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This year the *Journal of Chromatography* extends to 22 volumes, each of some 550 printed pages, and it seems as if the number of papers submitted is still increasing.

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GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC ANALYSIS OF BILE ACIDS AS TRIFLUOROACETYL-HEXAFLUOROISOPROPYL AND HEPTAFLUOROBUTYRYL DERIVATIVES

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(First received April 3rd, 1980; revised manuscript received August 4th, 1980)

SUMMARY

The gas chromatographic retention times on QF-1 of 38 bile acids in the form of their trifluoroacetyl-hexafluoroisopropyl (TFA-HFIP), trifluoroacetyl-methyl, and heptafluorobutyryl derivatives are given. In general hexafluoroisopropyl ester trifluoroacetates proved superior with regard to simplicity of preparation, absence of artifacts, and resolution on QF-1. The main disadvantages of heptafluorobutyrates were the production of artifacts with some ketonic bile acids and the impossibility of separating any of the dihydroxy bile acids with substituents in the 3,6- and 3,7-positions. Mass spectra of TFA-HFIP derivatives were recorded with both direct and gas chromatographic inlet systems. The spectra of these derivatives are easily comparable with those of methyl ester trifluoroacetates and they enable the identification of positional isomers.

INTRODUCTION

For gas chromatographic (GC) analysis bile acids are usually converted into methyl esters, methyl ester trimethylsilyl (TMS) or trifluoroacetyl (TFA) derivatives [1—6]. Among other derivatives only methyl ester acetates have gained any importance, because of their superior stability [7, 8]. Recently, however, the GC analysis of bile acids as hexafluoroisopropyl (HFIP) ester trifluoroacetyl derivatives [9] and most recently as heptafluorobutyrates (HFB) [10] has been proposed, mainly on the grounds of superior derivatization techniques and increased sensitivity by electron-capture detection. We checked the suitability of these new procedures for routine assays, and also the resolution on QF-1, which is the most promising liquid phase [5] for separation of these compounds. Furthermore we used TFA-HFIP derivatives of bile acids for combined gas chromatography—mass spectrometry (GC—MS) and compared their fragmentation patterns with those of methyl ester trifluoroacetates [11].

EXPERIMENTAL

Materials

5β-Cholanoic acid, lithocholic acid (3α-hydroxy-5β-cholanoic acid), hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid), and 3α,7α-dihydroxy-12-keto-5β-cholanoic acid were obtained from Roth (Karlsruhe, G.F.R.), and deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid), cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), dehydrocholic acid (3,7,12-triketo-5β-cholanoic acid), and cholesterol from Merck (Darmstadt, G.F.R.). Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid) was purchased from Shuchardt (Munich, G.F.R.), while chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid), 7α,12α-, and 3-keto-7α,12α-dihydroxy-5β-cholanoic acids were supplied by Calbiochem (Giessen, G.F.R.). Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid) was from Serva (Heidelberg, G.F.R.), 3β-hydroxy-5α-, 3β-hydroxy-3-keto-, 3α,6β-dihydroxy-, 3α-hydroxy-6-keto-, 3α-hydroxy-7-keto-, 3α-hydroxy-12-keto-, 3,6-diketo-, 3,7-diketo-, 3,12-diketo-, 7,12-diketo-, 3α,12α-dihydroxy-7-keto-5β-cholanoic acids and 3β-hydroxy-Δ^5-cholenoic acid, and 3α-hydroxy-12-keto-$\Delta^{9(11)}$-5β-cholenoic acid were obtained from Steraloids (Pawling, NY, U.S.A.) and 3α-hydroxy-6-keto-5α- and 3,6-diketo-5α-cholanoic acids from Makor (Jerusalem, Israel). 3α-Hydroxy-7,12-diketo-5β-cholanoic acid and apocholic acid (3α,12α-dihydroxy-$\Delta^{8(14)}$-5β-cholenoic acid) were available in the laboratory from previous studies. 3β,7α-Dihydroxy- and 3,7-diketo-12α-hydroxy-5β-cholanoyl methyl esters were generous gifts from Dr. J. Sjövall (Stockholm, Sweden), 3β,12α-dihydroxy-, 3-keto-7β-hydroxy-, and 3-keto-12α-hydroxy-5β-cholanoyl methyl esters were from Dr. P. Szczepanik (Argonne, IL, U.S.A.), and 3-keto-7α-hydroxy-5β- and 3α,7α,12α-trihydroxy-5α-cholanoyl methyl esters from Dr. W.H. Elliott (St. Louis, MO, U.S.A.). Trifluoroacetic anhydride and hexafluoroisopropanol were supplied from Merck—Shuchardt (Munich, G.F.R.), QF-1 phase was from Serva.

