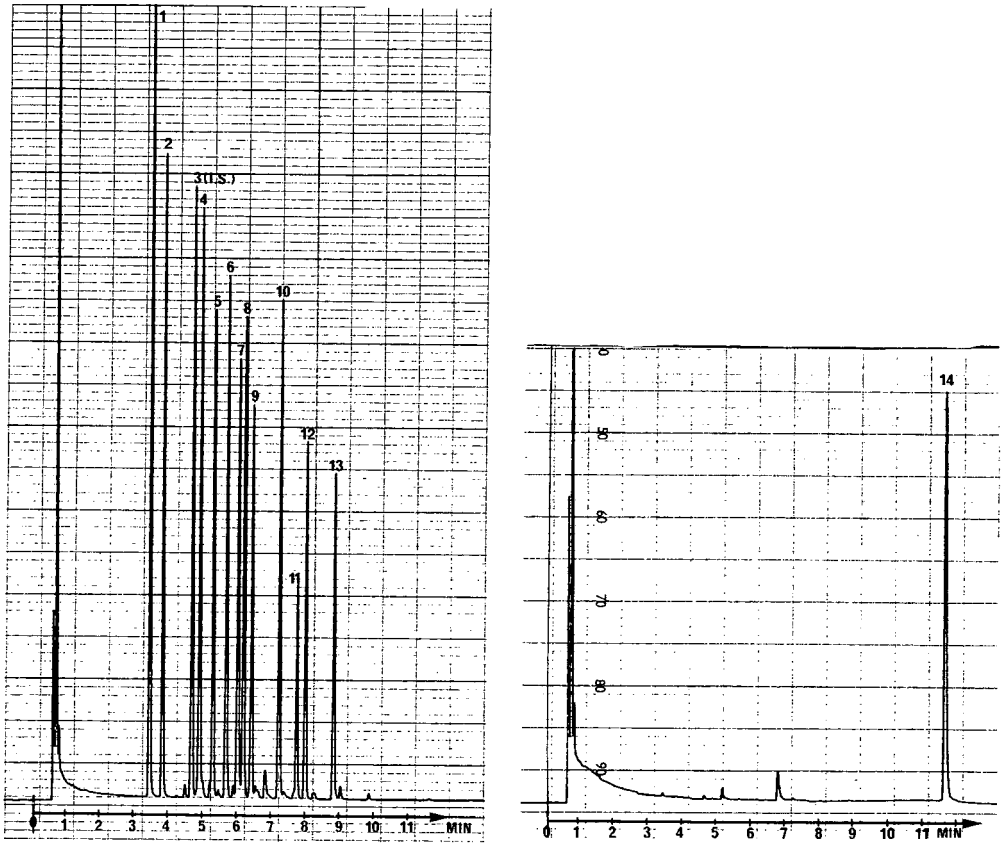

Fig. 1. The left chromatogram shows the results of an analysis of a serum barbiturate standard (concentration of each barbiturate = 20 μg/ml) obtained by the GC/GC—NPD procedure. Peaks: 1 = metharbital; 2 = barbital; 3 = allobarbital (internal standard); 4 = aprobarbital; 5 = butalbital; 6 = amobarbital; 7 = pentobarbital; 8 = vinbarbital; 9 = secobarbital; 10 = hexobarbital; 11 = phenobarbital; 12 = cyclobarbital; 13 = heptabarbital. Retention times are given in Table II. The right chromatogram shows the results for a serum blank (GC/GC—NPD procedure) without addition of internal standard (allobarbital). Peak 14 = unknown peak that is often found in the serum extracts.

DISCUSSION

The rapid extraction procedure chosen in this study is not selective for barbiturates. However, speed and simplicity of the sample preparation methods are great assets in routine clinical chemistry work. Further, when analysing serum samples from intoxicated patients valuable information may be lost if the extraction technique is selective for a group of drugs. Many methods

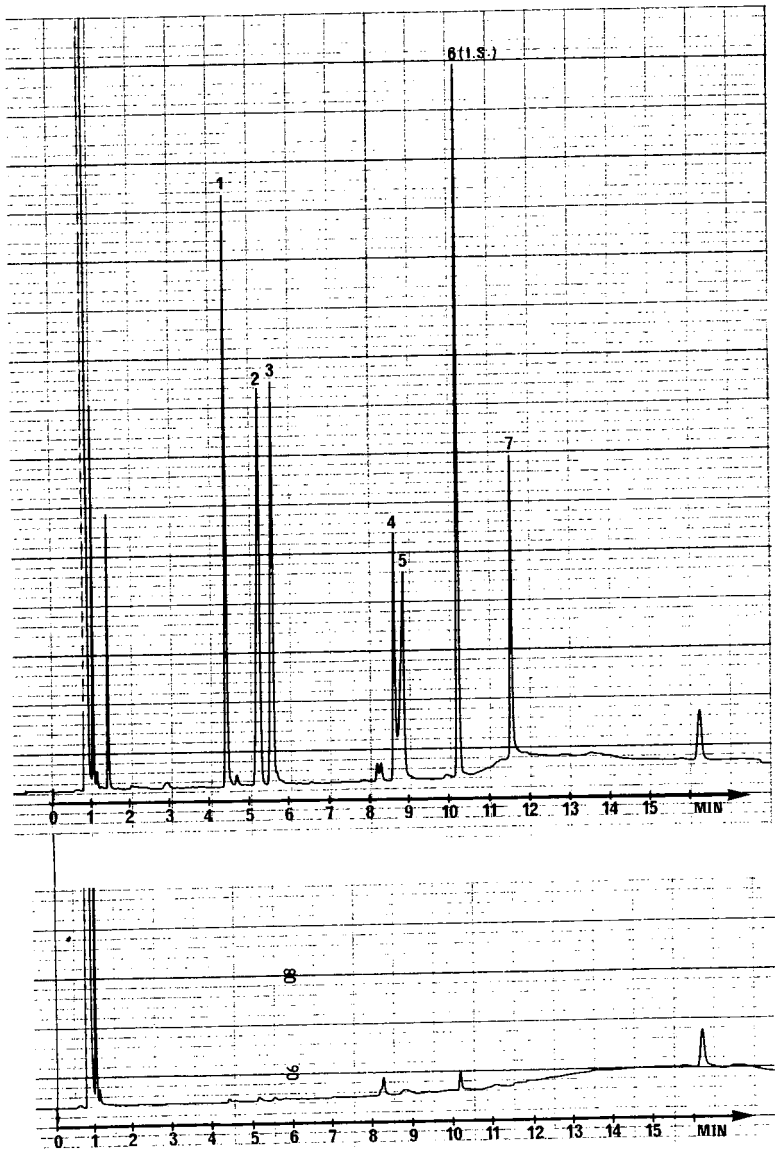

Fig. 2. The upper chromatogram shows the results of an analysis of a "serum neutral drug standard" obtained by the GC/GC—FID procedure. Peaks: 1 = hexapropymate; 2 = methyprylon; 3 = persedon; 4 = phenazone; 5 = meprobamate; 6 = eicosane (internal standard); 7 = methaqualone. Allobarbital addition was omitted (retention time of allobarbital is approximately 7.0 min and thus it does not interfere with the neutral drugs if added). Retention times are given in Table III. The lower chromatogram shows a serum blank (GC/GC—FID procedure) without addition of the internal standards eicosane and allobarbital.

TABLE I

PRECISION DATA FOR DETERMINATION OF BARBITURATES BY GC/GC–NPD PROCEDURE

Compound	Level 1 (n = 5)		Level 2 (n = 10)		Level 3 (n = 5)	
	Mean (μg/ml)	C.V. (%)*	Mean (μg/ml)	C.V. (%)*	Mean (μg/ml)	C.V. (%)*
Metharbital	9.8	11.4	19.5	7.6	39.8	5.6
Barbital	9.4	8.3	19.9	2.1	39.8	3.5
Aprobarbital	10.1	6.6	21.2	4.6	40.4	2.2
Butalbital	10.2	5.9	21.0	3.4	40.1	2.5
Amobarbital	10.2	5.3	21.1	4.4	40.4	2.9
Pentobarbital	9.8	7.5	21.4	5.5	41.3	3.4
Vinbarbital	9.9	8.2	21.5	4.6	41.6	2.5
Secobarbital	9.9	7.9	21.6	5.0	41.8	4.5
Hexobarbital	9.8	5.1	24.4	6.6	43.3	6.0
Pentobarbital	10.3	7.6	21.9	6.3	41.2	2.9
Cyclobarbital	10.1	7.4	23.7	5.8	42.1	3.6
Heptabarbital	9.8	8.9	23.8	8.7	43.9	5.9

*C.V. = coefficient of variation.

TABLE II

RETENTION DATA FOR THE GC/GC–NPD PROCEDURE (ETHYLATED DRUGS)

Peak No.	Compound	Retention time (min)	Relative retention
1	Metharbital	3.51	0.74
2	Barbital	3.88	0.82
3	Allobarbital (internal standard)	4.73	1.00
4	Aprobarbital	4.96	1.05
5	Butalbital	5.35	1.13
6	Amobarbital	5.73	1.21
7	Pentobarbital	6.07	1.28
8	Vinbarbital	6.23	1.32
9	Secobarbital	6.47	1.37
10	Hexobarbital	7.30	1.54
11	Phenobarbital	7.81	1.65
12	Cyclobarbital	8.06	1.70
13	Heptabarbital	8.90	1.88
—	Ethosuximide	1.88	0.40
—	Persedon (2 peaks)	3.66	0.77
		4.34	0.92
—	Methyprylon	4.27	0.90
—	Caffeine	6.83	1.44
—	Phenazone	7.25	1.53
—	Methaqualone	9.98	2.11

of analysis must then be applied to screen for drugs that could be the cause of intoxication.

Some of the neutral drugs may be detected underivatized with the NPD, but we chose the FID because we required a more general detection method

TABLE III

RETENTION DATA AND LINEARITY DATA FOR THE GC/GC—FID PROCEDURE (UNDERIVATIZED DRUGS)

Peak No.	Compound	Retention time (min)	Relative retention	Range of linearity of calibration graph (μg/ml)
1	Hexapropymate	4.43	0.43	2—40
2	Methyprylon	5.26	0.51	2—40
3	Persedon	5.59	0.55	2—40
4	Phenazone	8.63	0.84	1—40
5	Meprobamate	8.84	0.86	Not studied, severe peak tailing
6	Eicosane (internal standard)	10.23	1.00	—
7	Methaqualone	11.57	1.13	1—40
—	Caffeine	8.28	0.81	—
—	Glutethimide	8.63	0.84	2—40
—	Barbiturates	—	—	Severe peak tailing

instead of a sensitive method for some neutral compounds. If a drug was detected only with the GC/GC—FID procedure and not with the GC/GC—NPD procedure, the drug is not a barbiturate. Underivatized barbiturates are subject to adsorption and catalytic phenomena on the quartz capillary column, resulting in badly tailing peaks. A comparison of the retention data from the two modes of gas chromatography gives valuable information that could help in the identification of peaks. Of course, as Dünges et al. [2] pointed out, alkyl derivatives other than the ethyl derivatives could be prepared in order to assist with the identification.

The methods described here are adequate for the determination of any one of the investigated drugs, except meprobamate, in serum from patients on known medication programmes.

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Journal of Chromatography, 224 (1981) 457—464
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 887

SIMULTANEOUS DETERMINATION OF DISULFIRAM AND TWO OF ITS DITHIOCARBAMATE METABOLITES IN HUMAN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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(First received December 22nd, 1980; revised manuscript received March 9th, 1981)

SUMMARY

A simple, sensitive, reversed-phase liquid chromatographic assay is reported for the simultaneous determination of disulfiram, diethyldithiocarbamate (DDC) and its methyl ester (MeDDC) in human plasma. A single-step extractive ethylation converts DDC to its ethyl ester which is then separated from endogenously produced MeDDC and parent disulfiram on an alkylphenyl column. The method is sufficiently sensitive (25 ng/ml) to permit DDC and MeDDC determinations in patients receiving therapeutic doses of disulfiram.

INTRODUCTION

Disulfiram (tetraethylthiuram disulfide, Antabuse®) (DSF) is widely used clinically in the treatment of alcoholism. The drug renders the patient sensitive to ethyl alcohol although the underlying mechanism by which the drug—ethanol reaction (DER) occurs is still poorly understood. Little is known about the pharmacokinetics of DSF and its metabolites in man. It also remains to be determined whether relationships exist between the plasma levels of parent drug or metabolites and the intensity of the DER induced by the drug. Toxic side effects such as neuropathy have also not been related clearly to the drug's disposition. Such relationships might serve as the basis for the rational design of long term disulfiram delivery systems. A major obstacle to resolving these questions has been the lack of availability of sensitive analytical methods for the determination of DSF and its major metabolites (Fig. 1) in human plasma.

While spectrophotometric, polarographic and gas chromatographic methods have been reported [1—6] a liquid chromatographic technique would seem more appropriate because of its potential convenience, selectivity, sensitivity

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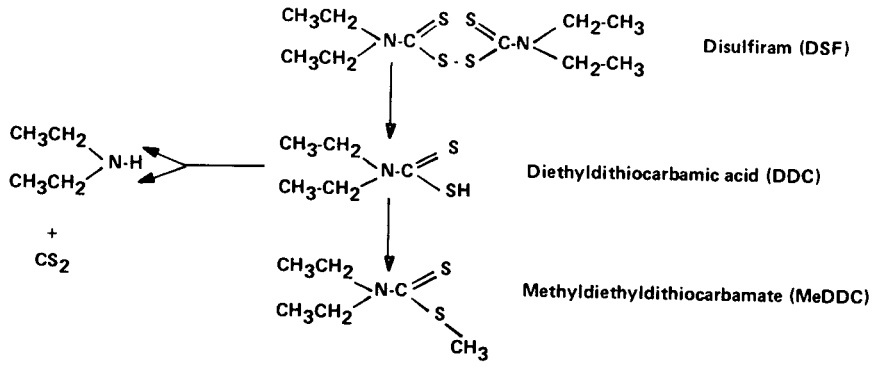

Fig. 1. Pathways for metabolism of disulfiram to its first degradation products in man.

and suitability to the quantitation of labile drugs and metabolites. Jensen and Faiman [7] and Pedersen [8] have determined DSF and its endogenously-produced metabolite methyldiethyldithiocarbamate (MeDDC) by normal-phase high-performance liquid chromatography (HPLC). They utilized extractive methylation of diethyldithiocarbamate (DDC) to determine this metabolite after removing and quantitating endogenously-produced MeDDC. Our objective was to develop a simple reversed-phase chromatographic technique that would quantitate both metabolites simultaneously rather than through two different extraction procedures.

EXPERIMENTAL

Chemicals

DSF was a gift of Ayerst Labs. (New York, NY, U.S.A.). DDC was purchased as the sodium salt from Sigma (St. Louis, MO, U.S.A.) and used without further purification. Methyl, ethyl and propyl esters of DDC were synthesized in our laboratory (see below). Methyl iodide and ethyl iodide (Aldrich, Milwaukee, WI, U.S.A.) were distilled from glass before use. Organic solvents were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). All other chemicals were reagent grade and used without further purification. Water was deionized and double distilled in glass.

Apparatus

A Model 5000 gradient liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) and a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) each equipped with a Model 440 absorbance detector (280 nm), a Phenyl/Corasil Bondapak precolumn (5 cm × 3.9 mm I.D. and a 10-μm alkylphenyl μBondapak column (30 cm × 3.9 mm I.D.) (Waters Assoc.) were used for gradient and isocratic separations, respectively. Mobile phase was a mixture of acetonitrile—water (52:48) for isocratic separations and was programmed to increase from 51:49 to 75:25 over 10 min for gradient separations. Mobile phase flow-rate was maintained at 1.5 ml/min and column temperature at 37°C.

Substitution of 0.01 *M* acetate buffer (pH 4) for the water did not affect the chromatography.

Synthesis of diethyldithiocarbamate esters

Approximately 0.025 mole of alkyl halide were added with constant stirring to an equimolar quantity of sodium DDC dissolved in approximately 7 ml of absolute ethanol. After 20—30 min reaction at room temperature an equal volume of water was added. The lower yellow oily layer was separated, dried over calcium chloride, filtered and vacuum distilled (Table I). Infrared and NMR spectroscopy were used to confirm the identity of the product.

TABLE I

PARAMETERS FOR DIETHYLDITHIOCARBAMATE ESTER SYNTHESIS

Alkyl halide	Ester	Distillation temperature (°C)	Distillation pressure (mm Hg)
Methyl iodide	MeDDC	89	1.5
Ethyl iodide	EtDDC	112	2.6—4.0
n-Propyl bromide	PrDDC	99	0.6

Preparation of standards

Stock solutions of DDC esters, sodium DDC and DSF (about 1 mg/ml) were prepared by dissolving the pure compound in acetonitrile. Dilutions of 1 : 50 and 1 : 100 of these stocks provided working solutions for spiking of plasma samples. Stocks of the esters were stable at 4°C for several months, but sodium DDC and DSF had to be freshly prepared prior to use.

Calibration standards containing between 50 and 500 ng/ml were prepared by addition of an appropriate quantity of working stock solution to drug-free plasma using *n*-propyl-DDC as the internal standard. Standards and samples were extracted as described below.

Extraction procedure

A 2-ml aliquot of plasma was placed in a 16 × 125 mm test tube containing an equal volume of 0.05 *M* Tris buffer (pH 8.5) to which has been added EDTA (0.01 *M*). Ethyl iodide (20 μl) was added and, following 30 sec of vortex mixing, the mixture was incubated at 40°C for 30 min and cooled to room temperature. Zinc sulfate (500 mg) and diethyl ether (4 ml) were added and the tubes gently inverted by hand for 3 min. After centrifugation for 6 min at 1200 *g*, the ether layer was removed and placed in a clean test tube. Nine milliliters of carbonate buffer (pH 11, 0.1 *M*) were added followed by 3 min of inversion and 6 min of centrifugation. The carbonate-washed ether layer was transferred to a clean tube, concentrated to 100 μl under nitrogen, diluted to 1 ml with acetonitrile and reconcentrated to about 200 μl. A 15—20 μl aliquot was injected onto the column for chromatographic analysis. Fig. 2 provides a schematic summary of the procedure.

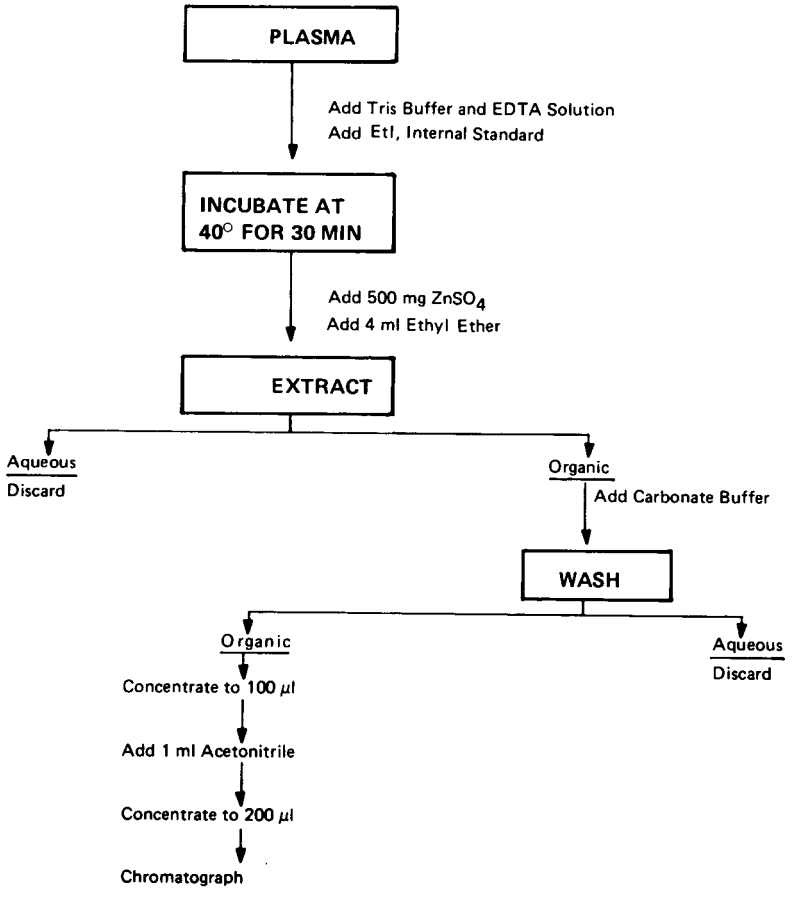

Fig. 2. Schematic of plasma sample workup for disulfiram and two of its diethyldithiocarbamate metabolites.

Standard curves

Standard curves were prepared by adding known quantities of DDC or MeDDC to a fixed concentration (400 ng/ml) of propyl-DDC in drug-free plasma. Samples were analyzed as described above and peak height ratios of drug to internal standard were plotted against drug concentration. Peak height ratios of unknown samples were similarly determined and concentrations calculated from the standard curve.

Recovery

Recoveries of the methyl and ethyl esters of DDC were determined by comparison of peak height ratios determined for an extracted plasma sample with those obtained for an unextracted standard in acetonitrile. In each case propyl-DDC was added as an injection volume standard just prior to chromatography. Efficiency of the ethylation reaction was also determined by comparing samples of DDC which were extractively ethylated with those containing equimolar amounts of the pure ethyl ester.

Human study

Three male alcoholic volunteers were utilized. Each was hospitalized on a daily regimen of 250 mg DSF as a single oral dose. Two of these were given their normal dose of drug at 8 a.m. and plasma samples were drawn 2 h post dosing. At this time breath tests using McKees reagent [9] were positive for carbon disulfide. A third subject was given a 500-mg dose of DSF and similarly sampled. Plasma was separated and analyzed immediately. Plasma aliquots were also maintained at room temperature for 2 h and reassayed to determine the in vitro stability of the analytes.

RESULTS AND DISCUSSION

Fig. 3a and b shows chromatograms depicting the isocratic separation of the methyl, ethyl and propyl esters of DDC and DSF from a neat mixture of the components in acetonitrile and an extracted plasma sample, respectively. DSF degraded rapidly upon addition to plasma and at the low concentrations (less than 500 ng/ml) used was in evidence only as a less than quantitative amount of DDC when the plasma was subsequently analyzed. Fig. 4 depicts the results of our gradient separation of components from acetonitrile solution. The only apparent chromatographic advantage over isocratic separation is a somewhat sharper and taller disulfiram peak for this lipophilic and highly retained material. Calibration curves for DDC and its methyl ester from plasma over the 50—500 ng/ml concentration range were linear (r = 0.999). Coefficients of variation (within-run) were 4.3% and 6.1% for DDC and MeDDC, respectively, at 100 ng/ml (n = 6) and 3.8% and 6.0% at 400 ng/ml (n = 6). DDC and MeDDC could be accurately quantitated in plasma down to a concentration of 25 ng/ml, and each appeared to be independent of concentration within the range studied.

Percentage conversion of DDC to its ethyl ester under the derivatization conditions invoked in our procedure was 78 ± 3.4% (mean ± S.D., n = 6). Recoveries of the ethyl-DDC formed and of MeDDC were 67 ± 6.2% (n = 6) and 68 ± 3.5% (n = 6), respectively. Both conversion and recovery were independent of concentration over the concentration range studied.