Gas chromatography and gas chromatography—mass spectrometry

A Varian Model 1700 gas chromatograph equipped with dual flame-ionisation detectors and silanized glass columns (1.8 m × 3 mm I.D.) packed with 3% QF-1 on Chromosorb W AW DMCS (100—200 mesh) were used for all determinations. The operating conditions were: injection temperature 240°C, column temperature 230°C, detector temperature 300°C, nitrogen flow-rate 30 ml/min. For GC—MS analysis an instrument combination, consisting of a Varian Model 1700 gas chromatograph with a two-stage helium separator by Biemann-Watson, a Varian MAT mass spectrometer, Model CH 7A, and a Varian Spectro System 100 data system were used. Helium was substituted for nitrogen. The operating conditions were: separator temperature 220°C, accelerating voltage 3 kV, electron energy 70 eV, trap current 60 μA, and the ion-source temperature 180°C. Mass spectra of bile acid TFA-HFIP derivatives were obtained mostly with both direct and gas chromatographic inlet systems.

Derivatization procedures

Bile acid TFA-HFIP derivatives were prepared by dissolving each bile acid in 300 μl of a mixture of 200 μl of trifluoroacetic anhydride and 100 μl of hexafluoroisopropanol [9]. The mixture was incubated at 37°C for 30 min, evaporated under reduced pressure at room temperature and the dry residue dissolved in about 200 μl of acetonitrile. Aliquots of this solution were subjected to GC or GC—MS. Substances available as methyl esters only were first saponified with methanolic KOH, acidified with HCl, and extracted with diethyl ether (five times). Bile acid TFA-methyl derivatives were prepared in two steps. First methyl esters were prepared by the reaction of the acid, dissolved in diethyl ether—methanol (9:1), with diazomethane in an ethereal solution added in excess. Trifluoroacetates were obtained by dissolving the methyl ester in trifluoroacetic anhydride and subsequent treatment at 37°C for 30 min. Excess reagent was evaporated under reduced pressure and the residue dissolved in acetonitrile. Bile acid HFB derivatives were prepared according to the method of Musial and Williams [10].

RESULTS

Gas chromatography

Table I summarizes the relative retention times (RRT) of 38 bile acids, tested as trifluoroacetates of hexafluoroisopropyl or methyl esters and as heptafluorobutyrates. The retention behaviour was characterized by RRT, to allow direct comparison of the values obtained with previous data. All but four bile acids were 5β-cholanoic acids. Separation of the 5α and 5β epimers was impossible for the 3,6-diketo-cholanoic acid because of poor resolution. The epimeric 3α-hydroxy-6-keto-cholanoic acids, however, could be separated as TFA-HFIP or HFB derivatives. 3α-Hydroxy-6-keto-5α-cholanoic acid was another rare exception from the general rule [5] that selective phases such as QF-1 retain the 5α-cholanoates longer than the corresponding 5β-cholanoates. It should be stressed that all dihydroxy bile acids with substituents in the 3,6- and 3,7-positions, isomers and epimers alike, were indistinguishable when analyzed as heptafluorobutyrates. However, separation of bile acids with epimeric hydroxyl functions as HFB derivatives was not impossible, as can be seen from 3α,12α- and 3β,12α-dihydroxy-5β-cholanoic acids.

The long retention times of ketonic acids are characteristic for the QF-1 phase. Most of them may be separated as esters or heptafluorobutyric anhydrides. It is of particular value that all ketonic bile acids tested were resolved as TFA-HFIP derivatives. As HFB or TFA-methyl derivatives, however, the two monoketo oxidation products of deoxycholic acid could not be separated, whereas the oxidation products of cholic acid with one keto group at carbon 3 or 12 were inseparable as methyl ester trifluoroacetates.