Several variables in the extractive alkylation procedure were optimized in the course of developing the procedure. The conversion of DDC to its ethyl ester was independent of the amount of ethyl iodide added above 5 μl/ml plasma and independent of reaction time beyond 20 min. The reaction temperature of 40°C was deemed adequate to provide an increased rate of reaction and solubility of ethyl iodide above that obtained at room temperature with minimal exposure of the other components of the system to heat. Zinc sulfate was selected as a plasma protein precipitant because it provided extremely clean plasma extracts. While chlorinated hydrocarbons such as methylene chloride and chloroform work well as the extraction solvent, they are inconvenient because they tend to form gels on shaking and generally sequester the drug in a difficult to separate lower phase. It must be noted that the esters of DDC were found to be sufficiently volatile to render evaporation to dryness inappropriate. We found the small quantities of residual diethyl ether present in the injected sample to have no effect on the chromatography. Only very small amounts of

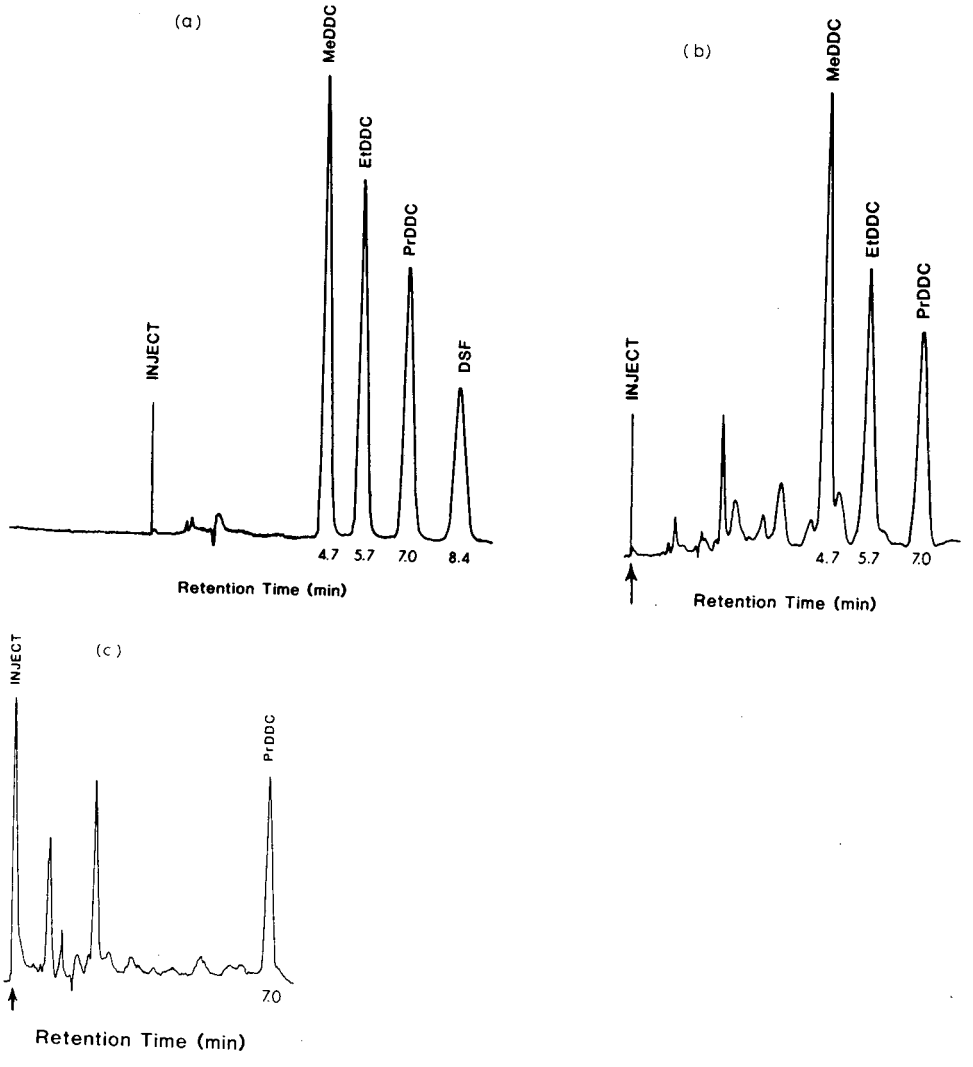

Fig. 3. Isocratic separation of disulfiram (DSF) and two of its diethyldithiocarbamate metabolites on a μBondapak alkylphenyl HPLC column. (a) Neat mixture of components in 52% acetonitrile in distilled water. Conditions: injection volume 20 μl; mass of each of the esters 27.2 ng/20 μl; mass of DSF 40 ng/20 μl. (b) Components extracted from human plasma at 200 ng/ml. (c) Blank plasma. Attenuation 0.01 a.u.f.s.

unreacted ethyl iodide were found in the sample applied to the column when diethyl ether was used whereas a large ethyl iodide peak eluting just prior to MeDDC was evident when methylene chloride or chloroform was substituted.

Fig. 5 shows a chromatographic analysis of the plasma from a male alcoholic drawn 2 h after a 500-mg oral dose of DSF. Plasma levels of MeDDC and DDC were 49 and 43 ng/ml, respectively. Reanalysis of the above sample after 2 h at 25°C resulted in an 8% decrease in the level of DDC while MeDDC remained

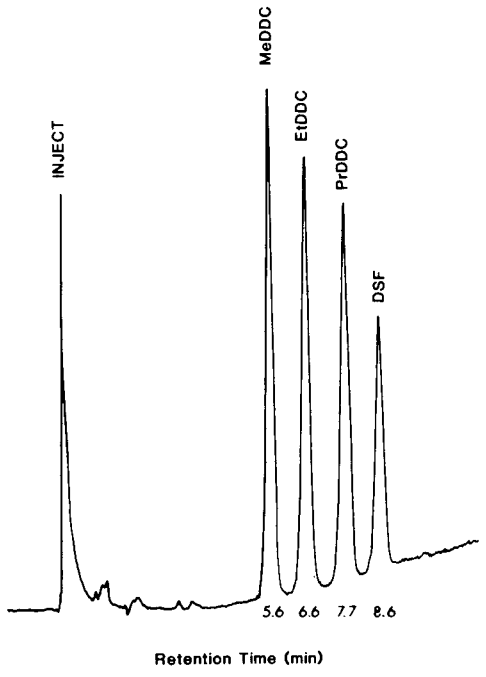

Fig. 4. Gradient separation of disulfiram (DSF) and two of its diethyldithiocarbamate metabolites on a μBondapak alkylphenyl HPLC column. Conditions: gradient from 51% acetonitrile in distilled water at 0 min to 75% acetonitrile in distilled water at 10 min; injection volume 20 μl; mass of each of the esters 27.2 ng/20 μl; mass of DSF 40 ng/20 μl; attenuation 0.01 a.u.f.s.

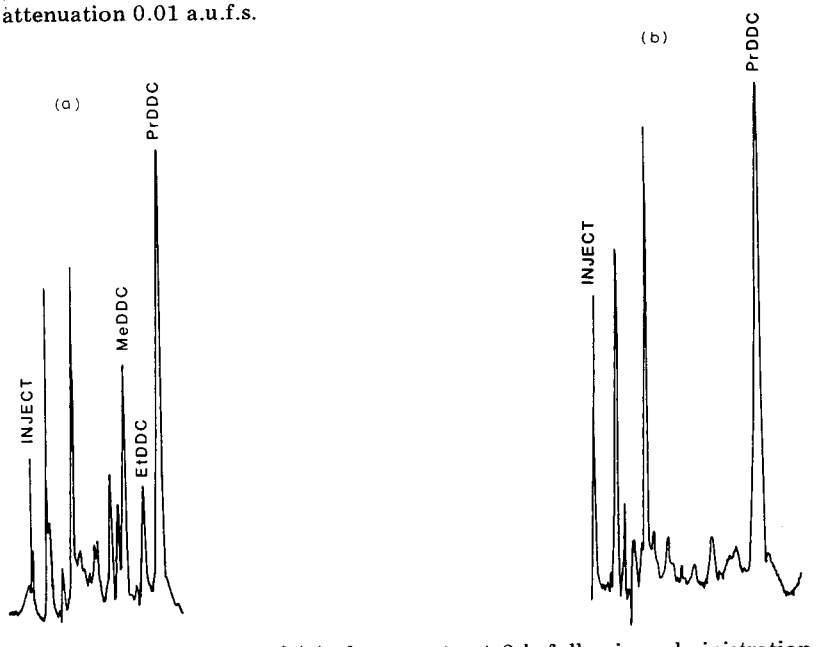

Fig. 5. Chromatograms of (a) plasma extract 2 h following administration of a 500-mg oral dose of disulfiram to a male alcoholic; MeDDC = 49 ng/ml, DDC = 43 ng/ml; (b) blank plasma. Attenuation 0.005 a.u.f.s.

unaffected. Two other patients who had been taking 250-mg daily doses of DSF did not achieve quantitable levels of DDC, MeDDC or DSF 2 h following their morning dose of DSF.

Our observations on the instability of DSF in plasma seem to confirm those of Pedersen [8]. He found that the recovery of DSF from plasma was a function of concentration and was able to recover only 5% of added drug at the 500 ng/ml level. Our results showed the compound to be essentially unrecoverable below 500 ng/ml. Pedersen also found negligible quantities of parent DSF and very low levels (generally less than 75 ng/ml) of DDC and MeDDC in a volunteer given an 800-mg oral dose of DSF. The levels reported are in good accord with those found in the present study following a 500-mg oral dose. Since Pedersen found only small quantities of protein-disulfide bound drug in plasma, it is apparent that DDC and its methyl ester in plasma account for only a very small percentage of absorbed DSF during times when the drug is exerting its maximum pharmacological effects. Their apparently rapidly disappearance from the blood stream [8] relative to the duration of action of DSF also suggests that if they are central to the drug's action they may be acting irreversibly.

The assay developed here is easy to perform, precise and sufficiently sensitive to permit determination of DDC and MeDDC levels following therapeutic doses of DSF. The sensitivity might well be increased still further if additional cleanup of the plasma extracts could be achieved. This has not been accomplished because of the combined lipophilicity of the compounds being analyzed and their lack of an ionizable group which might permit their back-extraction into aqueous solvents. The single-step extractive alkylation offers the added advantage of permitting simultaneous determination of DDC and its methyl ester on a reversed-phase system, obviating the need for prior removal of methyl ester.

ACKNOWLEDGEMENTS

This work was supported by funds from National Institutes of Health grants Nos. RR09078 and AG01168. We wish to acknowledge the invaluable assistance of Lloyd Alderson, Dennis Chapron, Dr. Michael Phillips, Dr. James O'Brien and Ms. Lorraine White.

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Journal of Chromatography, 224 (1981) 465—471
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 880

DETERMINATION OF METRONIDAZOLE, MISONIDAZOLE AND ITS METABOLITE IN SERUM AND URINE ON RP-18 HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC PLATES

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(First received November 28th, 1980; revised manuscript received February 26th, 1981)

SUMMARY

A method for the determination of radiotherapeutic concentrations of metronidazole, misonidazole and its metabolite is described. The biological fluid (serum or urine) was deproteinized with acetone containing 2-nitroimidazole as internal standard, centrifuged and the supernatant evaporated under vacuum. The residue, dissolved in acetone, was applied to an HPTLC-RP-18 layer and, after development, quantitation was achieved with a scanning densitometer. The response was linear up to 180 μg/ml for all tested compounds and the detection limit was 0.5 μg/ml. Owing to its rapidity and sensitivity the method can be considered to be equivalent to high-performance liquid chromatography.

INTRODUCTION

Metronidazole and misonidazole are widely employed as radiosensitizing drugs in the radiotherapy of hypoxic tumour cells. As both the radiosensitizing effect and the toxicity of these drugs are dose-dependent, their determination in biological fluids is important. Many analytical techniques are available for the quantitation of these compounds, such as UV spectrometry [1], polarography [2], gas—liquid chromatography [3], thin-layer chromatography (TLC) [4] and high-performance liquid chromatography (HPLC) [5, 6]. Spectropho-

tometric and polarographic techniques are rapid and sensitive but the resolution of misonidazole and its metabolite is, in practice, not satisfacory [3]. This separation can be achieved by chromatographic methods, of which HPLC is considered, at present, to be the most suitable for routine analyses [7].

This paper describes the application of bonded-phase TLC to the determination of metronidazole, misonidazole and its O-demethylated metabolite in biological fluids and demonstrates that this method is as suitable as HPLC for routine analyses.

EXPERIMENTAL

Chemicals

All chemicals and solvents were of analytical-reagent grade. Metronidazole was obtained from Farmitalia (Milan, Italy) and misonidazole and desmethylmisonidazole from Hoffmann-La Roche (Basle, Switzerland). 2-Nitroimidazole was supplied by Aldrich Europe (Beerse, Belgium).

Apparatus

All measurements were carried out with a Camag (Muttenz, Switzerland) TLC/HPTLC 76500 scanner reading the absorbance of the spots at 320 nm. In all determinations the instrument was zeroed on a blank area of the layer and a uniform baseline was always observed. The peaks obtained were quantitatively integrated by a Spectra Physics (Darmstadt, G.F.R.) Minigrator.

Sample preparation

The following procedure was found to be suitable for samples of urine and blood serum. The sample (1 ml) was mixed with acetone (2 ml) containing 2-nitroimidazole as an internal standard (22.6 μg/ml) and shaken for 1 min. After centrifugation for 10 min at 1200 *g* (Hettich refrigerated centrifuge) the supernatant was removed and dried in a rotary vacuum evaporator. The residue was dissolved in 0.5 ml of acetone.

Chromatography

A 1-μl sample was spotted quantitatively on an HPTLC-RP-18 bonded-phase layer (Merck, Darmstadt, G.F.R.) with a Camag micro-applicator. The layers were developed at room temperature using the ascending or horizontal technique according to Kristensen [8]. The best developing solvent system was *n*-hexane—acetone—96% ethanol (19:6:1). The layer was air dried and the spots were quantitated by scanning densitometry.

RESULTS AND DISCUSSION

HPTLC-RP-18 layers were chosen for the separation of the nitroimidazole radiosensitizers because the resolution obtained on conventional silica gel plates was not satisfactory with any of the solvent systems used. The optimal mobile phase was selected by performing preliminary chromatographic runs with some of the commonly used solvent mixtures for reversed-phase layers, viz., methanol—water, acetonitrile—water and isopropanol—water. With these

developing solvents no separation was achieved and all spots were close to the solvent front. A slight separation and a slight decrease in the R_F values occurred when the eluent mixture was isopropanol—*n*-hexane (1:1) (R_F values: misonidazole 0.85, desmethylmisonidazole 0.85, metronidazole 0.70 and 2-nitroimidazole 0.93).

Taking these results into consideration, it was necessary to decrease further the elution strength of the mobile phase to obtain good separations. The solvent system that gave the most satisfactory results for the separation of the nitroimidazole compounds and compactness of the spots was *n*-hexane—acetone—96% ethanol (19:6:1).

Fig. 1A shows a chromatogram of a mixture of desmethylmisonidazole, metronidazole, misonidazole and the internal standard 2-nitroimidazole obtained on the HPTLC-RP-18 layers with the selected eluent. Under these conditions all R_F values are reproducible, as can be seen in Table I. The four peaks were completely resolved with this solvent system, whereas Marques et al. [9] did not succeed in separating metronidazole from misonidazole with their HPLC method. The nitroimidazoles can be also separated using the solvent system *n*-hexane—acetone (3:1), although metronidazole and desmethylmisonidazole are not completely resolved. However, a better separation can be achieved by double development. Comparison of Fig. 1A and B shows that the blank serum, taken from a patient with a bladder tumour

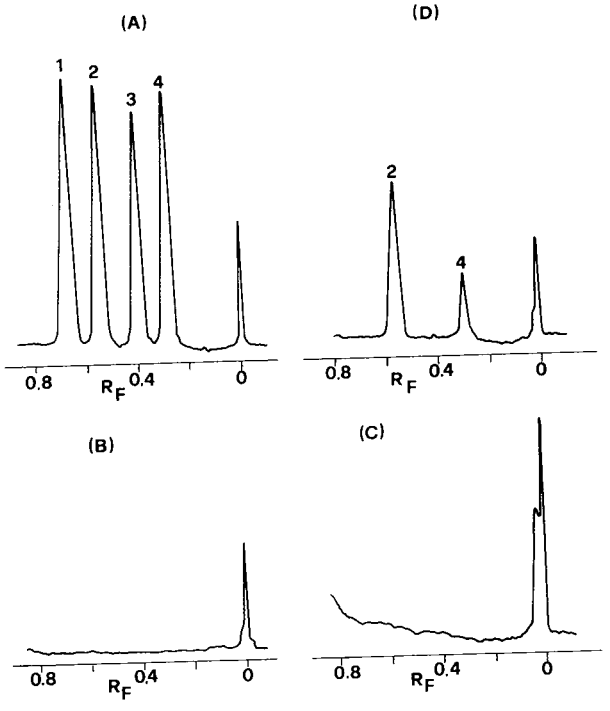

Fig. 1. Chromatograms of (A) a mixture of 0.28 μg of internal standard (peak 1), 0.50 μg of misonidazole (peak 2), 0.43 μg of metronidazole (peak 3) and 0.47 μg of desmethylmisonidazole; (B) serum blank; (C) urine blank; and (D) serum sample of a patient taken 5 h after oral administration of 0.6 g/m² of misonidazole.

TABLE I

$R_F \times 100$ VALUES FOR 8-cm RUNS AND RESOLUTION FACTORS

Each value is the mean (± S.D.) of five determinations.

Compound	$R_F \times 100$	Resoltuion factor*
Desmethylmisonidazole	31 ± 1	
Metronidazole	41 ± 1	1.4
Misonidazole	58 ± 1	2.4
2-Nitroimidazole	68 ± 1	1.5

*Defined as $\frac{2\Delta x}{w_2 + w_1}$ where Δx is the distance between two peaks and w_2 and w_1 are peak widths.

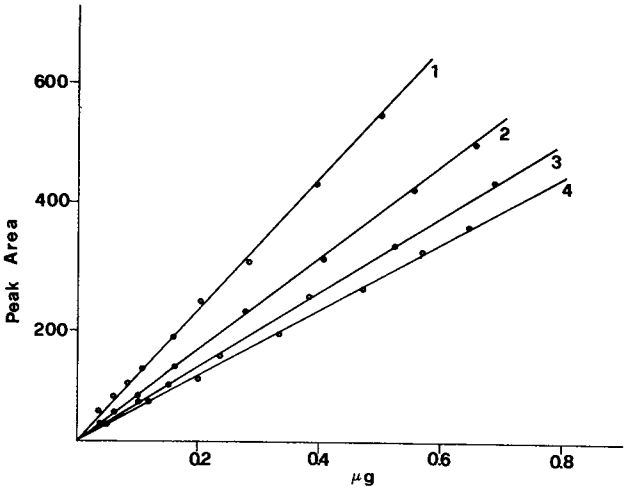

Fig. 2. Calibration graphs for (1) internal standard, (2) metronidazole, (3) desmethylmisonidazole and (4) misonidazole. Each point is the mean of five determinations.

immediately before administration of misonidazole, contains no components capable of interfering with peaks 1—4. A similar result was obtained for extracts of control urine (Fig. 1C). In contrast, when extracts of urine samples are chromatographed on silica gel some UV-absorbing constituents move with tailing, thus interfering in the quantitation of desmethylmisonidazole. Fig. 1D shows a chromatogram of an acetone extract of serum taken from the same patient 5 h after oral administration of misonidazole (0.6 g/m^2), and misonidazole (peak 2) and its O-demethylated metabolite (peak 4) are present.

Calibration graphs for the determination of 1—4 by TLC were prepared by spotting different amounts of a mixture of the nitroimidazoles and plotting peak areas against concentration. As shown in Fig. 2, all calibration graphs were linear up to 3.7 nmole (corresponding to about 180 μg/ml in the sample). The precision was determined for misonidazole, desmethylmisonidazole and metronidazole at 65, 20 and 65 μg/ml, respectively, and gave $\overline{x}$ = 64.7 μg/ml (C.V. = 2%, n = 5), $\overline{x}$ = 19.8 μg/ml (C.V. = 3.3%, n = 5) and $\overline{x}$ = 65.2 μg/ml

TABLE II

RECOVERIES ON EXTRACTION OF MISONIDAZOLE, METRONIDAZOLE AND DESMETHYLMISONIDAZOLE FROM SERUM AND URINE

Each value is the mean of five determinations.

Compound	Added (μg/ml)	Recovery from serum (%)	Recovery from urine (%)
Misonidazole	30.0	97.3	98.2
	70.0	98.4	98.9
	100.0	94.8	97.1
	130.0	96.5	95.8
Mean ± S.D.		97.1 ± 1.4	
Metronidazole	30.0	93.2	94.5
	50.0	94.2	94.8
	80.0	92.9	94.3
	110.0	93.6	93.5
Mean ± S.D.		93.9 ± 0.8	
Desmethylmisonidazole	5.0	94.1	95.8
	10.0	95.9	93.9
	20.0	95.2	94.4
	30.0	94.7	93.5
Mean ± S.D.		94.7 ± 1.2	

(C.V. = 1.7%, n = 5), respectively. Allowing a minimum signal-to-noise ratio of 2, the detection limit was ca. 0.5 μg/ml (in serum or urine samples) for each compound. Recovery experiments were carried out by adding known amounts of each nitroimidazole to serum and urine samples and processing the samples as described under Experimental. As shown in Table II, the overall recoveries were good. The concentration of misonidazole in serum after oral administration of 0.6 g/m^2 of misonidazole to a patient with a bladder tumour determined by the present method was compared with that obtained by polarography. Good agreement was observed, indicating that the proposed method can be utilized in routine analyses to determine the levels of nitroimidazole radiosensitizers in biological fluids.

A comparison of the proposed technique with the HPLC and polarographic techniques currently employed to determine the levels of misonidazole and desmethylmisonidazole in tissues and body fluids is shown in Table III. It can be seen that the TLC and HPLC methods are equivalent as far as sensitivity and specificity are concerned. Also, the analysis times are similar for these two methods, as eight samples can be processed in about 2 h when the TLC method is employed. After a measurement the layer can be developed with 96% ethanol to obtain complete elution of the sample compounds and then re-utilized for a new measurement. The washed layer gives reproducible results and this procedure can be utilized until the baseline remains sufficiently uniform. Another disadvantage with silica gel plates is that washing with ethanol is not adequate to remove completely interfering substances in the urine extracts, thus precluding further runs on the same plate.

TABLE III

COMPARISON OF RESULTS OBTAINED IN ASSAYING MISONIDAZOLE AND DESMETHYLMISONIDAZOLE IN SERUM SAMPLES BY TLC, HPLC AND DIFFERENTIAL-PULSE POLAROGRAPHY

Compound	TLC			HPLC*	
	R_F	Resolution	Detection limit (μg/ml)	Retention time (min)	Resolution
Misonidazole	0.58	good	≈0.5	4.1	good
Desmethylmisonidazole	0.31		≈0.5	2.3	

*Data taken from ref. 5.
**Experimental conditions: 7 ml of ethanol were added to 1 ml of serum; after centrifugation, the supernatant was diluted with an equal volume of 0.1 *M* potassium chloride solution.
***Same conditions as above, but with addition of Tylose up to a concentration of 0.1% (w/v).

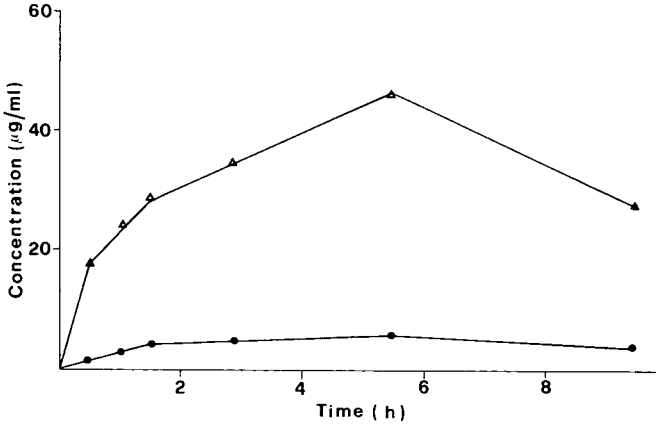

Fig. 3. Serum time course of misonidazole (△) and desmethylmisonidazole (●) for a patient with a bladder tumour after oral administration of 0.6 g/m² of misonidazole.

An application of the proposed TLC method is demonstrated in Fig. 3, which shows the time course of misonidazole and desmethylmisonidazole for a patient with a bladder tumour receiving 0.6 g/m² of misonidazole orally.

ACKNOWLEDGEMENTS

This study was supported in part by the Italian National Research Council (C.N.R.), Finalized Project "Controllo della Crescita Neoplastica", Grant No. 80.01486.96, and is published with C.N.R. permission. The authors thank R. Balducci for his help with the polarographic measurements.

	Differential-pulse polarography				
Detection limit (μg/ml)	$E_{1/2}$** vs. SCE (mV)	$E'_{1/2}$***	$E''_{1/2}$***	Resolution	Detection limit (μg/ml)
≈0.5	−620	−690	−950	poor	≈0.1
≈0.2	−640	−700	−965		≈0.1

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Journal of Chromatography, 224 (1981) 472—476
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 894

Note

Hydrophilic ion-pair reversed-phase chromatography of biogenic peptides prior to immunoassay*

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(First received December 11th, 1980; revised manuscript received March 16th, 1981)

The determination of biologically relevant peptides in human plasma is difficult, mainly owing to their very low concentrations, the presence of structurally related compounds and the insufficient specificity of the antisera used in radioimmunoassay (RIA) as commonly applied. Consequently, the results for these peptides are correctly defined as "peptide-like immunoreactivities". Gel filtration techniques, sometimes applied to the purification of plasma peptides [1, 2], fail to separate sufficiently peptides of closely related structure. Reversed-phase systems in high-performance liquid chromatography (HPLC) are being used increasingly to separate peptides with high resolution. Molnár and Horváth [3] and O'Hare and Nice [4] reported that adequate resolution is strongly dependent on the pH and ionic strength of the mobile phase. However, the non-volatile mobile phases recommended by these workers render these systems unsuitable for the subsequent determination of immunological or biological activity which is, as yet, the only tool for the quantitation of the minute amounts of peptides in peripheral blood.

We report here about our studies on two reversed-phase systems using hydrophilic ion-pairing reagents with particular respect to their suitability for subsequent immunoassay. The applicability of one system is demonstrated for the chromatographic profiling of adrenocorticotrophin (ACTH) immunoreactivities arising in human plasma.

*This paper was presented in part at the 24th Symposium of the Deutsche Gesellschaft für Endokrinologie, Berlin, 1980.

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Instrumentation

A Hewlett-Packard Model 1084B high-performance liquid chromatograph equipped with a variable-wavelength spectrometer was used. Octadecyl-coated silica (LiChrosorb RP-18; Knauer, Berlin, G.F.R.) was used as the stationary phase and stepwise gradient elution was performed. Different eluents were studied as the mobile phase. Eluted HPLC fractions were collected for subsequent RIA in plastic tubes using an UltroRac 7000 collector (LKB, Stockholm, Sweden). A Riedel Model RI 240 gamma spectrometer was used for monitoring ^{125}I radioactivity.

Chemicals

Phenylalanine and tryptophan were obtained from Merck (Darmstadt, G.F.R.). Synthetic human $ACTH_{1-39}$ and $ACTH_{1-24}$ were a gift from Ciba-Geigy (Basle, Switzerland). All other peptides were obtained from Serva (Heidelberg, G.F.R.). Acetonitrile (LiChrosolv grade), ammonium formate and trifluoroacetic acid (TFA) (all analytical-reagent grade) were obtained from Merck. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.). Charcoal (Norit A) was obtained from Serva.