A few hydroxy bile acids [3α-hydroxy- and 3β-hydroxy-, 3α,6α- and 3α,7β-dihydroxy-cholanoic acids, and 3β,12α-dihydroxy-cholanoic acid and 3α,12α-dihydroxy-$\Delta^{8(14)}$-5β-cholenoic acid (apocholic acid)] were not resolved by any of the three methods, but most other bile acids were resolved well. Hyocholic acid could not be analyzed as the TFA or HFB derivative

TABLE I

RELATIVE RETENTION TIMES OF BILE ACID HEXAFLUOROISOPROPYL AND METHYL ESTER TRIFLUOROACETATES AND OF HEPTAFLUOROBUTYRATES ON 3% QF-1 AT 230°C

All relative retention times are referred to the corresponding deoxycholate derivatives. Mean absolute elution times were: TFA-HFIP derivative 642 sec; TFA-methyl derivative 1007 sec; HFB derivative 811 sec. The deoxycholate derivative was injected simultaneously with each of the esters. All data tabulated are mean values from 5—6 estimations. Mean standard deviation was 0.003 up to RRT = 1.00, 0.015 up to RRT = 2.5, 0.025 up to RRT = 5.0 and 0.04 for higher RRT values.

Component*	Relative retention time		
	TFA-HFIP	TFA-methyl	HFB
Cholesterol	0.49	0.32	0.47
Cholanoic	0.20	0.25	0.19
3α	0.60	0.69**	0.54
3β	0.59	0.68**	0.56
3β-Δ^5	0.66	0.73	0.66
3β,Δ^5-22,23-bisnor	0.35	0.36	***
5α,3β	0.74	0.81	0.73
3α,6α	1.48	1.37**	1.22
3α,6β	1.38	1.24	1.21
3β,7α	1.05	1.02**	1.25
3α,7α	1.29	1.22**	1.21
3α,7β	1.49	1.40	1.20
3β,12α	0.86	0.88	0.76
3α,12α	1.00	1.00	1.00
7α,12α	0.62	0.63**	0.60
3α,12α,$\Delta^{8(14)}$	0.86	0.85	0.85
3-keto	1.37	1.49	1.19
3α,6-keto	2.69	2.69	2.33§
3α,7-keto	2.30	2.24**	1.93§
3α,12-keto	1.93	2.03**	1.68
3-keto,7α	2.62	2.37**,§	2.10
3-keto,7β	2.74	2.84	2.32
3-keto,12α	2.07	2.00**	1.66
3α,12-keto,$\Delta^{9(11)}$	2.12	2.55	1.82
5α-3α,6-keto	2.46	2.63§	2.01§
3,6-diketo	5.89	5.96	4.99
3,7-diketo	4.01	4.34	3.40
3,12-diketo	3.63	4.29	3.10
7,12-diketo	1.72	2.18	1.46
5α-3,6-diketo	5.91	6.06	5.08
3α,6α,7α	2.00§ (2.69)	1.84§§	2.31§§
3α,7α,12α	2.22	1.90**	2.58
5α-3α,7α,12α	2.44	2.02**	2.80

TABLE I (continued)

Component*	Relative retention time		
	TFA-HFIP	TFA-methyl	HFB
3α,7α,12-keto	3.77	3.56**	3.31
3α,12α,7-keto	3.28	2.93**	3.02§
3-keto,7α,12α	4.30	3.59**	3.71§
3α,7,12-diketo	5.50	6.35	4.70
3,7-diketo,12α	5.16	5.28	3.96§
3,7,12-triketo	8.24	9.70	6.89

*The cholanoic acids are identified by the location (3, 6, 7 or 12) and configuration (α or β) of the hydroxyl groups as well as the location of the keto groups and double bonds (Δ). They are all 5β-acids if another ring junction of the A/B rings is not indicated by 5α.
**Data from ref. 6.
***No peak visible, probably because of unsuccessful derivatization procedure.
§The compounds give two peaks.
§§The compounds often give two peaks.

because of thermal decomposition, which is critical for most 3,6,7-trihydroxy acids on account of the vicinal 6- and 7-hydroxyl groups [6]. Thermal decomposition was observed occasionally with HFB derivatives of other hydroxy bile acids too. With the ketonic acids indicated in Table I, however, we always saw a second peak. This artifact accounted for up to about 30% of the original compound, but we do not know if it is a product of derivatization or of decomposition in the gas chromatograph. With TFA-HFIP derivatives we have not observed any artifacts so far.