The following reagents were used for the RIA of ACTH: [^{125}I]-*p*-$ACTH_{1-39}$ and anti-*p*-$ACTH_{1-39}$ were obtained from CEA (Gif-sur-Yvette, France); h-$ACTH_{1-39}$ (Ciba-Geigy) was used as a standard; 0.02 *M* barbital buffer (pH 8.6) containing 1% bovine serum albumin (BSA) was used as the incubation medium.

Methods

For chromatographic studies, polypeptide standards were dissolved in the solvent initiating the gradient run. Depending on the number of aromatic residues within the peptide molecule, amounts between 0.5 and 12 μg were subjected to HPLC. The eluted peptide standards were detected by their UV absorbance at 275 and 254 nm. The flow-rate was 1.3 ml/min throughout. Ammonium formate and trifluoroacetic acid were studied as hydrophilic ion-pairing reagents, and acetonitrile as organic modifier. As the final system, 0.05 *M* TFA and acetonitrile were used for the immunoassay studies.

Peptides were extracted from plasma using a method originally outlined by Bennett et al. [5]. In brief, 4 ml of plasma were passed through a Sep-Pak C_{18} cartridge, then the silica matrix was washed with 5 ml of 0.05 *M* TFA and eluted slowly with 2 ml of acetonitrile—0.05 *M* TFA (80:20, v/v). The eluate was lyophilized, reconstituted with 150 μl of 0.05 *M* TFA and subjected to HPLC. The fractions eluted by HPLC were partly evaporated, then lyophilized and reconstituted in 450 μl of BSA—barbital buffer. Volumes of 200 μl were subjected to RIA in duplicate. The RIA data were evaluated using the "spline approximation" as a standard curve model [6].

RESULTS AND DISCUSSION

The influence of ionic strength and pH on the separation efficiency of

the reversed-phase system is demonstrated in Fig. 1, using tryptophan, phenylalanine and its polymers as the test mixture. Nice and O'Hare [7] emphasized that both acidic conditions and a suitable ionic strength in the primary solvent are essential for efficient reproducible chromatography and that neglect of either results in a marked impairment of peak shape and a decrease in resolution. Our experiments under neutral conditions and at an ammonium formate concentration of 0.05 *M* (Fig. 1b), however, yielded separations equivalent to those obtained under acidic conditions (pH 1.3) and at the same ionic strength (Fig. 1c). Hence, ionic strength is the only prerequisite for effective peptide separations, and the proton concentration may only affect the polar-

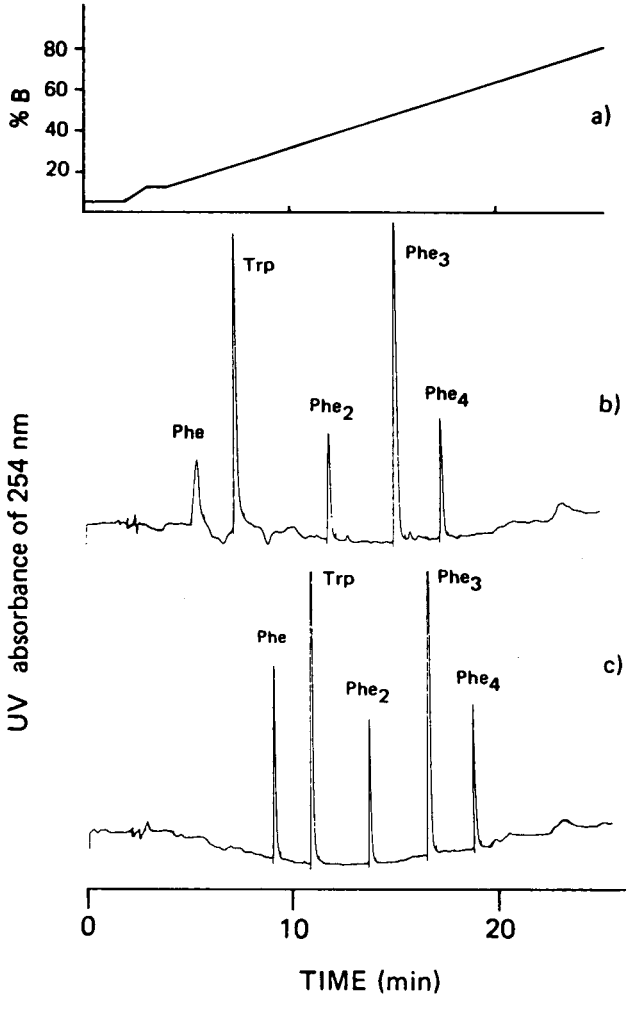

Fig. 1. HPLC of tryptophan (Trp), phenylalanine (Phe) and its polymers (Phe_2—Phe_4). Column, RP-18 (250 × 4.6 mm I.D.) (Knauer); diameter of particles, 5 μm; oven temperature, 40°C; amounts injected, 0.1—0.5 μg; the gradient applied is outlined in (a); attenuation, $6.4 \cdot 10^{-3}$ a.u./cm. The organic modifier was acetonitrile (solvent B). Solvent A in (b), 0.05 *M* ammonium formate; in (c), 0.05 *M* trifluoroacetic acid (pH 1.3).

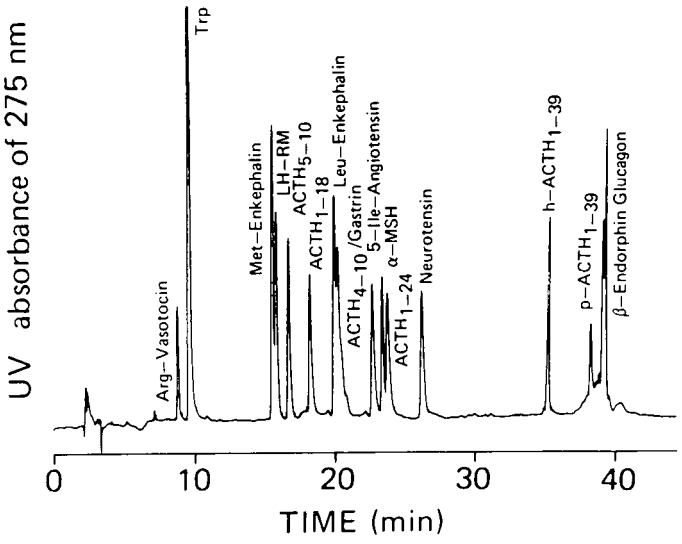

Fig. 2. HPLC of biogenic peptides. Amounts injected ranged from 0.5 to 12 μg. The TFA—acetonitrile system (Fig. 1c) was used. The gradient was increased stepwise from 5% to 80% B within 60 min. Other conditions as in Fig. 1.

ity of the amphoteric amino acids and peptide molecules [8]. Both of the hydrophilic ion-pairing reagents studied [ammonium formate (Fig. 1b) and trifluoroacetic acid (Fig. 1c)] are suitable for subsequent lyophilization and immunological quantitation. As lyophilization of the trifluoroacetic acid phase is more rapid, priority was given to this system, which is based on that outlined by Bennett et al. [5]. Reproducibilities (coefficients of variation) of the retention times ranged from 0.48% for phenylalanine to 0.09% for the tretramer of phenylalanine (n = 12). Such accuracy is essential for measuring peptides that are not detectable by direct classical detection techniques, but by sensitive immunological quantitation in fractions eluted after calibrated retention times.

The efficiency of the system in separating diverse biogenic peptides is demonstrated in Fig. 2. The orders of retention of the peptides are almost completely in agreement with those found by Nice and O'Hare [7] using the non-volatile phosphate system.

The conservation of bio- and immunoactivity of peptides after HPLC was established by assaying ACTH immuno- and bioactivity in fractions eluted by HPLC from a commercially available porcine pituitary ACTH preparation [7]. Owing to the acid and salt conditions used in this system, neutralization of the acid and dilution of the buffer 1:4 was necessary in order to provide immunoassayability of the post-HPLC fractions. Hence, a detection limit of only 200 pg per fraction was achieved, rendering this system unsuitable for monitoring immunoactivities of low-concentration peptides, e.g., ACTH in blood.

The suitability of the present ion-pair reversed-phase system for such purposes is demonstrated in Fig. 3. The volatile nature of the mobile phase provides a detection limit of about 2 pg per fraction. The chromatogram of the

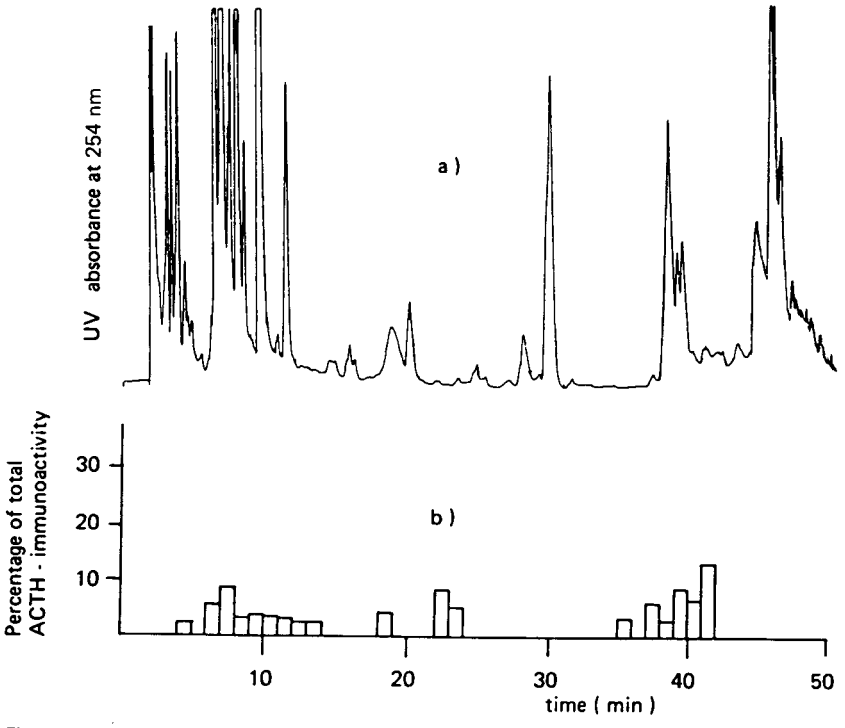

Fig. 3. HPLC of an extract of (a) human plasma and (b) ACTH immunoreactivities arising in the corresponding 1-min fractions. The total amount of ACTH immunoreactivity was 55.5 pg if 200 μl of the total fraction volume (450 μl; see Methods) were radioimmunoassayed. The TFA—acetonitrile system (Fig. 2) was applied.

extract of a normal human plasma sample obtained with UV detection (Fig. 3a) indicates considerable amounts of UV-absorbing materials in plasma that are not attributable to peptides of biological interest. The corresponding chromatogram of ACTH immunoactivities arising in 1-min HPLC fractions (Fig. 3b) displays a considerable variety of ACTH-like compounds, the chemical nature of which is as yet unknown.

In conclusion, the HPLC system described here provides sensitive immunoassayability of low-concentration peptides separated by efficient ion-pair reversed-phase chromatography. It may contribute to the further elucidation of the nature of peptide-immunoactive materials in the human circulation.

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Journal of Chromatography, 224 (1981) 477—480
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 893

Note

Ultramicromethod for the measurement of ascorbic acid in plasma and white blood cells by high-performance liquid chromatography with electrochemical detection

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Ascorbic acid content in plasma, serum and leucocytes was determined using high-performance liquid chromatography (HPLC) with electrochemical detection [1—3]. The method is highly sensitive and can be used to accurately detect as low as 10 ng of ascorbic acid in as little as 5 μl of serum or plasma.

EXPERIMENTAL

The materials, equipment and instrumentation were as described by Pachla and Kissinger [2, 3]. All HPLC data were obtained using commercially available components and an amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.).

Preparation of plasma samples

Each 10 μl of plasma was deproteinized with 30 μl of cold 6% trichloroacetic acid; after being shaken vigorously the deproteinized sample was allowed to stand for 10 min in ice to ensure complete deproteinization. Then 50 μl of cold 50 m*M* perchloric acid were added to the sample which was agitated again. At this time, the samples were centrifuged at 1520 *g* for 15 min at 4°C. The acidified supernatant was removed from the denatured protein for analysis. Aliquots of 2—20 μl were injected into the chromatographic column.

Preparation of white blood cell samples

To isolate the white blood cells from whole blood, the Ficoll—Hypaque method [4, 5] was used. The purified leucocytes were suspended in physiological saline. A small aliquot of the leucocyte suspension was removed in order to

carry out the white cell count. Another measured aliquot was centrifuged at 1520 *g* at 4°C for 15 min and the supernatant discarded. The pellet was taken up in trichloroacetic acid, homogenized, diluted with 50 m*M* perchloric acid and analyzed by HPLC.

RESULTS

The ascorbic acid content of plasma samples from seven human subjects was measured by the HPLC—electrochemical detection method and by the colorimetric method using 2,4-dinitrophenylhydrazine [6—8]. The results are listed in Table I. Comparison of the two sets of data shows that results obtained by the two methods are highly consistent. However, the ascorbic acid content obtained by the colorimetric method is systematically higher than that obtained by the present method. This suggests that the electrochemical method is probably more selective than the colorimetric method.

TABLE I

ASCORBIC ACID IN HUMAN PLASMA

Human plasma*	Ascorbic acid (mg/100 ml plasma)	
	Present method	Colorimetric method
1	1.30	1.47
2	1.10	1.18
3	1.03	1.20
4	1.89	2.05
5	1.03	1.38
6	0.62	0.67
7	1.15	1.25

*Blood samples were obtained from American Red Cross Blood Services, Central California Region, Palo Alto, CA, U.S.A.

The ascorbic acid content of leucocytes and that of the buffy coat from human subjects have been measured. The ascorbic acid concentration is expressed as μg per 10^8 white blood cells (WBC). Results listed in Table II are shown to compare well with literature values.

Plasma samples with ascorbic acid concentration ranging from 0.2—3 mg per 100 ml plasma have been satisfactorily analyzed in our laboratory using a variation of the procedure described in the Experimental Section. Furthermore, the detector system can easily detect one-hundred-fold differences by varying sample size, recorder span and detector sensitivity. Our results also show that the ascorbic acid level in a plasma sample of 2 μl can be estimated with this technique.

The precision of this assay method was checked by multiple analyses on a single plasma sample. Typically, the standard deviation for five measurements is calculated to be better than 4%. When 200 ng of ascorbic acid were added to 20 μl of plasma, 99% of the vitamin was recovered. We have also analyzed a series of 6 micro samples (10 μl each) and have found excellent agreement com-

TABLE II

COMPARISON OF ASCORBIC ACID CONTENT OF HUMAN WHITE BLOOD CELLS AND BUFFY COAT OBTAINED BY THE HPLC METHOD WITH LITERATURE VALUES

Subject	Ascorbic acid ($\mu g/10^8$ WBC)*			
	Leucocyte		Buffy coat	
	Present method	Lit. values	Present method	Lit. values
1	18.7			
2	21.4			
3	17.5			
4	25.9			
5	18.0			
6	25.2			
7	25.0			
8	21.8			
9	22.2			
10	16.8		35.7	
11	30.5		57.4	
12	19.6		43.8	
13	20.2		41.5	
Range	16.8—30.5	11—21 [9] 12—27 [10]** 8—38 [11]**	35.7—57.4	21—53 [10] 21—57 [9] 16—76 [11]

*White cell counts were performed by Diagnostic Laboratory Services, Clinical Pathology Laboratory, Stanford University, Stanford, CA, U.S.A.

**Calculated from values of buffy coat ascorbate content; a conversion factor of 2 was used [9].

pared to results obtained from larger samples (6 samples, 1 ml) from the same subject (ascorbic acid content 1.32 ± 0.05 and 1.40 ± 0.04 mg/100 ml plasma ± S.D., respectively).

ACKNOWLEDGEMENTS

We wish to express our thanks to Professor Ralph N. Adams and Dr. Ivan Mefford for their valuable advice during the course of this investigation.

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Journal of Chromatography, 224 (1981) 481—487
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 878

Note

Determination of noncatecholic phenylethylamines and monomethylated derivatives of phenylethylamine

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(First received October 24th, 1980; revised manuscript received February 27th, 1981)

There is growing evidence supporting the involvement of noncatecholic phenylethylamines (NCPEA), e.g., phenylethylamine (PEA), phenylethanolamine (OHPEA), the tyramines (TRMs) and octopamines (OCPs) in the pathophysiology of a number of disease states (refs. 1, 2 and references cited therein) including the major psychoses [3, 4], diabetes mellitus [5], Parkinsonism [6], hepatic encephalopathy [7], migraine [8], and phenylketonuria [9, 10]. Elucidation of the mechanisms by which these amines exert their physiological role and pharmacological effects has been, however, slowed by technical problems encountered in the development of relatively simple, sensitive, specific and reproducible methodologies for their determination in biological samples [11—15].

An analysis of the literature shows that despite the variety of techniques used in measuring these compounds such as spectrophotometry [16, 17], chromatography [10, 18—20], enzymology [11, 14, 21], radioimmunoassay [7] and mass spectrometry [13, 15, 22, 23], their actual levels in mammalian fluids and tissues is still a matter of great controversy (refs. 1, 2 and references cited therein). Similar discrepancies can be observed in the reported levels of these amines in invertebrates [11].

Results obtained using either gas—liquid chromatography (GLC), GLC—mass spectrometry or enzymatic isotopic (N-methylation) techniques are being actively reevaluated as several workers have questioned their specificity [11, 14, 15, 24]. These investigators have stressed the need for rigorous isolation of these substances from the original biological sample before their actual measurements [14, 15].

In this paper, we have addressed this problem particularly with regard to the parent NCPEA and to the biologically active monomethylated derivatives of PEA.

0378-4347/81/0000—0000/$02.50

MATERIALS AND METHODS

Reagents, solvents and gases used in these experiments were of the highest purity commercially available and were used without further purification. They were obtained from different sources; PEA (K & K, Labs., Plainview, NY, U.S.A.), the monomethylated PEAs, methylamine and nitroethane (Aldrich, Milwaukee, WI, U.S.A.), OHPEA, OCP, and *p*-TRM (Regis, Chicago, IL, U.S.A.), *m*-TRM (Vega-Fox Biochem., Tucson, AZ, U.S.A.), acetic acid, acetone, ammonia, benzene, chloroform, ethanol and sodium tetraborate (Sigma, St. Louis, MO, U.S.A.), ninhydrin (Fischer Scientific, Chicago, IL, U.S.A.); and 2,4-dinitrobenzenesulfonic acid (DNBS) and phenylacetic acid (PAAc) (Eastman Kodak, Rochester, NY, U.S.A.). Radioactive PEA, *p*-TRM, and *p*-OCP were purchased from New England Nuclear (Boston, MA, U.S.A.) and labeled PAAc was obtained from ICN (Irvine, CA, U.S.A.).

Thin-layer chromatography (TLC) plates were obtained either from Brinkman Inst. (Des Plaines, IL, U.S.A.; systems I and II) or Analtech (Newark, DE, U.S.A.; systems III, IV and V). Samples (5—10 μg standard compounds) were detected after ninhydrin spraying. N-Acetyl PEA was visualized under UV light (254 nm), whereas R_F values for PAAc were obtained by using autoradiography of plates spotted with ^{14}C-labeled acid.

For GLC analysis, whether individual or amine mixtures, these substances were reacted with DNBS and the resultant derivatives injected into a Beckman GC-65 equipped with flame ionization detector and coiled glass (U turn) 0.54 m × 4 mm I.D. column, packed with Diatoport S 80—100 mesh with a liquid OV-17 coating. Samples were run at the following temperatures: injection block, 250°C; detector line, 260°C; detector, 280°C; and oven, 220°C (all samples were run under isothermal conditions). Maintenance of the flame was obtained using hydrogen and air (42 ml/min and 300 ml/min, respectively); the carrier gas was nitrogen (80 ml/min). Amines were derivatized as previously described [18—20, 25, 26]. Briefly, an excess of freshly prepared DNBS reagent (0.25 *M* in saturated tetraborate, pH 7.86) was added to standard amine(s) (range 0.1—500 ng each, 0.001 *N* hydrochloric acid solution in glass test tube with screw top). The tubes were sealed, placed into a boiling water bath and reacted for 15 min. After cooling at room temperature, the corresponding dinitrophenyl (DNP) derivative(s) were extracted into benzene by twice adding 1 ml of benzene, shaking gently for 2 min, and centrifuging at 5000 *g* for 5 min. The organic layers were pooled, evaporated to dryness under a stream of dry nitrogen and the residue redissolved in benzene (10—100 μl) and analyzed by GLC (1—10 μl injections). Areas under peak-response were plotted as a function of amine concentration.

Studies to ascertain the optimal conditions (pH and reaction time) for amine derivatization (100 ng pure compound plus trace amounts of the corresponding labeled amine, either PEA, *p*-TRM or *p*-OCP) were carried out by varying the pH of the amine solution (pH range, 0.76—13.08; pH range of the resultant reaction mixture 0.83—9.94, respectively) and the reaction time (range 5—50 min). The extent of the reaction was followed by TLC of the benzene phase (alumina-gel TLC plates; chloroform—ethanol (9:1); 2 h, 27°C). After identifying the radioactive area corresponding either to unreacted amine or to its

derivative [Packard radiochromatogram scanner; also ninhydrin spraying (amine) or UV light (derivative; 365 nm, yellowish color)] these compounds were scraped off the TLC plate and counted. The stability of the DNP derivatives of the above labeled amines (up to 14 h in benzene solution) was followed in a similar fashion.

RESULTS

As can be seen from Table I, the basic NCPEA, PEA, OHPEA, *p*-TRM and *p*-OCP can be separated from each other using either one of the TLC systems I—IV. All of these compounds reacted promptly with ninhydrin giving either a reddish-blue (PEA and *p*-TRM) or reddish-gray (OHPEA and *p*-OCP) coloured spot. Separation of the *m*- and *p*-TRM isomers is better accomplished by using system II. As could be expected from their chemical structures the monomethylated derivatives of PEA behave quite similarly in most TLC systems. System III provides, however, a clear separation for PEA and N-MePEA, whereas TLC system II shows significantly different R_F values for PEA, N-MePEA and *o*-MePEA. In this system *p*- and α-MePEA behave as PEA itself, whereas β-MePEA has an R_F value similar to that of *o*-MePEA. Table I shows a range for the R_F

TABLE I

TLC OF PHENYLETHYLAMINE AND RELATED COMPOUNDS

System I: cellulose-coated (0.1 mm thickness) glass plates. Solvent system: nitroethane—acetic acid—water (9 : 2.8 : 1.2), 2 h, 30°C.

System II: silica gel-coated glass plates (0.25 mm thickness, activated at 110°C for 40 min). Solvent system: acetone—1 *N* ammonia (10 : 3), 3—4 h, room temperature.

Systems III, IV and V: microcellulose-coated glass plates (0.25 mm thickness). Solvent systems were, respectively, nitroethane—acetic acid—water (45 : 14 : 16) 37°C; *tert.*-amyl alcohol—40% methylamine—water (8 : 1 : 1) and 96% ethanol—concentrated ammonia (20 : 1).

Amines were detected after spraying with ninhydrin; 0.2% in ethanol, at 100°C for 10 min.

Compound	R_F value				
	System I	System II	System III	System IV	System V
β-PEA	0.75—0.80	0.62—0.68	0.45—0.50	0.92—0.96	0.87—0.91
N-MePEA	0.79—0.83	0.20—0.24	0.75—0.80	—	—
o-MePEA	0.80—0.82	0.72—0.74	—	—	—
p-MePEA	0.81—0.82	0.62—0.64	—	—	—
α-MePEA	0.79—0.82	0.67—0.69	—	—	—
β-MePEA	0.79—0.81	0.71—0.73	—	—	—
OHPEA	0.67—0.69	0.82—0.84	0.35—0.40	0.80—0.84	0.86—0.88
p-Tyramine	0.51—0.61	0.54—0.61	0.23—0.28	0.66—0.70	0.82—0.86
m-Tyramine	0.60—0.62	0.63—0.65	—	—	—
p-Octopamine	0.43—0.45	0.78—0.80	0.18—0.20	0.50—0.53	—
N-Acetyl PEA*	0.89—0.91	0.87—0.89	—	—	—
PAAc**	0.95—0.97	0.77—0.78	0.01—0.02	0.01—0.02	0.29—0.35

*Visual detection under UV light (254 nm) on plates containing a fluorescent dye background.

**Detected by autoradiography utilizing [^{14}C]phenylacetic acid.

value of each amine in the various TLC systems, which possibly reflects the different sample composition. Similarly to the parent compound, PEA, these substances were easily detected with ninhydrin (reddish blue spot; β-MePEA gave a brown-charcoal colouration). R_F values for PAAc and N-acetyl PEA, the main PEA metabolites, are included for comparison.

Table II shows the relative and absolute retention times for DNP derivatives of PEA and related compounds. Non-derivatized amines are combusted along with the benzene solvent and therefore do not interfere with the DNP-amine peaks. The DNP derivatives of monomethylated PEA showed consistently a

TABLE II

GLC OF PHENYLETHYLAMINE AND RELATED COMPOUNDS*

Compound	Time** (min)	Relative retention time***
β-PEA	44	1.00
N-MePEA	20	0.45
o-MePEA	23	0.53
β-MePEA	29	0.66
α-MePEA	33	0.75
p-MePEA	35	0.79
OHPEA	97	2.23
m-Tyramine	148	3.36
p-Tyramine	160	3.64
p-Octopamine	260	5.91

*After reaction with 2,4-dinitrobenzenesulfonic acid (DNBS). Non-derivatized amines come off with the solvent peak.
**Retention times have a variation of no more than ± 2%.
***Relative to DNP-phenylethylamine (DNP-PEA).

shorter retention time than PEA itself, whereas the derivatized products of OHPEA, *m*- and *p*-TRM and *p*-OCP have a retention time substantially longer than DNP-PEA. When analyzed in mixture these compounds were easily distinguishable from each other. Conditions for derivatization were found to be optimal when the reaction mixture was boiled for 15 min at pH 7.74 (range of 92—98%, 73—87% and 70—78% completion respectively, for PEA, *p*-TRM and *p*-OCP). These derivatives were stable in benzene for at least 14 h at room temperature. This GLC technique could detect as little as 0.5 ng of the amines studied and the detector response was linear (area under the peak) for the range of concentrations studied (0.1—500 ng).

DISCUSSION

A growing body of clinical and animal observations suggest a role for the NCPEA in body function and dysfunction (refs. 1, 2 and references cited therein). Several workers have proposed the use of the levels of these amines in biological samples (blood, urine, cerebrospinal fluid) as markers for a number of diseases, specially neuropsychiatric disorders [3, 6, 16, 27, 28]. Initial work in this field, mostly involving the basic NCPEA, PEA, *p*-TRM, *p*-OCP and OHPEA, has now been extended to include other related amines showing

biological activity. Similar to their parent compounds, some of these derivatives are endogenous substances, e.g., the *o*- and *m*-isomers of TRM [13] and OCP [12], and N-MePEA [29] whereas others, e.g., α-, *o*-, *m*- and *p*-MePEA, do not appear to be normally present in the species so far studied. However, the possibility of their formation in pathological states should not be entirely discarded. In fact, we have recently described rather striking behavioral and analgesic properties for these monomethylated PEAs [30, 31]. Although work on these substances, which easily cross the blood—brain barrier and are rapidly metabolized by MAO type B [31, 32], is only of a preliminary nature, these studies may be crucial for the understanding of the mechanisms involved in some of the actions of α-methyl PEA (amphetamine) and of PEA itself. So far, only N-MePEA has been shown to be present in the brain of pargyline administered rabbits, and to be synthesized in vivo by rabbit liver and brain preparations [29]. However, one should keep in mind that perhaps other monomethyl derivatives of PEA could also be present in mammalian tissues.

Progress in the elucidation of the possible physiological role and pharmacological actions of these compounds has been slowed by several factors, such as the behavioral and toxic effects reported after their administration to laboratory animals, which appears to be also the case for humans [1, 2, 30, 31], their short half-life [32, 33] and technological problems involved in their measurement in biological samples (refs. 1, 2 and references cited therein). Earlier techniques, e.g., visual estimation from spots on thin-layer or paper chromatograms [34], UV absorption or fluorescence spectrophotometry [17, 35], have been substituted by newer methodologies claimed to be highly sensitive and specific. However, and despite the elaborate amine separation and quantitation procedures involved in some of these assays, e.g., integrated ion-current mass spectroscopy [23], GLC—mass fragmentography [22], enzymatic isotopic assay [21], derivatization followed by GLC [19], and radioimmunoassay [36], the actual levels of noncatecholic PEAs in biological samples remain a conflictive issue [1, 2].

There is agreement among most workers in the field that the main remaining problem with these techniques is specificity [11, 13—15, 21, 22, 24, 36]. For example, accurate estimation of either *p*-TRM or of *p*-OCT in the presence of their *o*- and *m*-isomers and/or their N- and N,N-methylated derivatives has proven to be a formidable task [14, 15]. The same can be said for the methylated derivatives of PEA and OHPEA [29].

Using a combination of TLC plates and solvent systems, we have been able to clearly separate from each other the four endogenous, biologically active basic NCPEA as well as *m*-TRM and N-MePEA (Table I). These systems also allowed for the separation of the different monomethylated PEAs suggesting their possible use to isolate the methylated derivatives of the other basic NCPEA.

Isolation of these compounds from biological samples by selective solvent extraction procedures [17, 26, 29, 37], followed by further separation by TLC, derivatization and GLC analysis provides a relatively simple, specific, sensitive and reproducible technique to determine their concentration in biological samples. GLC analysis of DNP derivatives of a number of related amines and amino acids, e.g., dopamine, epinephrine, norepinephrine, serotonin, *p*-chloro PEA,

phenylalanine, tyrosine and others, show that these substances do not interfere with the accurate estimation of the compounds used in this study [26] (Table II). When using the correct derivatization pH, reaction time and temperature, the corresponding derivatives are produced with consistent, relatively high yields (see Results). These conditions are critical as small variations in the pH of the reaction mixture or increased reaction times (> 20 min) result in sharply lower yields of DNP-amine derivatives [23]. With the use of appropriate radioactive internal standards to correct for amine recoveries from biological samples and completeness of DNP-derivatization, the present technique could prove a useful tool in the elucidation of the biological role of NCPEA.

ACKNOWLEDGEMENTS

This work was supported in part by the National Migraine Foundation and by the School of Graduate and Post-Doctoral Studies, University of Health Sciences/The Chicago Medical School. The authors appreciate fruitful discussions with Drs. Borison, Silkaitis, Mason and Hsu. Dr. Hsu kindly donated a sample of N-acetylphenylethylamine.

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Journal of Chromatography, 224 (1981) 488—491
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 898

Note

Measurement of 2-chloroprocaine in plasma by selected ion monitoring

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(Received January 20th, 1981)

2-Chloroprocaine (2CP) (Nesacaine®) has become a popular and widely used short acting local anesthetic agent. One reason for its popularity is its rapid hydrolysis to inactive metabolites by plasma cholinesterases; it is therefore unlikely to reach toxic levels in plasma. Due to this rapid breakdown, it is also difficult to measure in plasma.

However, 2CP is not completely broken down immediately and low levels are measurable in plasma. We have recently reported that plasma levels of 2CP can be measured following epidural anesthesia in obstetric patients [1]. In addition, we have reported a very high plasma level in a patient who accidently received an apparent intravenous injection of 2CP through an epidural catheter [2]. One might also expect that other patients with decreased cholinesterase activity for genetic or toxicological reasons could also accumulate high plasma levels of 2CP. Consequently, there is a need for a rapid and specific analytical method for measuring 2CP.

We have reported that with elaborate collection and extraction procedures, gas chromatography with nitrogen—phosphorus detection can be used to measure 2CP in plasma [1]. We would now like to report an easier, more rapid and sensitive technique using selected ion monitoring (SIM) gas chromatography—mass spectrometry.

MATERIALS AND METHODS

Reference compounds

Reference 2-chloroprocaine hydrochloride was provided by the Pennwalt Corp. (Rochester, NY, U.S.A.). The internal standard, procaine hydrochloride, was purchased from Applied Science Labs. (State College, PA, U.S.A.). A stock solution of standard 2CP was made up to the equivalent of 1 mg/ml free base in 0.01 *N* hydrochloric acid. The internal standard stock solution was also made up to the equivalent of 1 mg/ml free base in 0.01 *N* hydrochloric acid.

Apparatus

A Hewlett-Packard 5995A quadrupole table-top mass spectrometer equipped with a direct probe inlet was used to obtain 70-eV electron impact mass spectra of the reference compounds. For selected ion monitoring the gas chromatograph was interfaced to the mass spectrometer with a glass jet separator. The chromatograph was fitted with a 1.0 m × 2 mm I.D. AW DMCS treated glass coil packed with 3% OV-1—OV-17 (6:1) coated on an 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The instrument conditions were: carrier gas flow-rate, 20 ml/min; injection port temperature, 250°C; oven temperature, programmed from 220°C (0 min) at 16°C/min to 240°C; and the entire run time was 2 min. The optics of the mass spectrometer were optimized by autotuning at *m/z* 100. The ion intensity at *m/z* 86 was monitored for both 2-chloroprocaine and procaine with a window width of 0.1 a.m.u.

Procedures

Blood was obtained from pregnant patients undergoing epidural anesthesia for Cesarean section. Samples were collected in heparinized Vacutainers to which 0.3 ml of a 0.2 g/ml solution of cholinesterase inhibitor, echothiophate iodide (Ayerst Labs., New York, NY, U.S.A.) had been added. Standard curves were prepared using echothiophate-inhibited blood bank plasma after careful checking for interfering contaminants. Patient blood samples were separated by centrifugation and the plasma was stored frozen until analyzed.

Patient plasma samples (0.3—1 ml) and spiked plasma were extracted using a procedure similar to that described by Lesko et al. [3]. Following addition of 25 ng of procaine, the internal standard, the samples were made basic with 0.5 ml of 2 *M* sodium carbonate solution saturated with sodium chloride. Following extraction with 5 ml of diethyl ether, the samples were centrifuged and the organic layer transferred to a clean tube. The ether was carefully evaporated under nitrogen at room temperature and the extract reconstituted with 30 μl of benzene or toluene. A 2-μl aliquot of the final solution was injected into the gas chromatograph—mass spectrometer system.

Standard curves were prepared and the samples were quantitated using the Hewlett-Packard software for automatic quantitation of selected ion monitoring data by area normalization on the *m/z* 86 peak for procaine [4]. Standard curves ranged from 1.5—200 ng/ml.

RESULTS AND DISCUSSION

The mass spectra of the 2CP and procaine standards are shown in Fig. 1. The *m/z* 86 peak was chosen for selected ion monitoring because it was the base peak in both compounds and permitted increased sensitivity by monitoring only one ion.

The selected ion chromatogram of a plasma extract containing 6.25 ng/ml 2CP and 25 ng/ml internal standard is shown in Fig. 2. The retention times of procaine and 2CP are approximately 1.0 and 1.6 min, respectively. Frequently a small non-interfering unknown contaminant peak can also be seen between the two peaks of interest.

Calibration curves were linear over the ranges studied. The least squares linear regression line which describes a typical curve is $y = 0.25x + 1.63$. Using this curve, samples containing less than 2 ng/ml 2CP could be quantitated.

The precision of the method was determined by repeated analysis of spiked samples containing low (6.28 ng/ml) and high (100 ng/ml) concentrations of 2CP. The relative standard deviation (coefficient of variation) was 8 and 4% for five low and high samples, respectively.

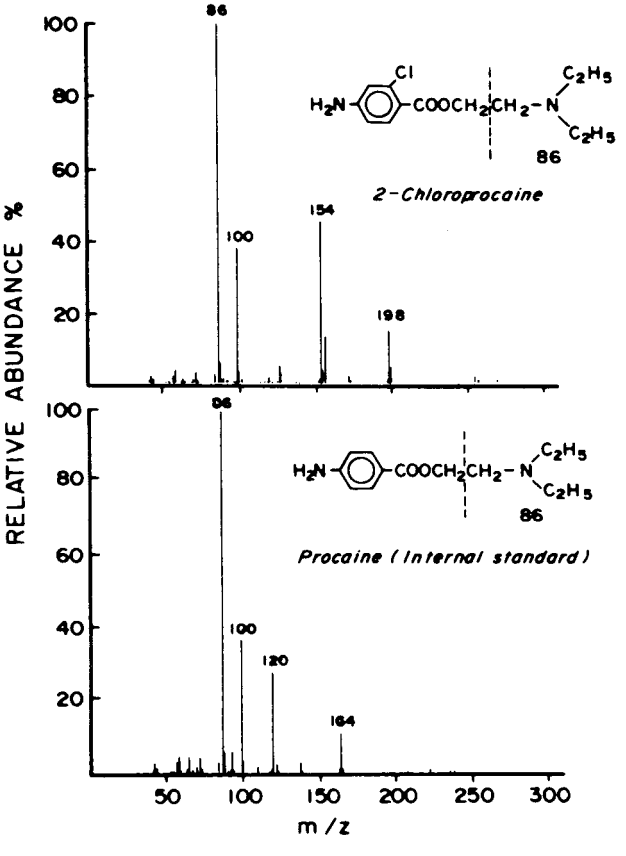
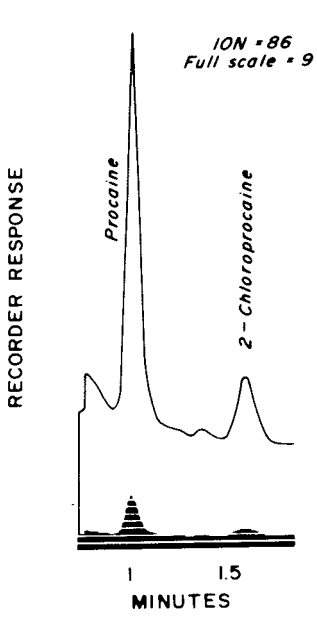


Fig. 1. Mass spectra of 2-chloroprocaine and procaine.



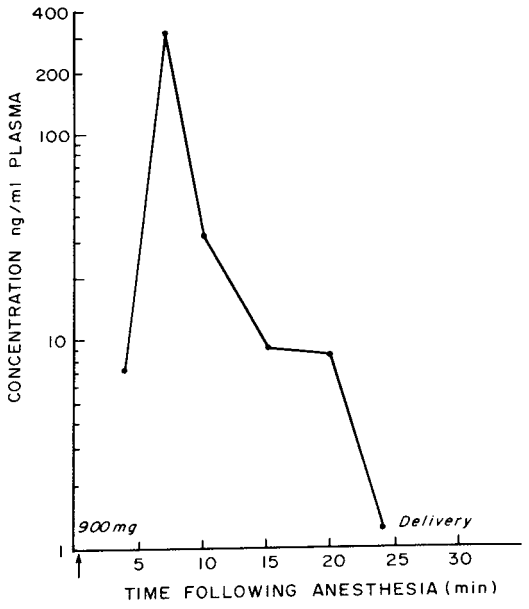


Fig. 2. Selected ion chromatogram of a plasma extract containing 6.25 ng/ml 2CP and 25 ng/ml procaine (internal standard).

Fig. 3. 2-Chloroprocaine in maternal plasma following epidural anesthesia for Cesarean section.

Using the method described, 2CP elimination curves in plasma were obtained during labor or Cesarean section (Fig. 3). Also, 2CP levels could be determined in umbilical cord vein and artery at delivery.

This method is very sensitive, quantitative, easy to perform, and it utilizes mass spectrometry equipment increasingly common in hospital chemistry and other laboratories. In addition, the automatic quantitation feature of the HP 5995A system greatly decreases analysis time; one technician can extract, analyze and quantitate 35 samples and standards in one day.

ACKNOWLEDGEMENTS

Supported in part by NIH USPHS Grants Nos. 5M01-RR-00210, 1R01 HD13359, and 1R28 RR01239, The Cuyahoga County Hospital Foundation and the Pennwalt Corporation.

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Journal of Chromatography, 224 (1981) 492—495
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 879

Note

Measurement of pyrimethamine in human plasma by gas—liquid chromatography

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(First received November 7th, 1980; revised manuscript received February 25th, 1981)

Pyrimethamine is an antimalarial drug which is sometimes used alone but often used in combination with dapsone as a chemoprophylactic. A method based upon high-performance liquid chromatography (HPLC) for determining pyrimethamine and dapsone simultaneously in human plasma was recently reported from these laboratories [1]. This method, using a UV detector, is conveniently applicable to both compounds but the lower limit of detection is about 5 ng injected for pyrimethamine and 2.5 ng for dapsone. Dapsone however, can be measured at much greater sensitivity by the use of a fluorescence detector [2], a technique not applicable for pyrimethamine which is only poorly fluorescent. The recommended dose of pyrimethamine when used on its own for malaria prophylaxis is 25 mg weekly but no data have been published for the plasma concentrations at this dose level. It was proposed to determine by direct measurement the plasma concentrations in volunteers so dosed and to correlate these with in vitro antimalarial activity as determined by means of a Plasmodium falciparum culture in human erythrocytes.

For this, a method for measuring pyrimethamine at greater sensitivity than hitherto was therefore developed using gas—liquid chromatography (GLC). A GLC assay method for a closely related compound, metoprine [2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine] (BW197U) has been published from these laboratories [3] and this served as a useful basis for developing the method. The results from the volunteer study will be reported separately.

MATERIALS AND METHODS

Reagents and materials

Pyrimethamine and BW197U were used as reference compounds as stock

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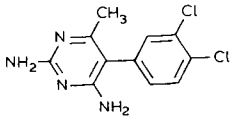
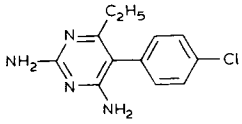
solutions at 1 mg/ml in methanol and these were diluted 1:100 with water before use.



BW 197 U



Pyrimethamine

Toluene AR grade was distilled before use. Amyl acetate (mixture of isomers; laboratory reagent grade, BDH, Poole, Great Britain) was distilled before use.

Human plasma was used for the validation experiments and was spiked with the diluted stock solutions of pyrimethamine and BW197U before extraction.

Glassware

Screw-capped 10-ml glass tubes were used in the extraction. Capped 200-μl vials were used for the autosampler.

Extraction procedure

Replicate 1-ml plasma samples or control plasma samples spiked with a range of standard quantities of pyrimethamine and with 20 μl BW197U solution as internal standard were mixed thoroughly and left at 4°C for at least 4 h to ensure equilibration. These samples were diluted with 1 ml water, 0.5 ml 1 *M* sodium hydroxide was added and roller-mixed for 20 min (1100 turns) with two 5-ml portions of toluene. After centrifugation at 1600 *g* for 10 min the pooled organic layers were transferred to clean tubes and dried under a stream of nitrogen gas at room temperature. The residue was redissolved in 100 μl amyl acetate and transferred to the GLC microvial which was then capped ready for chromatographic analysis.

Gas—liquid chromatography

Previous experience with the analysis of metoprine [3] indicated that 10% OV-17 on Chromosorb W HP (100—120 mesh) on a 2 m × 2 mm I.D. glass column would be suitable for the analysis of pyrimethamine and the conditions were optimised to separate the drug and internal standard from endogenous material extracted from plasma. The injection port of the gas chromatograph was maintained at 300°C, the detector at 350°C and the column at 235°C. After the BW197U had eluted, the column temperature was programmed to rise at 16°C/min to 280°C for 4 min, in order to elute late plasma peaks and reduce the overall analysis time for each sample to 26 min. Carrier gas was nitrogen at a flow-rate of 35 ml/min, and under these conditions pyrimethamine and BW197U had retention times of 7.3 and 10.8 min, respectively (Fig. 1).

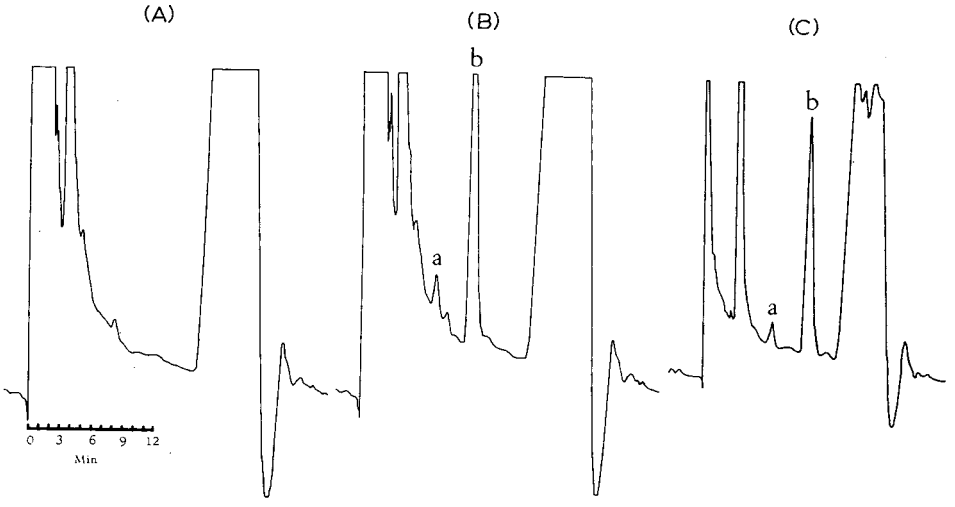


Fig. 1. GLC traces of plasma extracts assayed for pyrimethamine. (A) Unspiked plasma extract. Attenuation × 32. (B) Plasma extract spiked with pyrimethamine at 5 ng/ml (a) and internal standard at 200 ng/ml (b). Attenuation × 32. (C) Volunteer plasma extract containing pyrimethamine at 12 ng/ml (a) and spiked with internal standard at 200 ng/ml (b).

Instrumentation

The gas chromatograph was a Hewlett-Packard 5735A equipped with a constant-current electron-capture detector, with a 7671A automatic injector (5-μl injection used throughout) and linked to a Hewlett-Packard 3352B data system for controlling sample injection and processing chromatograms.

RESULTS AND DISCUSSION

The Hewlett-Packard 3352B data system identified by retention time, measured peak areas of pyrimethamine and BW197U, and calculated peak area ratios. Analysis of quadruplicate samples spiked with pyrimethamine over the range 5—400 ng/ml gave a coefficient of variation of 5.5%, being essentially constant over the whole concentration range. Recoveries of pyrimethamine and BW197U, which were also independent of concentration, were 75.6 ± 0.9% S.D. (n = 6) and 74.7 ± 1.4% S.D., respectively.

Regression of the logarithm of peak area ratios versus logarithm of pyrimethamine concentrations gave a correlation coefficient of 0.9994 (n = 23). The minimum detectable quantity of pure compound (signal-to-noise ratio = 2) was 50 pg injected on column and this allowed plasma concentrations ranging from 5—400 ng/ml to be comfortably assayed from volunteers receiving this drug. These results will be reported separately. The only other determination for pyrimethamine using GLC which appears to have been published [4] had a lower limit of sensitivity of 100 ng per g tissue. Using quantitative thin-layer chromatography, DeAngelis et al. [5] achieved a limit of 10 ng/ml as did Jones and Ovenell [1] using HPLC. In order to measure the lowest concentrations by the HPLC method it was necessary to inject over half of the extract. Using this

GLC method the errors are smaller and because only one twentieth of the total extract is injected at one time, replicate injections are possible. By this means, plasma levels can be monitored in volunteers receiving a single prophylactic dose (25 mg) of pyrimethamine for several weeks after dosing.

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Journal of Chromatography, 224 (1981) 496—499
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 882

Note

Determination of minaxolone (CCI 12923) by gas chromatography with nitrogen-sensitive detection

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(First received October 7th, 1980; revised manuscript received March 13th, 1981)

Minaxolone (Mx) is a new water-soluble steroid intravenous anaesthetic which has undergone early clinical trials in different centres [1—3]. A sensitive procedure for its estimation in plasma was required for its pharmacokinetic studies. This paper describes a procedure for the estimation of Mx in the plasma of anaesthetized patients.

MATERIALS AND METHODS

Reagents were of analytical grade and solvents had been distilled in glass. All glassware was silylated, rinsed with methanol and dried. Plasma standard, 1000 ng ml^{-1} was prepared by diluting 0.5 ml of 1 mg ml^{-1} methanolic solution of Mx base with drug free plasma. Plasma standards of 800, 400, 200, 100 and 50 ng ml^{-1} of Mx were prepared by serial dilutions. Plasma standards were divided into 2-ml aliquots and frozen.

Working internal standards, 500 ng ml^{-1} of N-7084 were prepared by diluting a methanolic stock solution of 1 mg ml^{-1} of N-7084 with 2 *N* hydrochloric acid containing 5 mg ml^{-1} of trimethylamine hydrochloride.

Blood was collected from patients anaesthetized with intravenous Mx by a heparinized line inserted into the opposite arm. Plasma was stored in plastic test tubes and kept frozen until analyzed. Standards and test samples were thawed at room temperature. To 1 ml of each specimen in PTFE-lined screw-capped culture tubes (16 × 100 mm), 1 ml of working internal standard and 6—7 ml of pentane were added. The contents of the tubes were mixed on a rotary mixer for 10 min. The tubes were centrifuged and the upper layer discarded by suction. To each tube, 1 ml of 2.5 *N* sodium hydroxide solution and 6—7 ml of pentane were added. The tubes were shaken on a rotary mixer for

10 min. The upper pentane layer was collected into tubes, taking care not to collect any emulsion or aqueous droplets. The aqueous phase was once again extracted with 6—7 ml of pentane and the upper layer collected. The combined pentane layers were evaporated to dryness at 45—50°C. The residue in each tube was dissolved in 20 μl of methanol by vortex mixing. The tubes were kept well stoppered until their contents (1—2 μl aliquots) were analyzed by gas chromatography (GC).