Mass spectrometry

All mass spectra of bile acid TFA-HFIP derivatives were interpreted with the help of known fragmentation patterns of TFA or trimethylsilyl (TMS) derivatives of bile acid methyl esters [11]. Arithmetic considerations showed that the known fragmentation pathways can be distinctly seen with HFIP esters as well. It should be stressed that neither detailed mechanistic studies

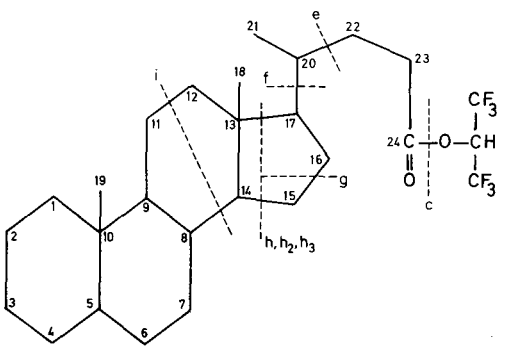

Fig. 1. Schematic illustration of diagnostically significant fragmentations of the carbon skeleton of hexafluoroisopropyl cholanoates according to Sjövall et al. [11].

with labeled acids nor high-resolution mass determination of particular ions were made. For an easier comparison of both published and measured data we used the same signs for fragment ions as Sjövall et al. [11]. Fig. 1 and Table II show, in a simplified way, these fragmentation patterns. Hexafluoroisopropyl cholanoates differ only by 136 mass units at ions containing the ester group.

TABLE II

SCHEMATIC REPRESENTATION OF THE TENTATIVE ORIGIN OF IONS FORMED IN THE FRAGMENTATION OF SUBSTITUTED METHYL AND HEXAFLUOROISOPROPYL CHOLANOATES

Fragmentation	Methyl cholanoates*		Hexafluoroisopropyl cholanoates	
	Lost fragment	Mass	Lost fragment	Mass
a	H_2O	18	H_2O	18
a_1	CF_3COOH	114	CF_3COOH	114
a_4			CF_3COO	113
b	CH_3	15	CH_3	15
c	CH_3O	31	$(CF_3)_2CHO$	167
e	22—24	87	22—24	223
f	20—24	115	20—24	251
g	16—17; 20—24	142	16—17; 20—24	278
i	AB—11	166**	AB—11	166**
h	15—17; 20—24	157	15—17; 20—24	293
h_2	15—17; 20—24	155	15—17; 20—24	291
h_3	ABC	220***	ABC	220***

*According to ref. 11.
**With no substituents in rings A, B.
***With no substituents in rings A, B, C.

Dihydroxy bile acids. The trifluoroacetates of hexafluoroisopropyl cholanoates generally cleaved in the same way as the methyl cholanoates (Table III). Trifluoroacetic acid (fragments a_1, $2a_1$) and the side-chain (e, f) were gradually lost, and ring D was cleaved (g, h), but these ions differed, as expected, in their relative intensities. The most striking change was an enhanced intensity of the a_1 ion, formed by the loss of one trifluoroacetic acid molecule.

In both 3,7-substituted HFIP cholanoates this ion became the base peak, whereas its intensity was significantly lower in the methyl ester (Table III, [11]). The subsequent loss of the side-chain with carbons of the ring D (a_1g) resulted in the second prominent fragment. In the spectrum of the 3α,12α-substituted dihydroxy bile acid the base peak a_1f did not change, but the intensity of the fragment a_1 increased considerably, compared with the methyl ester (Table III, [11]).