A Varian 3700 gas chromatograph (Walnut Creek, CA, U.S.A.) equipped with a thermionic nitrogen—phosphorus detector and a glass column (1.8 m × 4 mm ID) packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh) was used. The oven, injector, and detector temperatures were 265, 280, and 300°C, respectively. The carrier gas was nitrogen at a flow-rate of 30 ml min^{-1}. The chromatogram was recorded with a Varian recorder and the areas of the peaks were computed with an Autolab System I integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

RESULTS AND DISCUSSION

In the last few years, many drugs present in blood at ng ml^{-1} level have been analyzed by GC with the use of nitrogen-selective detectors. With the improvements in the design of these detectors, it has become relatively easy to use them [4]. Since Mx has a tertiary amino group, we elected to use a nitrogen-selective detector for higher sensitivity and selectivity for the assay of Mx. Some brands of blood-collecting devices contain phosphorus compounds as lubricants which produce potentially interfering peaks when a nitrogen-selective detector is used. Devices free from such contaminants were used for collecting blood for this study. After trying a number of tertiary amines, N-7084 (Fig. 1) was found to be a suitable internal standard. As seen in Fig. 2, it is well separated from the solvent peak and from the Mx peak. Minor tranquilizers (e.g. diazepam), which are frequently administered prior to surgery did not interfere with either the Mx peak or with the internal standard peak. After the addition of internal standard, plasma is washed at a pH of about 2 with pentane to remove some of the neutral or acidic impurities. Mx and the internal standard are not extracted by pentane at this pH. After adjusting the pH to greater than 10, plasma is extracted twice with pentane. Pentane is a non-polar solvent and extracts endogenous impurities in poor yield. The uncorrected recovery of

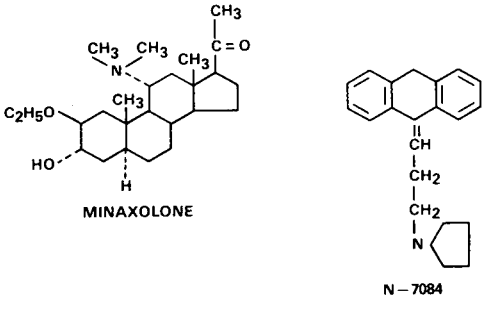

Fig. 1. Structural formulae of minaxolone (Mx) and N-7084.

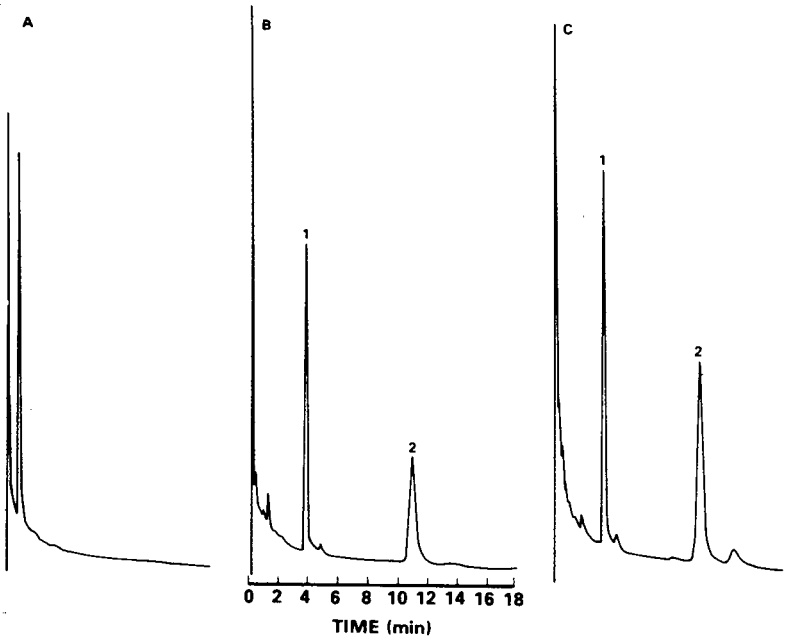

Fig. 2. Chromatograms of extracts of plasma (attenuation 32×10^{-12}). (A) Extract of drug-free pooled plasma; (B) extract with added Mx (200 ng/ml); (C) extract of plasma of a patient anaesthetized with Mx (320 ng/ml). Peaks: 1, N-7084 (internal standard); 2, Mx.

Mx is about 55% as determined by analyzing supplemented plasma without the addition of internal standard and comparing the peak areas with those obtained by injecting the same aliquots of non-extracted standards. The losses due to absorption during extraction are minimized by silylating the glassware and by addition of trimethylamine as a scavenger. During evaporation of the extract, trimethylamine is completely removed. Mx also has a secondary alcoholic group. Alcohols are polar compounds and have a tendency to adsorption on columns during GC analysis. However, the peak of Mx is sharp and symmetrical (Fig. 2) and the standard curve is linear for the range tested (50—1000 ng ml^{-1}) either by comparing the ratio of peak areas obtained by electronic integration or by comparing the ratios of peak heights for Mx and internal standard. The calibration curve passes through the origin. Haloperidol which has similar functional groups (a tertiary amino group, secondary alcoholic group and a keto group) has been analyzed in plasma in very low concentration by GC using a nitrogen-selective detector without any derivatization [5].

The coefficients of variation obtained by analyzing 10 replicate 50, 200, and 800 ng ml^{-1} plasma standards yielded values of 9.7, 4.6 and 9.8%, respectively. Prepared plasma solutions of Mx were found to remain unchanged after storage at −15°C for three months by extraction and comparison of concentrations of a similar set of freshly prepared samples.

This procedure was used to study the pharmacokinetics of Mx. An example of the plasma concentration curve for a female patient after receiving a bolus of 62.5 mg of Mx intravenously is shown in Fig. 3. This procedure allowed us to delineate plasma Mx concentration as low as 30 ng ml^{-1}.

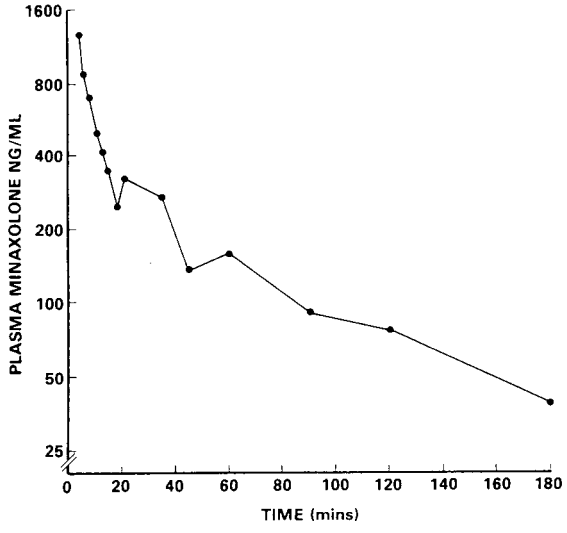

Fig. 3. Plasma profile of a female patient after administration of 62.5 mg of Mx intravenously.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. M. Gupta for technical assistance. This work was supported by a grant from Glaxo Canada.

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Journal of Chromatography, 224 (1981) 500—502
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 901

Note

Improved method for the determination of procetofenic acid in human plasma by gas—liquid chromatography

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(First received January 12th, 1981; revised manuscript received March 16th, 1981)

Procetofenic acid {2[4'-(*p*-chlorobenzoyl)phenoxyl]-2-methylpropionic acid} is the active metabolite of Procetofen (Lipanthyl) (LF 178; isopropyl ester of procetofenic acid). In order to be able to determine the level of this compound routinely in serum samples, we simplified the gas—liquid chromatographic (GLC) method described by Desager [1]. This improvement was achieved by the use of Meth-elute (0.2 *M* trimethylanilinium hydroxide in methanol) for the derivatization and of Bezafibrate (Cedur) (2-{4-[2(4-chlorbenzamido)-ethyl] phenoxyl}-2-methylpropionic acid) as an internal standard.

EXPERIMENTAL

Reagents

All chemicals were of analytical-reagent grade. Meth-elute (0.2 *M*) was obtained from Pierce Eurochemie (Rotterdam, The Netherlands), procetofenic acid from Fournier (Lyon, France) and Bezafibrate from Boehringer (Mannheim, G.F.R.).

Preparation of samples

To 1 ml of serum in a 12-ml stoppered glass tube were added 5 ml of diethyl ether, 100 μl of internal standard solution (Bezafibrate, 200 μg/ml) 200 μl of

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water and 100 μl of concentrated hydrochloric acid. After continuous mechanical stirring for 30 sec and centrifugation for 10 min at 3000 *g*, the upper layer was transferred to another tube. After evaporation to dryness at 50°C under nitrogen the residue was dissolved in 250 μl of 0.02 *M* Meth-elute and a 0.2-μl aliquot was injected into the gas chromatograph.

Gas chromatography

A Hewlett-Packard 5830A gas chromatograph equipped with a nickel-63 electron-capture detector was used. The column was a 1.6 m × 2 mm I.D. glass tube packed with 8% OV-101 on Gas-Chrom Q (80—100 mesh) (Boehringer) and was conditioned at 320°C for 24 h under nitrogen at a flow-rate of 20 ml/min. The oven temperature was 300°C, injection port temperature 320°C and detector temperature 320°C. The carrier gas was argon—methane (95:5) at a flow-rate of 50 ml/min.

RESULTS

A calibration graph of the peak-height ratio of procetofenic acid to internal standard versus amount of procetofenic acid was linear in the range from 2 to 200 μg/ml and passed through the origin. The accuracy and reproducibility of the method were evaluated by ten repeated determinations on three serum samples that contained 2, 20 and 200 μg/ml of procetofenic acid, respectively. The coefficients of variation were 4.7, 2.7 and 4.9%, i.e., below the 5% level that is generally accepted for quantitative analyses of drugs. The detection limit was 1 μg/ml.

A typical gas chromatogram is shown in Fig. 1. The retention time for proce-

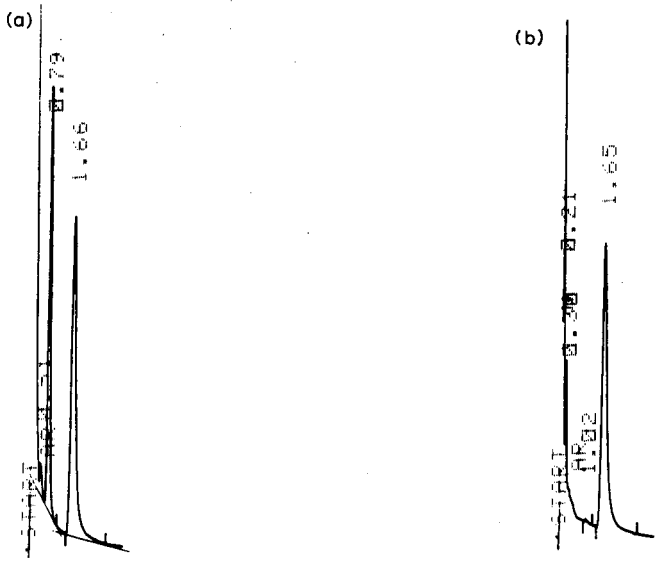

Fig. 1. Gas—liquid chromatograms of serum extracts: (a) procetofenic acid, retention time (RT) = 0.79 min, with Bezafibrate (RT = 1.66 min) as internal standard; (b) Bezafibrate alone (RT = 1.65 min).

tofenic acid (methyl ester) was 0.79 min and that of the internal standard was 1.66 min.

The specificity of the method was checked using a coupled GLC—mass spectrometric system. The mass spectrum was identical with that described by Elsom et al. [2].

DISCUSSION

In order to be able to establish a relationship between serum levels of procetofenic acid and its effect on the one hand and to control patients' compliance on the other, it was necessary to establish a method for specific and relatively rapid determinations. The method of Desager [1] could be simplified in two ways: diazomethane, which was used for the methylation, was replaced with Meth-elute, which need not be specially prepared and is less hazardous, and instead of Procetofen we used Bezafibrate as an internal standard. In comparison with Bezafibrate, Procetofen has two disadvantages: it cannot be excluded that some unmetabolized Procetofen may remain in the patients' serum [3], which would invalidate the evaluation, and there is a peak [1] which, being independent of the drug intake, has the same retention time as Procetofen and could therefore influence the evaluation. Under the conditions described here Procetofen will appear separately at a retention time of 1.11 min.

The proposed modification allows the specific and exact determination of procetofenic acid with good linearity in the range of serum levels that occur during continuous drug intake (2—20 μg/ml). Using this simplified method, one technician can handle about 120 serum samples per day if an automatic sampler is available.

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Journal of Chromatography, 224 (1981) 503—506
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 902

Note

Determination of chloroquine in blood by gas chromatography with nitrogen-selective detection using an internal standard

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(First received January 9th, 1981; revised manuscript received March 3rd, 1981)

For determining chloroquine in biological media, ultraviolet spectrophotometry and spectrofluorimetry are the commonest techniques, but they can exhibit a lack of sensitivity or selectivity. Several workers have used gas chromatography (GC) with a flame-ionization detector (FID) [1] and possible coupling with a mass spectrometer [2, 3] or with a ^{63}Ni electron-capture detector and O-ethyl O-(*p*-nitrophenyl) phenylphosphonothioate as an internal standard [4]; a few nanograms of chloroquine can be determined in rabbit urine by the last procedure. We obtained good sensitivity using a nitrogen-selective detector (NP FID) and medazepam as an internal standard [5], but interferences sometimes occurred and the volume of the samples was often very large. We attempted to overcome these problems by using another internal standard and improving the operating conditions. Whilst we were carrying out these studies, a technique for the determination of chloroquine and its monodesethyl metabolite in plasma and urine by high-performance liquid chromatography (HPLC) has been developed, with a sensitivity of 0.5 nmol/l using fluorescence detection [6].

EXPERIMENTAL

Reagents and standards

All reagents were of analytical-reagent grade. Diethyl ether and 1,2-dichloroethane were purified by using "Solvants, Documentation, Synthèses" (Peypin, France). Standard solutions of chloroquine sulphate (10 and 1 ng/μl of chloroquine base) and of papaverine (internal standard) (20 ng/μl of papaverine base) were prepared in methanol. They remain stable for at least 1 month when stored in a refrigerator at 4 °C.

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Blood samples

Blood samples were taken with potassium oxalate as anticoagulant and analysed immediately or stored at 4°C; in the latter instance the analysis must be carried out within 1 week.

Procedure

A whole blood sample (1—2 ml) was placed in a cylindro-conical centrifuge tube and 1 ml of distilled water and 1 ml of 60% potassium hydroxide solution were added. The mixture was boiled for 3 min with shaking, in order to destroy the protein bonds of chloroquine. After cooling, 100 μl of the papaverine standard solution (2 μg of internal standard) and, after vibrating for 14 sec, 10 ml of diethyl ether were added. The mixture was shaken mechanically for 15 min and centrifuged. The ether phase was transferred into another centrifuge tube and 1.2 ml of 0.1 *N* sulphuric acid were added. After shaking for 15 min and centrifuging, the ether phase was discarded. The aqueous phase, transferred in a centrifuge micro-tube, was alkalinized with one drop of 60% potassium hydroxide solution and 50 μl of a 1,2-dichloroethane—isoamyl alcohol (9:1) mixture were added. After shaking for 15 min and centrifuging, the upper aqueous phase was discarded and 2—3 μl of the organic phase were injected into the gas chromatograph.

Gas chromatography

A Hewlett-Packard Model 5170A gas chromatograph equipped with a nitrogen-specific detector was used. The glass column (1.2 m × 4 mm I.D.) was packed with 3% of OV-1—OV-17 mixture (1:3) on Gas-Chrom Q (100—120 mesh). The column temperature was 235°C and the injection port and detector temperatures were 300°C. The gas flow-rates were carrier gas (nitrogen) 50 ml/min, hydrogen 4 ml/min and air 30—40 ml/min. Under these conditions, chloroquine and the internal standard (papaverine) had retention times of 3 min 50 sec and 13 min 10 sec, respectively.

The chloroquine concentration was calculated from a calibration graph constructed by analysing blood samples spiked with chloroquine (20—300 ng/ml) and a fixed amount (2 μg/ml) of internal standard. The chromatographic response was linear in this range. The peak-area ratio of chloroquine to internal standard was plotted against the concentration of chloroquine. It would be possible in routine operation to incorporate only two standard points in each series of determinations.

RESULTS AND DISCUSSION

Papaverine was chosen as the internal standard because this quinoline alkaloid, in addition to its structural similarity to chloroquine, also has similar extraction characteristics. Moreover, papaverine is not often associated with chloroquine in therapeutic applications.

When applied to whole blood the technique gives an average recovery (mean of six determinations) of 67 ± 3.5% for 100 and 150 ng/ml of chloroquine and 65 ± 7% for 200 and 300 ng/ml. The recoveries from plasma and urine were 68 ± 3% and 78 ± 4%, respectively, for 200 ng/ml of chloroquine.

The within-run reproducibility (coefficient of variation 2%) was established using blood samples that contained 200 ng/ml of chloroquine. The determinations were repeated seven times. The between-run reproducibility (coefficient of variation 6.3%) was tested on plasma samples that contained 200 ng/ml of chloroquine. The samples were frozen for 4 weeks. The determinations were carried out each week.

The sensitivity limit for a quantitative determination was ca. 20 ng/ml in whole blood.

The specificity was tested with respect to possible interferences from other antimalarial drugs and from chloroquine metabolites. The peaks of amodiaquine (Flavoquine), pyrimethamine and quinine did not interfere in the determination of chloroquine. Sulfadoxine, associated with pyrimethamine in the commercial drug Fansidar, was not extracted. Chloroquine metabolites were extracted from the urine of a patient under chloroquine therapy, after enzymatic hydrolysis, separated by thin-layer chromatography and eluted from the spots with methanol. One of the metabolites isolated had a similar retention time to chloroquine, but on lowering the column temperature to ca. 220°C two separated peaks were obtained. The other urinary metabolites did not

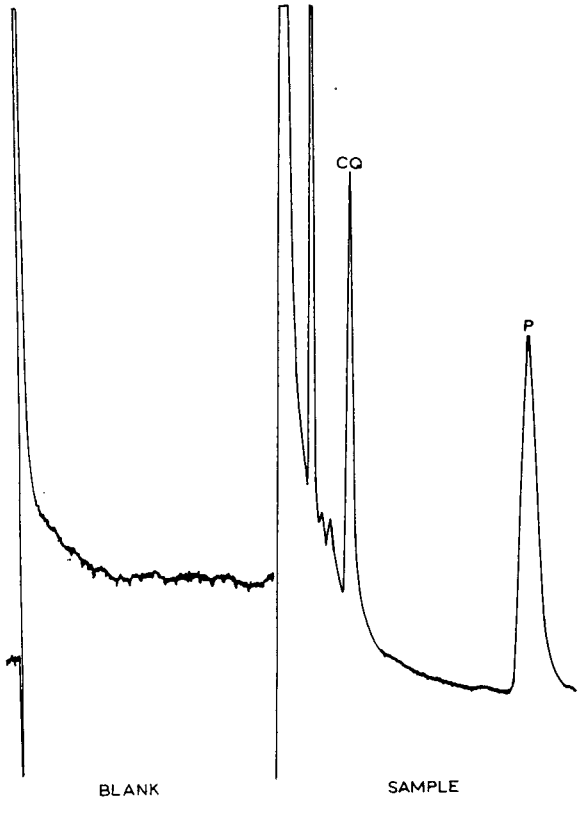

Fig. 1. Chromatogram obtained from the whole blood extract of a patient who had received 600 mg of chloroquine in tablets 24 h previously (attenuation = 16; recorder speed = 0.5 cm/min). CQ = chloroquine; P = papaverine (internal standard).

interfere. However, on analysing blood from patients on chloroquine treatment, no interfering substances (natural or metabolites of chloroquine) were found in the determination of the unchanged drug (Fig. 1).

The procedure is more sensitive and selective than spectrophotometry and spectrofluorimetry. It is less sensitive than the HPLC method described by Bergqvist and Frisk-Holmberg for human plasma and urine [6]. Its sensitivity is similar to that of the GC technique developed by Murayama and Nakajima for rabbit urine [4]. It gives a result within 1.5 h and seems to be more rapid than the other GC or HPLC methods.

The method is particularly suitable for the routine determination of chloroquine in whole blood from patients under treatment. In blood samples from two patients who had ingested 24 h earlier 600 mg of the drug in a single dose, we found 0.5 and 0.6 μg/ml of chloroquine. It can also be used for pharmacokinetic studies and therapeutic control. The method can be applied to the determination of chloroquine in plasma and urine; hydrolysis with potassium hydroxide solution is not necessary for urine samples, but the column temperature must be lowered to 220°C in order to prevent interference from one of the chloroquine metabolites. Its extension to the analysis of tissues should be possible. Finally, it is suitable for use in toxicology, for diagnostic or prognostic purposes in possible cases of poisoning.

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Journal of Chromatography, 224 (1981) 507—512
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 889

Note

Determination of α-methyldopa, α-methylnoradrenaline, noradrenaline and adrenaline in plasma using high-performance liquid chromatography with electrochemical detection

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(First received December 29th, 1980; revised manuscript received February 24th, 1981)

α-Methyldopa is widely used in the treatment of hypertension. The mechanisms underlying its hypotensive effect are still unclear. Although the most important action of α-methyldopa is thought to be a centrally mediated reduction in sympathetic activity [1], this cannot be reliably assessed by measurement of plasma catecholamines if there is also appreciable release of the α-methyldopa metabolite, α-methylnoradrenaline. Thus it is of interest to correlate changes in plasma concentrations of noradrenaline, adrenaline and α-methylnoradrenaline with changing levels of α-methyldopa.

Of the available methods, high-performance liquid chromatography (HPLC) with electrochemical detection was most suitable because its separation power permits determination of closely related catecholamine compounds with a high degree of specificity [2, 3]. Radioenzymatic assays which have been used for the determination of noradrenaline and adrenaline in human plasma [4, 5] do not allow discrimination between adrenaline and α-methylnoradrenaline.

The approach has been used previously for the detection of α-methyldopa in biological fluids [6—8]. It has also been shown to be capable of providing sufficient sensitivity to allow determination of concentrations of endogenous catecholamines in the plasma of normal subjects [9, 10]. The present paper describes a method for the simultaneous determination of noradrenaline, adrenaline, α-methyldopa and α-methylnoradrenaline in plasma. It is an extension of a method that has been used to measure noradrenaline and adrenaline in

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a wide range of patients and normal subjects. It involves a simple extraction procedure and a single, isocratic, chromatographic run.

EXPERIMENTAL

Reagents

Noradrenaline (NA), adrenaline (A) dihydroxybenzylamine (DHBA) and α-methyldopa (αMD) were obtained from Sigma (London, Great Britain). α-Methylnoradrenaline (αMNA) was a gift from Hoechst (U.K.) (Hounslow, Great Britain). Sodium octanesulphonate was obtained in solid form from Fisons Scientific Apparatus (Loughborough, Great Britain). All other chemicals used were of reagent grade and solutions were prepared using glass-distilled water.

Stock solutions of catecholamines and αMD, 1 mg/ml in 0.1 *M* hydrochloric acid were prepared and stored at 4°C. Working standards were prepared by making suitable dilutions of the stock solutions in 0.01 *M* hydrochloric acid.

Sample collection

At the beginning of each study, subjects (untreated hypertensives or patients receiving long-term αMD therapy) had indwelling catheters inserted into forearm veins. They then rested. Three samples were taken at 5-min intervals to establish basal plasma catechol concentrations.

Further blood samples were taken immediately after 5 min of sub-maximal exercise on a bicycle ergometer.

On each occasion, 10 ml blood was collected in a chilled lithium heparin tube and immediately centrifuged at 4°C. The plasma was collected and stored at −80°C. Prior to analysis, it was thawed and centrifuged for 5 min at 4°C.

Extraction of catechols

Plasma (1—4 ml) and 2 ng of internal standard (DHBA, in 20 μl of 0.01 *M* hydrochloric acid) were added to a chilled tube containing 75 mg alumina and 1 ml of 1 m*M* hydrochloric acid containing 0.1 m*M* EDTA. A 1-ml aliquot of 3 *M* Tris, pH 8.5 was added and the tube quickly stoppered and mixed for 10 min on a roller/shaker mixer.

The liquid was then aspirated and the alumina washed three times with 12 ml water. Elution of the catechol compounds was achieved by mixing the alumina with 200 μl of 0.091 *M* orthosphosphoric acid for 1 min.

Chromatography

The chromatograph comprised an Altex 100A pump (Altex Scientific, Berkeley, CA, U.S.A.), Altex 210 injection valve, Altex Ultrasphere Octyl (5 μm, C_8-bonded silica) column (150 × 4.6 mm I.D.) and precolumn (45 × 4.6 mm I.D.), B.A.S. TL5 glassy-carbon electrode and LC4 amperometric controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A sheet-aluminium Faraday cage enclosing column, electrode and controller were used to decrease electrical noise. The chromatograms were recorded on a Servoscribe 1S potentiometric recorder.

The mobile phase was composed of 70 m*M* KH_2PO_4, 1 m*M* EDTA, 6 m*M* sodium octanesulphonate and 14% methanol, pH 6.4 (using 5 *M* sodium hydroxide). Before use, the solution was filtered and then degassed by a flow of helium. Mobile phase flow-rate was 1.5 ml/min. Working electrode potential was +0.5 V.

The orthophosphoric acid eluate (100 μl) was mixed with 30 μl of 0.27 *M* tripotassium citrate solution containing sodium octanesulphonate, EDTA and methanol in amounts calculated to equalize concentrations between the mobile phase and the injected material. This has the effect of buffering the orthophosphoric acid eluate to approximately pH 5.5. A 100-μl aliquot of the buffered eluate was injected for chromatography.

Calculation of catechol concentrations

The concentrations of αMD, NA, A and αMNA in each sample were determined by calculating the ratios of heights of each catechol peak with that of the DHBA peak in that particular chromatogram. These ratios were then compared with equivalent ratios obtained from a chromatogram of known quantities of a standard mixture, extracted and separated using a similar procedure.

RESULTS

Sample chromatogram

Fig. 1 represents a chromatogram obtained from plasma from a patient on

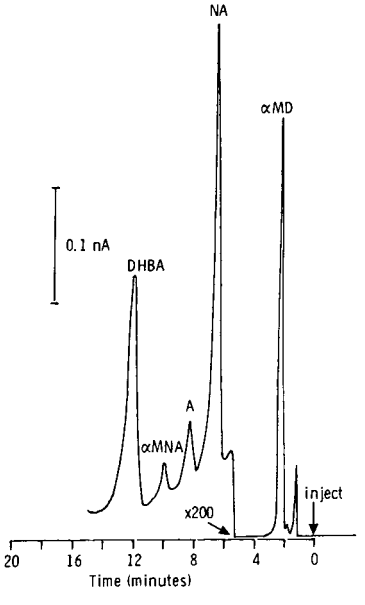

Fig. 1. Chromatogram of a plasma sample obtained from a patient on α-methyldopa therapy attending a regular hypertension clinic. Chromatographic conditions as described in Experimental. Determined concentrations: αMD, 0.275 μg/ml; NA, 0.770 ng/ml; A, 91 pg/ml; αMNA, 79 pg/ml; added internal standard (DHBA) concentration, 0.5 ng/ml.

αMD therapy, showing peaks corresponding to NA, A, the parent drug αMD, its metabolite αMNA and internal standard DHBA.

Standard curves

By comparing determined and added concentrations after the addition of various amounts of catechol standards to aliquots of a plasma pool, the method was shown to be linear in the range 20 pg/ml—20 ng/ml for NA and A, 40 pg/ml—20 ng/ml for αMNA and 10 ng/ml—2.5 μg/ml for αMD.

Precision

Repeated determinations of endogenous A and NA and added αMD and αMNA (in aliquots of pooled plasma) gave the following coefficients of variation (C.V.): A, C.V. = 4.7% at a mean calculated concentration of 51 pg/ml; NA, C.V. = 4.0% at 188 pg/ml; αMD, C.V. = 4.1% at 97.2 ng/ml; αMD, C.V. = 3.9% at 97 pg/ml.

Application in experimental subjects

Table I shows determined plasma catechol concentrations in four patients receiving αMD therapy, before and after exercise on a bicycle ergometer.

TABLE I

PLASMA CATECHOL CONCENTRATIONS IN αMD PATIENTS BEFORE AND AFTER EXERCISE

Limits of detection: αMD, 10 ng/ml; NA, 20 pg/ml; A, 20 pg/ml; αMNA, 40 pg/ml. N.D. = not detectable.

Subject		αMD (μg/ml)	NA (ng/ml)	A (ng/ml)	αMNA (ng/ml)
1	basal	1.36	0.107	N.D.	N.D.
	post-exercise	1.44	1.317	0.228	0.233
2	basal	0.22	0.248	N.D.	N.D.
	post-exercise	0.22	1.770	0.110	0.354
3	basal	0.74	0.151	N.D.	N.D.
	post-exercise	0.77	0.374	0.077	0.090
4	basal	1.06	0.063	N.D.	N.D.
	post-exercise	1.03	0.998	0.085	0.301

DISCUSSION

Interference from other substances (unidentified, but thought to include acidic catechol compounds co-extracted on alumina) was decreased by using a relatively high mobile phase pH (6.4) and sodium octanesulphonate concentration (6 m*M*). Increasing pH in the range 3.0—7.0 has the effect of selectively decreasing protonation of the acidic species, the fully protonated state of the basic species e.g. catecholamines, being largely unaffected. In the pH range 6.0—7.0, the latter are still capable of a high degree of ion-pair bonding with the octanesulphonate and thus have relatively long retention

times. Catecholamine retention times can effectively be further selectively increased by increasing the concentration of the ion-pair reagent [11].

An additional benefit obtained by using a mobile phase with high pH is improved detector response [11]. It is of note that new columns could not be used with pH 6.4 mobile phase immediately. It took some days before auto-oxidation of catecholamines (catalysed by new metallic surfaces) was reduced to a negligible level.

An operating potential of +0.5 V (relative to the B.A.S. RE1 Ag/AgCl reference electrode) proved to be optimal for achieving the maximal signal from the oxidation of catecholamines without oxidation of any other compounds with similar retention times. (Using potentials greater than +0.5 V, there is little further gain in signal from a fixed catecholamine concentration [12].)

The previously adopted system for the measurement of NA and A only, utilised mobile phase containing 5 m*M* sodium octanesulphonate and 12% methanol. 6 m*M* sodium octanesulphonate and 14% methanol were found to give better resolution of A and αMNA and were thus adopted for the present application, maintaining other factors constant. Resolution of these two substances was also found to be better using a C_8 column than a C_{18} column.

Using the present system, αMD has a very short retention time (see Fig. 1) running close to the solvent front where it may, in fact, co-chromatograph with other unidentified peaks. Although these components have not been investigated in any detail, they generally have associated peak heights which are negligible compared with those associated with αMD in the samples analysed (see e.g. Fig. 2 which shows a chromatogram obtained from plasma from a

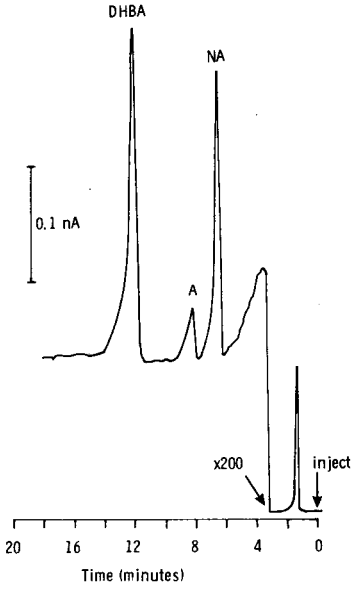

Fig. 2. Chromatogram of a plasma sample obtained from a normal subject. Chromatographic conditions as described in Experimental. Determined concentrations: NA, 0.349 ng/ml; A, 66 pg/ml; added internal standard (DHBA) concentration, 0.5 ng/ml.

normal subject). In patients on αMD therapy, determined plasma αMD concentrations were in the range 0.2—2.0 μg/ml. Comparable levels have been reported by other workers [8, 13]. These concentrations are three to four orders of magnitude higher than concentrations of endogenous catecholamines. Chromatographic problems associated with peak overlapping have been noted by Moyer et al. [14], who used a similar system, but by using an efficient column and ensuring sufficient difference in retention times between αMD and later eluting peaks, this problem is diminished. Using the present system, αMD peak tailing only becomes a problem at high plasma levels, > 2 μg/ml. In order to measure αMD, NA, A, αMNA and internal standard DHBA in the same chromatogram, it is necessary to employ large sensitivity changes in mid-run as illustrated in Fig. 1. These sensitivity changes do not lead to problems in quantitation, high plasma αMD concentrations are still within the linear range of the system.

The method described was shown to be capable of detection of plasma concentrations of NA in resting subjects and of NA and A in subjects after exercise. Although detection limits for αMNA were similar, it is of interest that the substance was not found in plasmas from resting αMD patients. However, its appearance on exercise demonstrates that it is clearly present in the sympathetic nerve endings of these patients.

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Journal of Chromatography, 224 (1981) 513—518
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 897

Note

High-performance liquid chromatographic determination of free resorcinol in plasma and in urine

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(First received December 29th, 1980; revised manuscript received March 20th, 1981)

Resorcinol is used in some topical antiacne preparations to produce keratolysis. Because the agent can penetrate human skin [1], this study was done in order to develop a suitable method for the analysis of free (unchanged) resorcinol in plasma and in urine. Such a method was considered to be essential to studies of the absorption and subsequent metabolic disposition of topically-applied resorcinol.

Previously reported techniques for the determination of resorcinol [2—4] lack specificity and sensitivity; in particular, the high-performance liquid chromatographic (HPLC) procedure for this agent in pharmaceutical dosage forms [5] is not sensitive enough for pharmacokinetic studies.

This paper describes a HPLC technique, which requires only a relatively simple extraction procedure. It permits sensitive and speedy determination of free resorcinol in human plasma and in urine at concentrations as low as 0.5 μg/ml. Recoveries are more than 90% of theoretical, with good reproducibility. So far as we know, this is the first report of an assay for resorcinol in biological fluids.

EXPERIMENTAL

Materials

Analytical grades of resorcinol and orcinol (internal standard) were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, HPLC grade, was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Glass-distilled water was filtered through Type EG 0.22 micrometer filter paper (Millipore, Bedford, MA, U.S.A.) before use. All other chemicals and solvents used were ACS grade or better.

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Apparatus

A high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) composed of a M6000 pump, a Model 450 variable-wavelength UV detector, and a U6K injector was used. A WISP (Waters Assoc.) automatic sample injection system was used in the analysis of large numbers of samples. Chromatography was performed on a 250 mm × 4.6 mm I.D., 5-μm particle size, Zorbax ODS C_{18} reversed-phase column (Dupont, Wilmington, DE, U.S.A.), preceded by an Altex 5 cm × 2.1 mm ODS pre-column. All analyses were performed isocratically at a flow-rate of 1.5 ml/min, with a mobile phase consisting of 15% acetonitrile in a pH 6.6 phosphate buffer. The detector wavelength was set at 280 nm. A Hewlett-Packard 5830A electronic integrator was used for data processing.

Extraction procedure

The plasma or urine sample (1.0 ml) was pipetted into a 16 × 125 mm culture tube provided with a PTFE-lined screw cap. Five ml of diethyl ether containing a known amount of internal standard (orcinol) were added. The closed tube was shaken for 3 min and then centrifuged at 2000 rpm for 3 min. The organic layer was transferred into another tube and the aqueous layer was again washed with 5 ml of diethyl ether. The pooled organic extracts were evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was taken up into 1 ml of mobile phase and 20 μl were injected into the liquid chromatograph.

Calibration and recovery

Calibration curves were constructed with plasma and urine samples containing added concentrations (0.5—10.0 μg/ml) of resorcinol. To all samples, 5 μg of orcinol were added as internal standard and the samples then were extracted and analyzed as described. The ratio of the height of resorcinol peak to the height of orcinol peak was plotted against the concentration of resorcinol. The concentrations of unknown samples were subsequently determined from the standard curve.

The efficiency of the extraction procedure was assessed by adding known amounts (0.5—10 μg) of resorcinol to 1.0-ml aliquots of plasma and urine. The samples were extracted as previously described and reconstituted into 1 ml of the mobile phase containing 5 μg of orcinol as internal standard.

Recovery and reproducibility

Good recovery and reproducibility of this assay were confirmed by replicate analyses of plasma and urine samples fortified with 1.0 and 5.0 μg/ml of resorcinol, respectively.

RESULTS AND DISCUSSION

Various chromatographic conditions (different columns and mobile phases) for the analysis of resorcinol in plasma and urine were investigated. Under the experimental conditions described, no interferences from the endogenous constituents of plasma or urine were observed. Chromatograms obtained

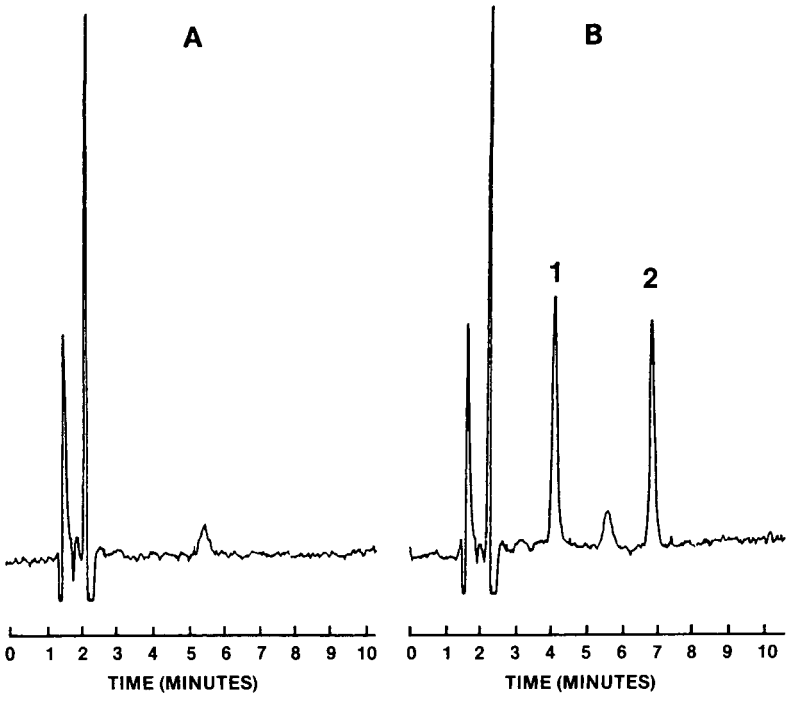

Fig. 1. Chromatograms of (A) blank plasma and (B) plasma spiked with 1.0 μg/ml resorcinol and orcinol (internal standard). Peaks: 1 = resorcinol; 2 = orcinol.

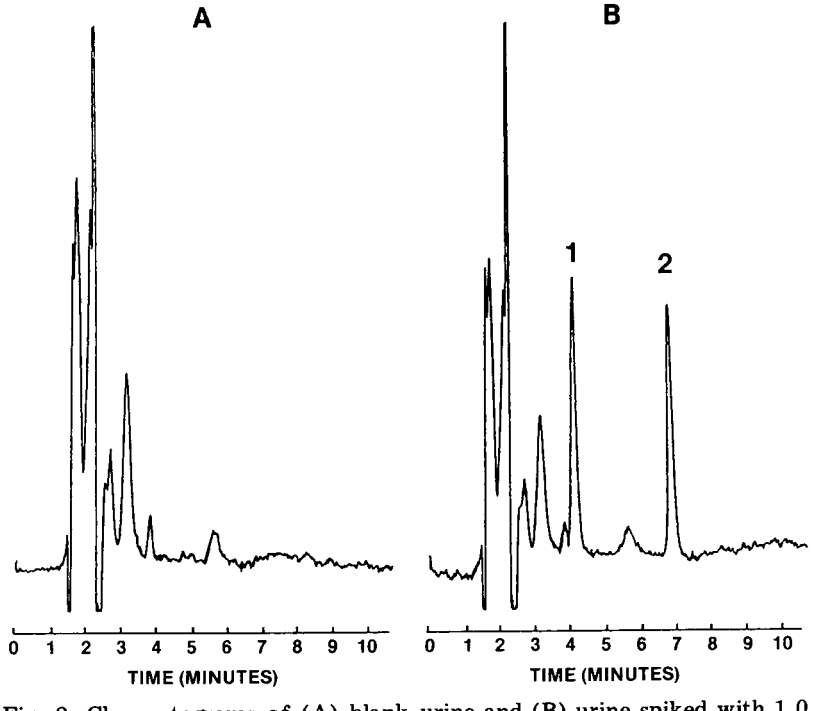

Fig. 2. Chromatograms of (A) blank urine and (B) urine spiked with 1.0 μg/ml resorcinol and orcinol (internal standard). Peaks: 1 = resorcinol; 2 = orcinol.

from the analysis of plasma and urine blanks, as well as those from samples spiked with resorcinol and orcinol, are shown in Figs. 1 and 2. These compounds were eluted with retention times of 4.19 and 7.04 min, respectively. The total time required for the analysis of each sample was 10 min.

The resorcinol calibration curves in plasma and urine were linear over the concentration range studied. Least-square regression analysis of the data resulted in good linearity in the range of 0—10 μg/ml with correlation coefficients of 0.9999 for plasma and 0.9993 for urine. The limit of detection of resorcinol with this method is 0.5 μg/ml.

The efficiency of the extraction procedure was confirmed by analyzing plasma and urine samples spiked with known amounts of resorcinol, ranging in concentrations from 0.5—10 μg/ml. Five replicates at each concentration were analyzed. As shown in Table I, the recovery of resorcinol in plasma throughout the concentration range studied varied from 87.6—98.3%, with

TABLE I

RECOVERY OF RESORCINOL FROM SPIKED BIOLOGICAL FLUIDS

Five trials for each level of resorcinol.

Biological fluid	Amount of resorcinol spiked (μg/ml)	Recovery (%) (Mean ± S.D.)	Coefficient of variation (%)
Plasma	0.5	87.6 ± 2.08	2.37
	1.0	94.3 ± 5.28	5.60
	2.0	93.9 ± 4.58	4.88
	3.0	98.3 ± 3.11	3.16
	5.0	96.3 ± 2.25	2.34
	Mean	94.1	3.67
Urine	0.5	89.6 ± 1.82	2.03
	1.0	88.8 ± 10.03	11.30
	2.0	96.0 ± 1.76	1.83
	3.0	98.0 ± 3.35	3.42
	5.0	94.6 ± 2.85	3.01
	Mean	93.4	4.32

TABLE II

REPRODUCIBILITY OF THE ANALYSIS

Sample	No. of trials	Conc. of resorcinol (μg/ml)	Standard deviation	Coefficient of variation (%)
Plasma	8	1.0	±0.019	2.4
	8	5.0	±0.088	3.9
			Mean	3.2
Urine	9	1.0	±0.029	4.0
	7	5.0	±0.056	2.5
			Mean	3.3

TABLE III

PLASMA AND URINARY LEVELS OF RESORCINOL OF THREE SUBJECTS AFTER 2 WEEKS OF DAILY TOPICAL RESORCINOL ADMINISTRATION (800 mg/day)

Subject	Plasma	24-h urine	
		mg	% dose
Untreated Control	0*	0	0
1	0	3.75	0.47
2	0	12.65	1.58
3	0	22.98	2.87

*Less than 0.5 μg/ml.

a mean recovery of 94.1%. In urine, the recovery ranged from 88.8—98.0%, with a mean recovery of 93.4%.

The mean measured concentration, standard deviation and coefficient of variation of the assay are shown in Table II. In plasma, the coefficient of variation was 2.4% at the lower concentration of 1.0 μg/ml, and 3.9% at 5.0 μg/ml. In urine, the coefficients of variation for samples of low and high resorcinol concentration were 4.0 and 2.5%, respectively.

Biological results

The percutaneous absorption and metabolic disposition of resorcinol was investigated following highly exaggerated repeated topical application of a 2% resorcinol in a hydroalcoholic vehicle to three volunteers. After 2 weeks of treatment, an average of 1.64% (range 0.47—2.87%) of the administered dose was excreted in 24-h urines (Table III). No resorcinol could be detected in any of the blood samples collected after 1, 2, 3 and 4 weeks of drug application.

CONCLUSION

The method described is simple, rapid, and specific; it has good recovery for the determination of free resorcinol in plasma and in urine. Moreover, the assay is sensitive — the detection limit is approximately 0.5 μg/ml — and reproducibility is very acceptable. In contrast, the more commonly used technique of deproteinization of plasma with acetonitrile, followed by the direct analysis of the filtrate, results in peaks with retention times similar to that of resorcinol, which interfere with the analysis. Even greater sensitivity might be achieved with this method by increasing the volume of plasma or urine extracted or by increasing the injection volume. The nondestructive nature of this technique allows the isolation and subsequent identification of free resorcinol and its metabolites in animal or human pharmacokinetics studies.

ACKNOWLEDGEMENT

The authors thank Mr. M.C. Tillery, Manager of R&D Publications, for editorial assistance.

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Journal of Chromatography, 224 (1981) 519—525
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 903

Note

Simultaneous determination of chlordiazepoxide and its metabolites in human plasma and urine by means of reversed-phase high-performance liquid chromatography

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(Received February 10th, 1981)

Chlordiazepoxide (Librium) is widely used for the relief of anxiety, tension and CNS depressions. A number of metabolites of chlordiazepoxide are possible, but so far only four metabolites and the parent compound could be identified in biological fluids.

Methods that have been described for determining the parent compound and metabolites include spectrofluorimetry [1, 2], gas chromatography—mass spectrometry [3, 4], radioimmunoassay [5], thin-layer chromatography [6—8], spectrophotometry [9], colorimetry [10], differential pulse polarography [11] and high-performance liquid chromatography [12—15]. These methods either are laborious or require large sample volumes.

The method described here permits the simultaneous determination of chlordiazepoxide and its metabolites N-desmethylchlordiazepoxide, demoxepam, N-desmethyldiazepam and oxazepam. The small plasma sample volume of 0.2 ml allows frequent sampling from laboratory animals and patients and from volunteers by self-sampling with fingertip puncture. The method has been applied to human pharmacokinetic studies, routine monitoring and cases of severe chlordiazepoxide overdose. Some examples of the pharmacokinetic behaviour of chlordiazepoxide and its metabolites in man will be shown.

EXPERIMENTAL

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph equipped with a variable-wavelength detector (Model SP 770), operating at 240 nm was used. The detector was connected to a 10-mV recorder (BD 40, Kipp & Zonen,

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Delft, The Netherlands). A stainless-steel column (10 cm × 4.6 mm I.D.) packed with LiChrosorb RP-8, particle size 5 μm, was used. The injection loop volume was 100 μl.

Solvent

The solvent was 0.01 *M* sodium acetate in water—methanol—acetonitrile (600:200:200) and the flow-rate was 2.0 ml/min at a pressure of about 270 bar. All reagents were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.).

Drugs

Chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, N-desmethyldiazepam and oxazepam were obtained from Hoffmann-LaRoche (Mijdrecht, The Netherlands).

Patients

Samples were taken from patients in the Departments of Neurology and Psychiatry and the Intensive Care Unit of the Sint Radboud Hospital.

Sample preparation

Plasma. A 10-μl aliquot of water containing 334 ng of diazepam as internal standard and 1 ml of diethyl ether was added to 0.2 ml of plasma and mixed on a Vortex mixer for 1 min. The mixture was then centrifuged for 5 min at 4000 rpm (2600 *g*) in a Heraus Christ centrifuge. The ether layer was removed and evaporated to dryness with a dry stream of air. The residue was dissolved in 0.2 ml of the eluent and 0.1 ml was injected on to the column.

Urine. A 0.1-ml urine sample was injected directly on to the column.

Deglucuronidation of plasma and urine

Plasma. A 0.2-ml volume of plasma was incubated with 15 μl of β-deglucuronidase (100,000 U/ml; Sigma, St. Louis, MO, U.S.A.), 0.2 ml of potassium dihydrogen orthophosphate buffer (0.067 *M*) and one drop of 0.2 *M* acetic acid and 10 μl of water containing 334 ng of diazepam for 16 h. A 100-μl volume of 1 *N* sodium hydroxide solution was added and the mixture was extracted with 1 ml of diethyl ether and treated for injection as described above.

Urine. A 0.2-ml volume of urine was incubated with 15 μl of β-deglucuronidase, 0.2 ml potassium dihydrogen orthophosphate buffer (0.067 *M*) and one drop of 0.2 M acetic acid for 16 h. After centrifugation of the mixture, 100 μl were injected directly on to the column.

Recovery

The recoveries of the extraction were 95 ± 6% for chlordiazepoxide, 93 ± 3% for N-desmethylchlordiazepoxide, 94 ± 4% for demoxepam, 89 ± 7% for N-desmethyldiazepam and 88 ± 2% for oxazepam (plasma). The detection limit for all derivatives was 20 ng/ml. The calibration graphs were linear for the concentration range 20 ng/ml—20 μg/ml.

RESULTS

Fig. 1 shows the possible pathways in the metabolism of chlordiazepoxide and the structural formulae of the metabolites that can be expected.

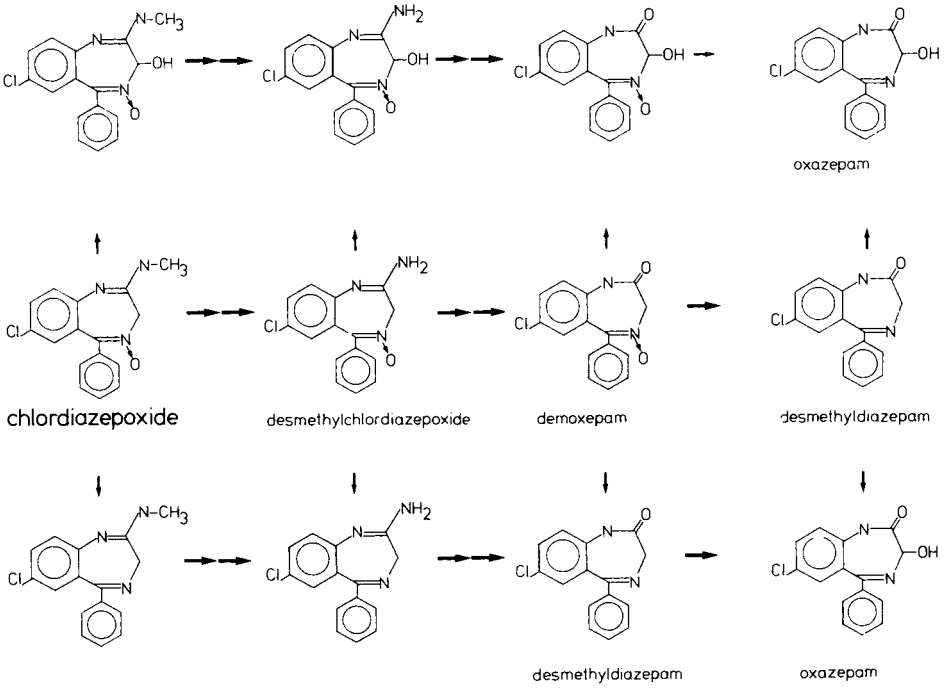

Fig. 1. Structural formulae of possible metabolites of chlordiazepoxide due to metabolic pathways of N-demethylation + N-deamination, C_3 hydroxylation and N → O reduction. Metabolites identified are indicated by their names. The thickness of the arrows indicates the relative rate of each metabolic process.

Fig. 2 shows the HPLC traces for chlordiazepoxide (peak 4) and its metabolites desmethylchlordiazepoxide (2), demoxepam (1), N-desmethyldiazepam (5) and oxazepam (3) in blank plasma, a standard plasma sample and a plasma sample from a patient receiving 10 mg of chlordiazepoxide (Librium) three times a day. The relative retention times (k') are shown in Table I.

TABLE I

RELATIVE RETENTION TIMES (k') OF CHLORDIAZEPOXIDE AND ITS METABOLITES

Compound	k'
Demoxepam	6.20
Desmethylchlordiazepoxide	8.10
Oxazepam	9.60
Chlordiazepoxide	11.20
Desmethyldiazepam	15.20
Diazepam (internal standard)	22.20

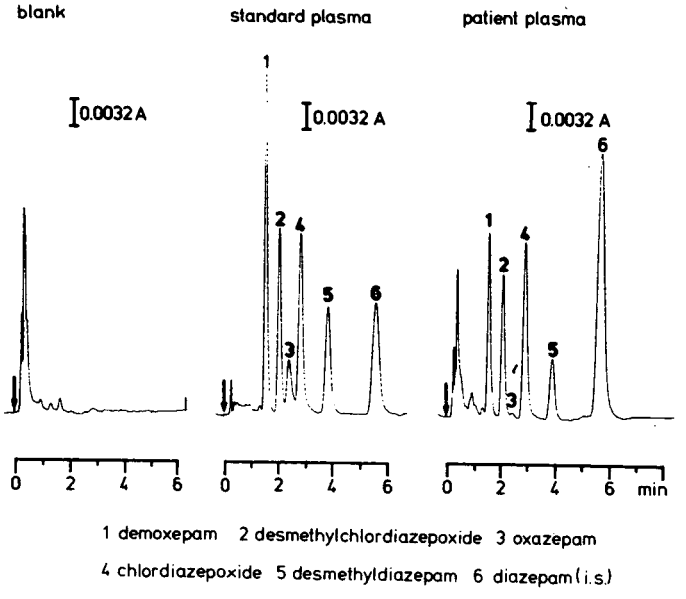

Fig. 2. HPLC traces for chlordiazepoxide (4) and its metabolites N-desmethylchlordiazepoxide (2), oxazepam (3), N-desmethyldiazepam (5) and the internal standard diazepam (6) in a blank human plasma sample (left), in a standard plasma sample (centre) and in a plasma sample from a patient receiving 10 mg of Librium three times a day (right).

Fig. 3 shows the plasma concentration—time profiles of chlordiazepoxide in a patient after ingestion of an apparent overdose of chlordiazepoxide. The plasma concentration of chlordiazepoxide starts with an extremely high value of 12 μg/ml, but declines rapidly with a half-life ($T_{½}$) of 6 h. Chlordiazepoxide is demethylated to N-desmethylchlordiazepoxide ($T_{½}$ 10 h), which in turn is deaminated to demoxepam ($T_{½}$ 28 h); this is subsequently reduced to N-desmethyldiazepam ($T_{½}$ 46 h), which is finally hydroxylated to oxazepam ($T_{½}$ 46 h).

Fig. 4 is an example of the plasma concentration—time profile of chlordiazepoxide and its metabolites in a patient receiving a normal treatment regimen of 10 mg of Librium three times a day. The purpose of the study was to compare the bioavailability of 10 mg of Librium three times a day with 30 mg of Librium CR administered once a day. It was found that the plasma concentrations of the parent drug and the four metabolites, including the low concentrations of the final metabolite oxazepam, can be measured with this HPLC method.

DISCUSSION

The HPLC method for chlordiazepoxide described here is a step forward towards an HPLC analysis that would make it possible to identify and measure all possible metabolites of chlordiazepoxide shown in Fig. 1 without interferences from endogenous substances as previously described for diazepam [16].

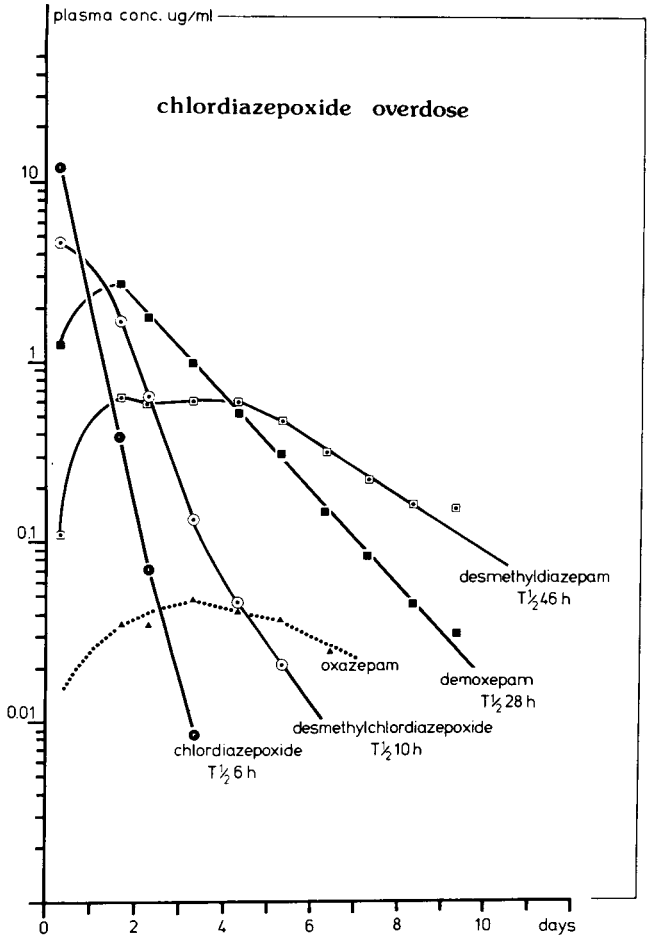

Fig. 3. Plasma concentration—time profiles of chlordiazepoxide and its metabolites in a patient who had taken an apparent overdose of Librium (about 1 g). The metabolites of chlordiazepoxide, desmethylchlordiazepoxide ($T_{½}$ 10 h), demoxepam ($T_{½}$ 28 h) and desmethyldiazepam ($T_{½}$ 46 h) all have higher $T_{½}$ values than chlordiazepoxide. Oxazepam, with its intrinsic $T_{½}$ of 3 and 10 h, will finally adopt the $T_{½}$ of N-desmethyldiazepam (46 h).

The advantage of this method over published ones is that, with a simple extraction, chlordiazepoxide and four of its major metabolites can be measured in one run. The small blood sample (0.2 ml of plasma) makes frequent sampling from patients and laboratory animals possible and human volunteers can take blood samples themselves by fingertip puncture. The detection limit of 20 ng/ml facilitates the determination of the plasma concentrations of chlordiazepoxide and its metabolites under conditions of a relatively normal dosage regimen of 10 mg of Librium three times a day, as shown in Fig. 4, and also in samples from patients suffering from an overdose of chlordiazepoxide.

Fig. 3 demonstrates the main metabolic pathway in Fig. 1 from the half-lives of the successive metabolites.

Several HPLC methods have already been developed, but most lack some of

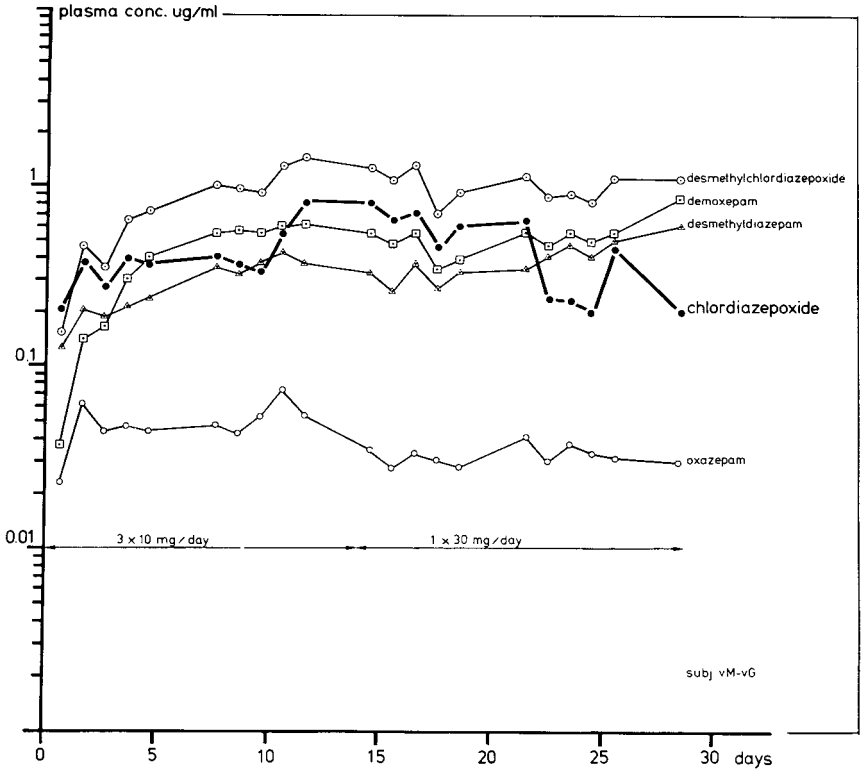

Fig. 4. Plasma concentration—time profile of chlordiazepoxide and its metabolites in a patient treated initially for 14 days with 10 mg of Librium three times a day, replaced in the following 14 days by 30 mg of Librium CR once a day.

the advantages achieved in this work, such as the possibility of showing all metabolites [14, 15]; although while Peat et al. [17], Strojny et al. [13] and Ascalone [12] described suitable HPLC methods, they did not allow the detection of the final metabolite oxazepam.

From the metabolic point of view, the method must contain the possibility of detecting as many metabolites as possible, whether the detection limit allows their actual detection or not. Only when the method of analysis allows the detection of a specific metabolite can one establish whether the metabolite is present in amounts higher than the detection limit or not.

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Journal of Chromatography, 224 (1981) 526—531
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 885

Note

Fluorescence assay of citalopram and its metabolites in plasma by scanning densitometry of thin-layer chromatograms

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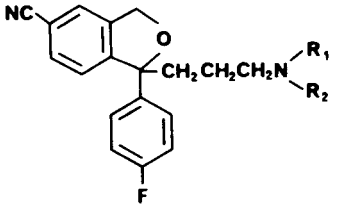
(First received December 17th, 1980; revised manuscript received February 25th, 1981)

The drug citalopram (Fig. 1, I), Lu 10-171, is a specific, potent serotonin re-uptake inhibitor [1—4], now under clinical trials as an antidepressant [5].

Pharmacokinetic studies have been performed in man [6, 7] and in various safety-related animal trials, one of which has been published [8]. The analytical technique used in these studies was based on the isolation and purification of citalopram (I) and its demethylated metabolite (II) by extraction and thin-layer chromatography (TLC) followed by fluorimetric quantitation by ion-pair formation with a fluorescent anion [6]. The method was fairly reproducible (standard deviation ca. 10%) and sensitive (limit of detection 20 ng of citalopram in 2-ml plasma samples), but lengthy. In addition, quantitation of the demethylated metabolite (II) was less reliable and less sensitive. Assay of the didemethylated metabolite (III), present in both animals and man, was not possible.



		R_1	R_2	
Citalopram	(I)	CH_3	CH_3	
Monodesmethyl metabolite	(II)	CH_3	H	
Didesmethyl metabolite	(III)	H	H	
Citalopram N-oxide	(IV)	CH_3	CH_3	$-N^{+}-O^{-}$

Fig. 1. Formulae of citalopram (I), a potent and specific serotonin re-uptake inhibitor, and its metabolites.

For these reasons, efforts have been made to improve the assay, especially with regard to quantitation of the metabolites present and sample throughput. A method involving direct scanning of the native fluorescence of drug and metabolite spots on the thin-layer plate is described here and compared with the previous technique.

EXPERIMENTAL

Extraction procedure

Plasma samples (2.0 ml) were adjusted to pH 10 with 100 μl of 1 *N* sodium hydroxide solution. As standards were added in ethanol solution, all samples were diluted with 75 μl of ethanol. The samples were extracted twice with 6-ml portions of *n*-hexane containing 1% of triethylamine by mechanical shaking for 10 min in stoppered glass tubes, after which the samples were centrifuged and placed in a dry-ice—ethanol mixture. The aqueous phase was frozen and the *n*-hexane phase transferred into 10-ml conical glass tubes and evaporated to dryness under a stream of air in a 50°C water-bath.

For each set of sixteen samples, four standards (0, 25, 100 and 250 ng of I, II and III added to drug-free plasma) were included.

Thin-layer chromatography

The residues were dissolved in 50-μl portions of chloroform by vigorous shaking and transferred together with a 50-μl rinse by autopipette into Sanz micro-tubes, ready for application onto 0.25 mm thick, pre-coated HPTLC silica gel plates without a fluorescence indicator (Merck, Darmstadt, G.F.R.).

Twenty samples were applied on a 10 × 20 cm plate with a Desaga Autospotter as spots of diameter about 2.5 mm. In addition, a reference mixture containing I, II and III was applied on each side of the plate. Ascending chromatography was then performed with dichloroethane—ethyl acetate—ethanol—acetic acid—water (15:26:12:8:7.5); the development time was about 45 min for a height of 70 mm. This system ensures the separation of citalopram (I) (R_F = 0.43) and its monodemethylated (II) (R_F = 0.51) and didemethylated (III) (R_F = 0.58) metabolites from each other and from endogenous impurities. The plates were dried in an oven at 80°C for 10 min. The references on the margins were made visible by spraying with Dragendorff reagent (No. 87 [9] after covering the sample part of the plate.

Florescence scanning

The intrinsic fluorescence of citalopram (I) and its metabolites (II and III) was then quantitated using a Perkin-Elmer MPF-3L fluorescence spectrophotometer, equipped with a TLC scanning device. The excitation and emission wavelengths were 240 and 295 nm, respectively. Slits of 12 nm were used with a sensitivity setting of 1; the background was corrected for by zero suppression. Sensitivity and zero suppression settings were chosen so as to provide a stable baseline (Fig. 2). The back face of the plate was wiped off with water and ethanol prior to scanning, which was performed along the path with a 3 × 6 mm slit at a scanning speed of 50 mm/min after optimization of the fluorescence of the spot.

Calculation

Amounts of drug and metabolites were calculated from calibration graphs generated by linear regression analysis of the peak height versus amount of standard (normally 0, 25, 100 and 250 ng of each compound, added to drug-free plasma prior to extraction and analysed on each plate). Two plates were normally run simultaneously and a common calibration graph was constructed.

RESULTS AND DISCUSSION

Calibration graphs

The calibration graphs obtained (Fig. 2) showed that the fluorescence emitted was linear with concentration of drug or metabolite in the range 10—300 ng and was identical for the three compounds. Fig. 2 shows regres-

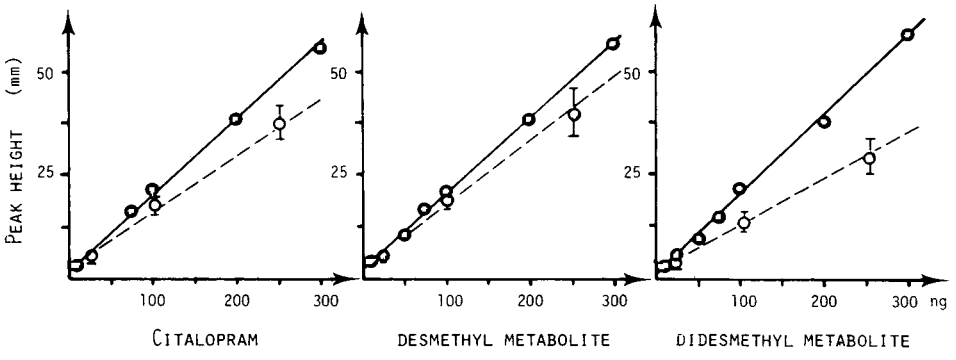


Fig. 2. Fluorescence of citalopram and its metabolites after application directly on HPTLC plates (•, single values) and after addition to drug-free plasma, extraction and TLC (○, mean ± S.D. from a series of 11 replicate experiments).

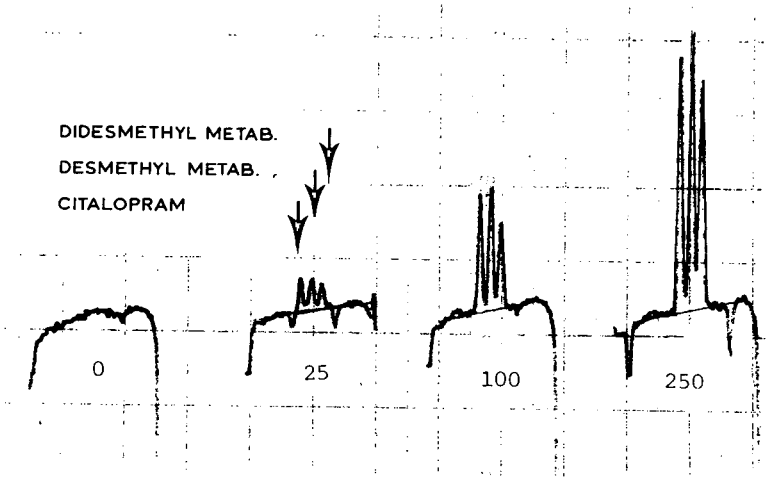


Fig. 3. Recorder trace from HPTLC scanning of fluorescence of citalopram and its mono- and didemethylated metabolites added to drug-free plasma in amounts of 0, 25, 100 and 250 ng.

sion lines for directly chromatographed standards and also for standards added to control (drug-free) plasma and assayed after extraction and chromatography. Typical chromatograms of authentic standards added to blank plasma are shown in Fig. 3. The correlation coefficients for the calibration lines were generally 0.99. With few exceptions they were reproducible from day to day or plate to plate as indicated (Fig. 2) by the variation of the average values from eleven replicate assays. The relative standard deviations were 10—15% for citalopram and 10—20% for the metabolites.

The recovery of citalopram and its demethylated metabolite (II) was 80—90%. The recovery of the more polar didesmethyl metabolite (III) was about 70%. Extraction of the more polar citalopram N-oxide (IV), identified in animals and man as a minor metabolite, was negligible.

Sensitivity and precision

The limit of detection, corresponding to peak heights of about 3 mm, was 10 ng. In 2-ml samples this corresponds to a concentration limit of about 5 ng/ml or 10—15 nmol/l.

The intra-assay variation as calculated from a series of duplicate determinations [10] of standards was 6—8% (standard deviation as a percentage of the mean) for 100- and 250-ng amounts of the three compounds, and higher (about 15%) at the 25-ng level. The duplicates were spotted on different plates, but otherwise processed simultaneously as is the routine procedure for standards and unknowns. From the calibration graph it can be calculated that a 6—8% variation in fluorescence corresponds to a 7—9% variation in concentration. This result agrees well with that from 25 identical samples (1.0 ml) containing 100 ng of citalopram (I), assayed frequently during several months, viz., 103 ± 12 ng/ml (mean ± S.D.).

Selectivity

The chromatographic separation of drug and extractable metabolites ensures complete selectivity in this respect. As regards potential interference from other drugs, only a few (hitherto encountered in clinical use) have been tested so far. Diazepam, nitrazepam, oxazepam, estazolam were all excluded on the plate. Dextropropoxyphene had an R_F value close to that of the didemethylated metabolite but showed no fluorescence at the given wavelength. In general one would expect little interference because of the TLC step and the specificity of fluorescence.

Advantages over previous technique

A comparison between the new and the previous technique was made by the assay of sixteen samples from a dog study at a sub-lethal dosage. The citalopram concentrations ranged from 1000 to 9000 nmol/l and the samples were diluted accordingly prior to assay. A single determination was performed with the previous technique and duplicates with the new scanning method. The correlation coefficient between the two methods was 0.93 and the regression line had a slope of 0.95 and an almost zero intercept (Fig. 4). The two methods were thus considered to produce identical citalopram data.

The advantages of the new assay are four-fold: greater simplicity, greater

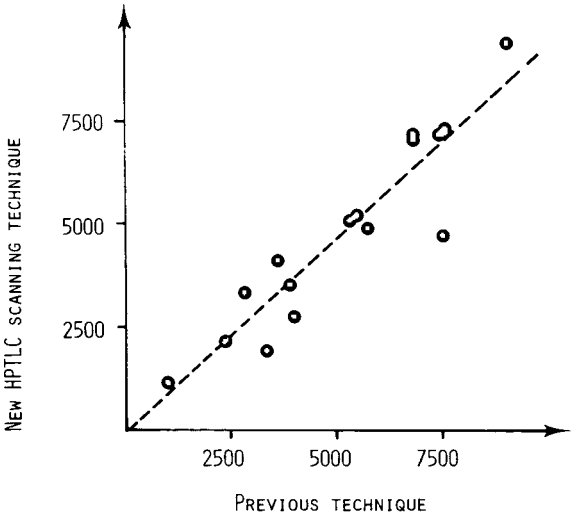


Fig. 4. Data from identical samples assayed by the coupling method (abscissa) and the new HPTLC method (ordinate). Concentrations in nmol/l.

accuracy, comprehensive metabolite assay and increased sensitivity. The method is considerably less time- and effort-consuming than the previous method, and 40 samples (including 8 standards) can be assayed by one assistant per day. Whereas with the coupling method the determination of the demethylated metabolite was less accurate and sensitive than that of citalopram, the scanning technique enables virtually the same accuracy and sensitivity for the drug and metabolites, including the didemethylated metabolite, which could not be determined by the previous technique. The limit of detection by the previous technique was stated [6] to be 10 and 25 ng/ml for citalopram and the demethylated metabolite, respectively. In comparison, the new scanning method is capable of detecting about 5 ng/ml of the drug and its metabolites. The improvement is thus limited for citalopram, but considerable for the metabolites. The sensitivity is satisfactory for clinical plasma levels and also for pharmacokinetic studies.

ACKNOWLEDGEMENTS

The skilful technical assistance of Susanne Hjerpsted Jønsson is gratefully acknowledged. Thanks are also due to Ruth Sparre Andersson for preparation of the manuscript.

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Journal of Chromatography, 224 (1981) 532—538
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 888

Note

Simple high-performance thin-layer chromatography method for the determination of disopyramide and its mono-N-dealkylated metabolite in serum

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(First received December 23rd, 1980; revised manuscript received February 19th, 1981)

Disopyramide is an antiarrhythmic agent used in the treatment of ventricular and supraventricular arrhythmias, whose plasma therapeutic range is 1.5—8 μg/ml [1]. Of an administered dose, 46—60% is excreted unchanged, predominantly in urine. A further 15—20% is metabolized to mono-N-dealkylated disopyramide (MND), which then also undergoes renal elimination [2]. After a single dose of disopyramide (3 mg/kg), serum concentrations of MND are usually very low (< 0.4 μg/ml) [3], but after long-term disopyramide therapy, MND concentrations amount to approximately 1 μg/ml [4]. A large increase in the ratio of MND to disopyramide concentrations has been observed in chronic renal insufficiency or in the case of simultaneous therapy with phenytoin, known as an enzyme inducer [4].

In animal experiments, MND is only slightly less active than the parent compound against supraventricular arrhythmia but is inactive against ventricular arrhythmia [5]. In addition, MND might exert a positive inotropic effect on isolated cardiac muscle [6].

Several methods for the determination of disopyramide in serum, plasma or urine have been described, which are based mainly on gas—liquid chromatography (GLC) [1, 3, 7—12] or on high-performance liquid chromatography (HPLC) [13—16]. In GLC, quantitation of MND requires derivatization of the molecule, because it usually breaks down and is eluted as three poorly separated peaks under GLC conditions suitable for disopyramide [1—3]. Simultaneous GLC determination of disopyramide and its metabolite requires a complex and tedious sample preparation; therefore, most GLC methods are not suited to measure MND concentrations [7—12]. HPLC quantitation does not require any derivatization of MND, so that sample preparation is simpler and more rapid than for GLC [13—16]. Usually, GLC and HPLC techniques require

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a relatively large serum sample (500–1000 μl). Fluorimetric assay of disopyramide is more rapid and the equipment simpler than that of GLC or HPLC, but it does not distinguish between the parent drug and the metabolite [17, 18]. Conventional thin-layer chromatography (TLC) has also been used for the determination of disopyramide and MND, but scraping of the plates and other steps make the method tedious and unsuitable for routine analysis [18]. Recently, a conventional TLC method using in situ quantitation of disopyramide and MND by fluorescence spectrophotometry has been described [19].

The method of high-performance thin-layer chromatography (HPTLC) described in this paper allows a simple, rapid, reliable and relatively inexpensive assay of disopyramide and its mono-N-dealkylated metabolite in serum, from a small sample (100 μl).

EXPERIMENTAL

Materials

Disopyramide, as disopyramide phosphate (Norpace® or SC 13957), mono-N-dealkylated disopyramide (SC 24566) and *p*-chlorodisopyramide (SC 13068) were supplied as pure compounds by Searle (Lausanne, Switzerland).

All reagents were of analytical grade and purchased from Merck (Darmstadt, G.F.R.).

All the glassware was washed with hot dichromate–sulfuric acid solution prior to use.

Equipment

Precoated HPTLC plates silica gel 60 F_{254}, 10 × 10 cm were obtained from Merck.

Linomat III, an automatic sample applicator for HPTLC plates, type 27804, HPTLC linear-developing chamber, type 28510, TLC/HPTLC Scanner, standard version, type 76500 were all from Camag (Muttenz, Switzerland).

A potentiometric recorder was connected to the 100-mV and the remote control scanner outputs.

Sample preparation

Extraction. Serum (100 μl) was placed into a 10-ml conical glass tube. Then, 100 μl of the internal standard solution (6 μg/ml of *p*-chlorodisopyramide in 0.1 *M* hydrochloric acid), 200 μl of saturated aqueous sodium carbonate solution and 1000 μl of chloroform were added. The tube was shaken vigorously for 15 sec, centrifuged for 15 min at 2000 *g*, then the organic layer was transferred into a second conical tube and centrifugation repeated. The organic phases were collected and evaporated to dryness under a stream of nitrogen.

Sample application. The dry extract was dissolved in 50 μl of chloroform and 30 μl of this solution were applied to the HPTLC plate by the Linomat as narrow, 5 mm long bands. The selected application speed was 0.25 μl/sec and the plate table speed 15 mm/sec. Usually, eighteen samples were applied to opposite sides of one plate by successive 5-mm displacement of the plate table.

Chromatography

The HPTLC plate was placed in the developing chamber, the silica layer being directed upwards; 2 ml of mobile phase (a 2% solution of concentrated ammonia solution in ethanol) were placed in each lane and development started immediately. The developing chamber was protected by a plastic hood (part of the equipment). After 25 min, both solvent fronts met and the plate was removed from the chamber and dried under a gentle stream of compressed air.

Quantitation

The HPTLC plates were inserted into the scanner for the UV absorption measurements of the bands in each track (λ = 254 nm, micro slit 3 mm). The scanning speed of the plate tracks was 0.5 mm/sec. Serum levels of disopyramide and MND were deduced from the peak height ratio to internal standard.

Calibration

Calibration graphs were obtained by adding known amounts of disopyramide and MND to blank human serum samples, which were then handled the same as patient sera. The regression lines were computed by the G02CAF program from the FORTRAN NAGLIB computer program library [20].

Reproducibility

The intra-assay reproducibility in serum was determined at three concentrations: 1.0, 4.0 and 8.0 μg/ml for disopyramide and 0.43, 1.7 and 3.4 μg/ml for MND, respectively. Eight different samples were prepared at each concentration.

Interferences

The R_F values of two typical basic compounds susceptible to interfere with the drug assay, i.e. amitriptyline and imipramine, were compared with those of disopyramide, MND and internal standard under the same extraction and chromatographic conditions.

Patients sera

Determination of serum concentrations of disopyramide and MND was performed in three patients on various preparations and dosages of disopyramide.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, MND, disopyramide and the internal standard gave symmetrical, well defined peaks, the R_F values of which were 0.26, 0.46 and 0.52, respectively (Fig. 1A).

The calibration graphs were linear in the range of 0.5—10.0 μg/ml for disopyramide and 0.25—5.0 μg/ml for MND (Fig. 2). Equations of the computed regression lines were $Y = -0.005 + 0.173\ X$ and $Y = -0.011 + 0.084\ X$ for disopyramide and MND, respectively. The correlation coefficients were 1.000 and 0.998, respectively.

In preliminary assays, the silica layer was facing downwards and a counterplate was used so as to realize the conventional sandwich configuration of the

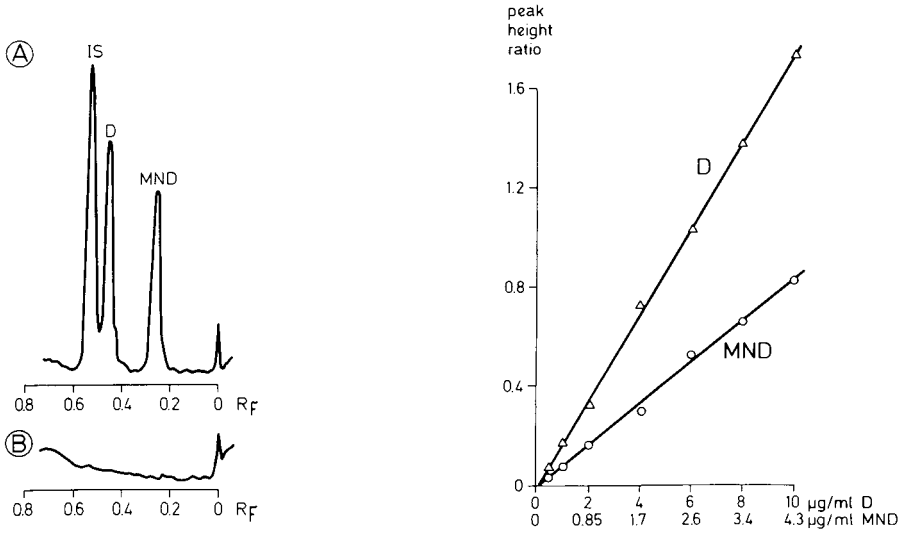

Fig. 1. (A) Typical HPTLC chromatogram from a serum sample containing mono-N-dealkylated disopyramide (MND) 1.7 µg/ml, disopyramide (D) 4.0 µg/ml and *p*-Cl-disopyramide, internal standard (IS) 6.0 µg/ml. (B) Blank serum (same extraction and chromatographic conditions as in A).

Fig. 2. Calibration graph for the determination of disopyramide (D) and its mono-N-dealkylated metabolite (MND) in serum. The peak height ratios of D or MND to internal standard are plotted against the serum concentrations of D or MND, respectively.

developing chamber. The development of the HPTLC plates yielded poor results, due to an incurvation of the chromatographic bands. The scanning of such bands revealed peak broadening and overlapping. Experimental conditions were modified in such a manner as to obtain straight bands perpendicular to the mobile phase flow. These experimental conditions were realized by changing the conventional operation of the developing chamber, that is by developing the HPTLC plate with the silica layer directed upwards. Under these conditions only, the peaks were clear-cut, reproducible and without overlapping.

From the data shown in Table I it may be seen that intra-assay coefficients of variation were in the range 2.4—7.8% for disopyramide and 7.8—17.7% for MND; this reproducibility is comparable with that of the GLC [1, 3, 7—12], HPLC [13—16] and TLC [19] methods. The coefficient of variation for MND is relatively large at the low serum concentrations of the metabolite. At high serum concentrations of MND, a situation that has been observed in some patients [4] and may be of clinical significance, the coefficient of variation is reasonably good.

No serum components, even if extracted simultaneously with disopyramide, interfered with the assay (Fig. 1B). The R_F values of amitriptyline and imipramine, two basic drugs that were co-extracted with disopyramide, were close to unity.

In the three patients whose relevant clinical data are listed in Table II, significant levels of disopyramide were measured in each case. The relatively low

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION OF HPTLC DETERMINATION OF DISOPYRAMIDE AND ITS MONO-N-DEALKYLATED METABOLITE (MND) IN SERUM

Eight samples were prepared at each concentration.

Compound	Serum concentration (μg/ml)		Coefficient of variation (%)
	Expected	Assayed	
Disopyramide	1.0	0.86	7.8
	4.0	4.2	2.4
	8.0	7.9	4.0
MND	0.43	0.33	17.7
	1.7	1.9	13.1
	3.4	3.4	7.8

TABLE II

CLINICAL CHARACTERISTICS AND SERUM CONCENTRATION OF CREATININE, DISOPYRAMIDE AND ITS MAIN METABOLITE IN PATIENTS ON THERAPY

	Subject		
	U.P.	L.B.	O.M.
Age (years)	48	73	76
Sex	M	M	F
Weight (kg)	70	73	51
Indication for therapy	VPB* after myocardial infarction	Sick sinus syndrome	Paroxysmal atrial tachycardia
Preparation and dosage**	Norpace® 150 mg twice each day	Rythmodan® 100 mg/day	Norpace® 100 mg three times each day
Duration of therapy	9 days	1 week	2.5 weeks
Concomitant diseases	Coronary heart disease Hyperthyroidism	Hypertension	Cerebral thrombosis
Concurrent medications	Isosorbide dinitrate Oxprenolol Bromazepam	Digoxin Debrisoquine Tienilic acid Cloxacillin Flunitrazepam	Digoxin Oxazepam
Serum creatinine (μmol/l)	94	147	71
Creatinine clearance (ml/min)		61	
Disopyramide serum concentration (μg/ml)	1.3	2.2	1.7
MND metabolite serum concentration (μg/ml)	—	—	0.5

*VPB = ventricular premature beats.
**Norpace® = disopyramide phosphate; Rythmodan® = disopyramide.

values obtained are consistent with the low disopyramide dosage they were receiving [1, 21]. A detectable concentration of MND was found in only one case, an elderly woman with the longest duration of therapy and with normal renal function.

In view of these data, the HPTLC method described here is suitable for routine analyses of disopyramide and MND. In comparison with a conventional TLC method such as that developed by Gupta et al. [19], it differs by the following features.

(1) The use of high-performance layers in conjunction with the band application technique contributes to achieve a high resolution and sensitivity and makes a UV spectrophotometric determination possible even with very small samples. A first advantage associated with UV spectrophotometry is that it does not require the step of dipping the plate into sulfuric acid and waiting for the appearance of fluorescence. The UV response remains constant in the course of time. In these conditions, the plates may be scanned again with the same accuracy. Moreover, there is a second advantage, in the sense that there is no difficulty in using an internal standard, such as *p*-chlorodisopyramide, that has an intense fluorescence, much greater than that of disopyramide or MND. The conditions described offer a good separation of *p*-chlorodisopyramide from disopyramide and MND.

(2) The use of chloroform instead of benzene allows a rapid application by bands, under safer conditions and without heating.

(3) Finally, the use of plates with fluorescent indicator contributes to easily optimizing the positioning of the plate in the scanner and to ensure constant chromatographic conditions from plate to plate.

CONCLUSION

The method described in this paper may prove suitable for the routine serum level determination of disopyramide and its mono-N-dealkylated metabolite, firstly, because it is rapid, simple, reliable and relatively inexpensive, and secondly because the serum sample is very small (100 μl). This assay requires only a single serum extraction and eighteen samples may be applied semi-automatically on the same HPTLC plate. Another advantage is the use of a new chromatographic support after every 18 samples, avoiding those problems related to column ageing and clogging encountered with the other methods. The low plate cost per sample analysed may rapidly compensate for the initial investment in the HPTLC scanner.

The possibility of simultaneously determining the concentration of disopyramide and its mono-N-dealkylated metabolite makes this method especially useful for monitoring disopyramide therapy in patients with impaired renal function. In addition, it may also prove to be a useful tool for the detection of those other clinical situations where there is a high concentration of metabolite, such as in hepatic microsomal enzyme induction.

ACKNOWLEDGEMENTS

We thank Mrs. F. Piotton for excellent technical assistance and Mrs. G. Tissot

for secretarial help. We are also grateful to Dr. M. Mühlemann, Searle, Switzerland, for his gift of reference compounds and to the Laboratoire cantonal de chimie, Geneva, for use of the HPTLC scanner.

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETING

BIOCHEMISCHE ANALYTIK 82

The International Conference on Biochemical and Instrumental Analysis "Biochemische Analytik 82" will be held from April 27 to 30, 1982, at the Exhibition Center, Munich, G.F.R. The Scientific Committee has selected the following topics for the conference: Trace analysis of organic compounds; Analysis of biological macromolecules (proteins, lipoproteins, glycoproteins, proteoglycans, nucleic acids and chromatin structure); Receptor analysis; Non-invasive methods in biochemical analysis of organ function; Pathobiochemistry of white blood cells; Monoclonal antibodies as analytical tools; Recent advances in enzymology (enzymes, substrates, inhibitors); Identification of drugs in foodstuffs; Perinatal diagnosis from the view of molecular biology; Pharmacokinetics and drug monitoring; Toxicological analysis; Application of stable isotopes in biochemical analysis; Nuclear magnetic resonance spectrometry; Thin-layer chromatography; High-pressure liquid chromatography; Fluorimetry; Bioluminescence; Nephelometry; Progress in immunoassays; Isotachophoresis.

All topics are eligible for poster presentations, while posters on other topics may also be submitted. In addition, there is a possibility of presenting, in combination with posters, new analytical apparatus developed in scientific institutions.

During the conference, practical courses on instrumental TLC, fluorimetry and bioluminescence, nephelometry, and isotachophoresis will be offered. The conference languages will be German and English with simultaneous translation from German into English.

In connection with the conference, the 2nd FECHEM Conference on Computer-Based Analytical Chemistry (COBAC) will be organized by the FECS Working Party on Analytical Chemistry in cooperation with the Analytical Division of the Gesellschaft Deutscher Chemiker. This conference will take place on April 28–29, 1982.

The preliminary programme of Biochemische Analytik 82 will be dispatched in August, 1981, with a request to submit posters (registration before November 11, 1981) and to register for practical courses (before February 2, 1982). Final programmes with registration forms and forms for hotel reservations will be dispatched in January, 1982. The conference fee will be DM 90.00 (DM 120.00 after March 1, 1982) for the entire conference, while day tickets will be available for DM 30.00 and DM 10.00 (students). Fees for practical courses will be DM 50.00 per day (plus the fee for the entire conference).

Further information can be obtained from the Secretary General, Dr. Rosemarie Vogel, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik der Universität München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel. (089) 15 14 19.

NEW BOOKS

Progress in pesticide biochemistry, Vol. 1, edited by D.H. Hutson and T.R. Roberts, Wiley, Chichester, New York, Brisbane, Toronto, 1981, XI + 346 pp., price £ 24.00, ISBN 0-471-27920-X.

New trends in antibiotics: Research and therapy, edited by G. Gialdroni Grassi and L.J. Sabath, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1981, X + 354 pp., price Dfl. 158.00, US$ 77.00, ISBN 0-444-80326-2.

MANUFACTURERS' LITERATURE

N-1574

ARC LAMPS CATALOGUE

In a 28-page brochure the Schoeffel Instrument Division of Kratos describes the company's line of arc lamps, housing, power supplies and related accessories. The catalogue gives information on the Schoeffel lamps for use in spectrophotometry, fluorescence spectrometry, UV exposure testing, solar simulation, reflectance studies, photochemistry and high intensity illumination.

N-1575

SEPARATION NEWS, 1 (1981) and PERISTALTIC PUMP P-1

Pharmacia's periodical, Separation News, has reports on the covalent chromatography of proteins, peptides, nucleic acids and on the plaque assay using protein A (*S. aureus*). The new peristaltic pump Model P-1 has an adjustable flow-rate between 0.6 and 500 ml/h and can be operated in ambient temperatures from 0–40°C. The pump is supplied with a supporting rod, 6 tubing connectors, silicon tubing (1.0, 2.1 and 3.1 mm I.D.), a flow-rate guide sticker, a 3-mm allen key and one O-ring.

N-1576

HAMILTON CATALOGUE

The new Hamilton catalogue H-81 gives full information on the products of the company in the field of handling small volumes. New products are the microliter syringe model 7000.5 with a capacity of 0.5 µl and the special 2-l super syringe. For the newly designed HV and HVP valves many re-designed fittings, connections and adapters are available.

N-1577

PEAK, Vol. 3, No. 2

This issue of Hewlett-Packard's Peak has a complete description of the B-version of the Model HP 1081 liquid chromatograph, including the possibilities of the instrument in combination with the various types of data processing equipment, including the HP-85 personal computer. Furthermore, Peak has short reports on single column compensation, parallel detection, preparative HPLC with the Model 1084B, the HP 8450 A UV–VIS spectrophotometer, winning the IR-100 Award, the analysis of fatty acid derivatives by HPLC and information on Hewlett-Packard courses in BASIC.

N-1581

BROCHURE ON LC/MS INTERFACE

A six-page brochure describes the Finnigan LC/MS interface for the analysis of sensitive organic compounds such as steroids, pesticides and drug metabolites with a combination of an HPLC instrument and a mass spectrometer. The Finnigan LC/MS interface can be installed in the field on any Finnigan LC/MS equipped with differential pumping and can be ordered with the purchase of a new Finnigan chromatography–MS system. Operation in either EI or CI modes is standard.

N-1583

NEW PRODUCTS AND PRICE LIST

A 16-page booklet forms a supplement to the Pharmacia Fine Chemicals 1980 catalogue. This supplement contains information on their recently introduced instruments and components for use in chromatography, electrophoresis and cell biology. The supplement also has additional information concerning their publications on analysis and research in these fields. Another 16-page booklet from the same manufacturer has complete information on prices of the instruments, components and chemicals in their sales program. Prices are given in US dollars.

N-1582

NACHTRAG ZUM KATALOG 1980

This 48-page booklet is a supplement to the 1980 general catalogue of the West German manufacturer of biochemicals, Serva Feinbiochemica GmbH & Co. The catalogue has short essential information on each product; the prices are given in DM, and the language is German.

N-1584

VARIAN INSTRUMENT APPLICATIONS, Vol. 14, No. 2, 1980

This issue of Varian's customer magazine, VIA, outlines a number of application reports from various fields of analytical research. One article is devoted to the use of high-performance liquid chromatography for the separation of proteins and peptides. Another deals with spectrophotometric measurements of protease activity with a *p*-nitroanilide peptide substrate. VIA also contains a report on the GC analysis of trihalomethanes in potable water with liquid–liquid extraction. Comprehensive reports describe Varian's new VISTA series of chromatography instruments and new equipment for NMR- and EPR-analysis.

N-1586

HPLC ANALYSIS REPORT

A 3-page lab report recently published by the Schoeffel Instrument Division of Kratos Inc. discusses the separation and detection of nucleosides and bases via HPLC. The determination of these substances in physiological fluids and in tissue extracts can be useful in the diagnosis of certain types of cancer and other biochemical disorders. The report presents detailed information on the necessary chromatographic procedures. Particular attention is paid to the effects of changes in mobile phase composition. Sufficient information is presented so that the reader can duplicate the separation with little or no alteration in separation conditions.

N-1587

BROCHURE ON AMINO ACID ANALYZER

A brochure describing the Model 3A29 Automatic Aminoacid Analyzer is available from the manufacturer, Carlo Erba Strumentazione. The 3A29 is a benchtop instrument that performs protein hydrolyzates in *ca.* 1 h; physiological fluids may be analyzed in 160 min. The detection limit of 100 picomoles is said to be easily achieved routinely. The instrument has a built-in sampler.

N-1589

LC APPLICATION NOTE, LC 117

Varian has published an application note entitled, "HPLC analysis of amino acids by post-column reaction and fluorescence detection". Note LC-117 acknowledges the difficulty of detecting amino acids and describes how they may be derivatized to increase detectibility. The Model 5000 liquid chromatograph is used to accomplish both a pre-column and a post-column derivatization.

N-1590

LC APPLICATION NOTE, LC 107

Varian has published an application note entitled, "Rapid estimation of polymer molecular weight using high-performance steric exclusion chromatography". This technique, gel permeation chromatography, has become a widely used method of characterizing polymers for molecular weight distribution. With this method, molecules are separated according to their effective size in solution. Separation results from the selective diffusion of sample molecules into and out of the mobile phase-filled pores in the column packing. The column used is Micropak TSK Gel, type GMH6.

N-1592

CHROMPACK NEWS, No. 34 (1981)

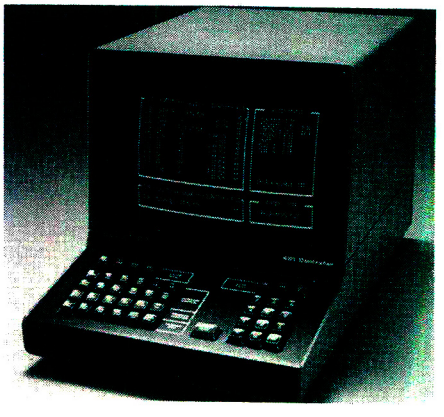
As usual Chrompack News has actual information on methods of analysis carried out with the company's columns and/or chemicals. No. 34 has reports on the analysis of light hydrocarbons, fatty acid methyl esters on packed columns, the use of squalane-coated stainless-steel capillary columns, and the on-column injection on 0.32-mm capillary columns. No. 34 also has information on new products such as a shrinkable teflon connection material for fused silica connection and a new gasatlas, a booklet containing condensed information on gas analysis.

NEW PRODUCTS

N-1573

NEW ISOELECTRIC FOCUSING SYSTEM

A new system of thin-layer agarose isoelectric focusing developed around a new form of electroendosmosis-free agarose has been developed by the Marine Colloids Division of FMC. Until now the agarose media were difficult to handle. With the introduction of Marine Colloids' IsoGel^{tm} the isoelectric focusing in agarose gels is made more convenient and useful for analysis. The IsoGel system consists of IsoGel agarose, GelBond support film, IsoGel Ampholite (pH 3.5–9.5) and the IsoGel Accessory Kit. The system is described in a brochure available from the manufacturer.



N-1578

EXPANDED SOFTWARE CAPABILITIES

Beckman Instruments has added a number of software capabilities to its Model 421 CRT controller for the Model 324 abd 334 HPLC systems. The new features include prompting of users to guide them through an analysis or operation of an entire program, simultaneous display of chromatographic functions and results of diagnostic testing on the CRT. Model 421 provides storage of up to 100 different programs and offers both file linking and file looping.

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

N-1571

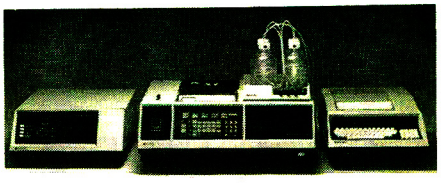
UV–VIS MONITOR

Gilson's Model HM Holochrome is a UV–VIS spectrophotometer which allows quantitative monitoring of column effluents in chromatography. The light source in the Holochrome is a deuterium source, and the monochromator is equipped with a holographic grating. The wavelength is adjustable from 190–600 nm. The Holochrome is supplied with two flow-cell assemblies, two air-reference aperture blocks, a recorder cable, an event marker cable and an accessory packet containing assorted fittings for connection to plastic or stainless-steel tubing. The spectrophotometric detector is available with flow cells for HPLC or low pressure LC applications.

N-1593

NEW ANTI-SERA

Shandon Southern Products have added a new range of anti-sera to their range of biochemistry instruments and consumables. These anti-sera are designed for immunological work and will provide a complement to their immunoelectrophoresis system and cellulose acetate membranes. The concentrate of human serum proteins which makes up the new anti-sera is stabilised to remove any unstable elements. The concentrate has also been carefully determined by use of nephelometric techniques in relation to reference proteins. The anti-sera contains immunogens to the following serum components: whole human serum, haptoglobin, C3-complement alpha 2 macroglobulin, albumin, transferrin and immunoglobulin A, G or M. The anti-sera are sold in 5-ml bottles.



N-1579

LIQUID CHROMATOGRAPH

The Autolab Division of Spectra-Physics has introduced the SP 8100 liquid chromatograph. The SP 8100 is a highly automated, modular built system and is an instrument easily used for both repetitive routine analyses and methods development. The new instrument includes the SP 8700 ternary solvent delivery system. The basic unit includes an oven, a heated autoinjector and pump module. The software of the system enables automation of all modules through 10 pre-programmable parameter files. Integral options include a fixed wavelength detector and an 80-sample intelligent autosampler featuring a bar coded label reader which not only transmits sample information to the data system, but can also call up any instrument parameters to analyse a particular sample.

ELECTROPHORESIS

A SURVEY OF TECHNIQUES AND APPLICATIONS

Part A: Techniques

Z. DEYL, Czechoslovak Academy of Sciences, Prague (editor)
F. M. EVERAERTS, Z. PRUSÍK, and P. J. SVENDSEN (co-editors)

JOURNAL OF CHROMATOGRAPHY LIBRARY 18

This first volume in a two part set, deals with the principles, theory and instrumentation of modern electromigration methods. The second volume will be concerned with details of applications of electromigration methods to diverse categories of compounds, although a few applications are already discussed in Part A.

Some electromigration methods have become standard procedures because of their extensive use in analytical and preparative separations. These are discussed together with newer developments in the field. Hints are included to help the reader to overcome difficulties frequently arising from the lack of suitable equipment. Adequate theoretical background of the individual techniques is included. A theoretical approach to the deteriorative processes is presented in order to facilitate further development of a particular technique and its application to a special problem.

In each chapter practical realizations of different techniques are discussed and examples are presented to demonstrate the limits of each method. The mathematical and physicochemical background is arranged so as to make it as coherent as possible for both non-professionals such as post-graduate students, and experts using electromigration techniques.

CONTENTS: Preface. Foreword. Introduction. Chapters: 1. Theory of electromigration processes. 2. Classification of electromigration methods. 3. Evaluation of the results of electrophoretic separation. 4. Molecular size and shape in electrophoresis. 5. Zone electrophoresis (except gel-type techniques and immunoelectrophoresis). 6. Gel-type techniques. 7. Quantitative immunoelectrophoresis. 8. Moving boundary electrophoresis in narrow-bore tubes. 9. Isoelectric focusing. 10. Analytical isotachophoresis. 11. Continuous flow-through electrophoresis. 12. Continuous flow deviation electrophoresis. 13. Preparative electrophoresis in gel-media. 14. Preparative electrophoresis in columns. 15. Preparative isoelectric focusing. 16. Preparative isotachophoresis. 17. Preparative isotachophoresis on the micro scale. List of frequently occurring symbols. Subject index.

1979 xvi + 492 pages US $83.00/Dfl. 170.00 ISBN: 0-444-41721-4

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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	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	The publication schedule for further issues will be published later.				
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2					

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. 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.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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

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

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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