The loss of the trifluoroacetyl group seemed to proceed in two ways, as CF_3COOH (a_1) and CF_3COO (a_4). The latter mode of elimination gave rise to the base peaks of the TFA-HFIP derivatives of chenodeoxycholic, ursodeoxycholic, and deoxycholic acids as reported by Imai et al. [9]. In our spectra

TABLE III

MASS SPECTRA OF THE TRIFLUOROACETATES OF METHYL AND HEXAFLUORO-ISOPROPYL DIHYDROXY-5β-CHOLANOATES

Ion	Methyl esters*			HFIP esters*			
	m/e	Intensity (%)		*m/e*	Intensity (%)		
		3α,7α (CDC)	3α,12α (DC)		3α,7α (CDC)	3α,7β (UDC)	3α,12α (DC)
M^+	598	—	—	734	5.5	—	—
a_1	484	27.9	7.3	620	100.0	100.0	64.6
a_1b	469	8.3	—	605	9.9	9.4	5.7
a_1a_4	371	28.3	13.6	507	19.3	19.4	14.2
$2a_1$	370	80.1	36.3	506	12.4	16.9	6.4
$2a_1b$	355	42.3	6.0	491	4.2	5.2	—
a_1c	453	—	2.8	453	11.0	5.6	7.8
a_4e	398	—	—	398	7.9	5.5	4.4
a_1e	397	—	—	397	4.9	4.5	4.8
a_1f	369	54.7	100.0	369	21.9	22.7	100.0
i	208	10.6	3.7	344	17.1	14.7	7.2
a_1g	342	7.4	20.4	342	43.8	26.3	14.1
a_4h	328	7.7	2.6	328	36.9	67.4	14.4
a_1h	327	12.9	—	327	18.5	17.3	6.7
h_3	154	38.1	—	290	19.3	17.0	41.0
$2a_1f$	255	53.7	42.9	255	13.5	18.3	7.3
a_1a_4g	229	13.2	3.8	229	10.7	9.1	5.4
$2a_1g$	228	12.5	3.7	228	12.8	10.8	—

*CDC = chenodeoxycholic acid; UDC = ursodeoxycholic acid; DC = deoxycholic acid.

of the hexafluoroisopropyl esters of these acids the fragments $2a_1$, a_1e, and a_1h were accompanied by intense ions, one mass unit heavier. These peaks were significantly higher than the corresponding isotopic contributions of ^{13}C atoms and they may arise by the loss of the trifluoroacetyl group without a ring hydrogen.

Spectra of positional isomers differed distinctly. A major reaction in bile acids having a 12-hydroxyl group was the loss of one molecule of trifluoroacetic acid and the side-chain, whereas 3,6- and 3,7-dihydroxy bile acids preferentially lost two trifluoroacetic acid molecules [11]. The replacement of the methyl ester group by the hexafluoroisopropyl ester group did not change this rule. The base peak of deoxycholic acid (a_1f) was about fifteen times more intense than the $2a_1$ ion. With chenodeoxycholic and ursodeoxycholic acid the ratio of the fragments a_1f:$2a_1$ was smaller than 2.

Trihydroxy bile acids. Table IV gives spectra typical of cholanoic acids with three hydroxyl groups. The elimination of trifluoroacetyl groups (a_1, $2a_1$, $3a_1$), of the side-chain (a_1f, $2a_1f$, $3a_1f$), and the cleavage of the ring D ($2a_1g$) were seen. The molecular ion was absent. The spectrum, obtained with the GC inlet, shows, as expected, a shift of dominant ions to lower mass units on account of thermal excitation. The ion $2a_1f$ became the base peak, whereas in the direct inlet spectrum this ion was less intense than its precursor $2a_1$. The intensity ratio of $2a_1f$ and $3a_1$ ions was in both spectra in agreement

TABLE IV

MASS SPECTRUM OF THE TRIFLUOROACETATE OF HEXAFLUOROISOPROPYL 3α,7α,12α-TRIHYDROXY-5β-CHOLANOATE (CHOLIC ACID)

m/e	Ion	Intensity (%)	
		Direct inlet	GC inlet
846	M^+	—	—
732	a_1	6.4	—
619	a_1a_4	31.6	17.4
618	$2a_1$	88.4	24.4
603	$2a_1b$	5.6	4.9
505	$2a_1a_4$	12.1	9.4
504	$3a_1$	8.0	7.2
489	$3a_1b$	4.9	6.3
481	a_1f	14.6	5.2
367	$2a_1f$	53.6	100.0
340	$2a_1g$	4.8	5.0
290	h_3	100.0	45.7
253	$3a_1f$	11.6	35.5

with the finding by Sjövall et al. [11]. In the presence of a trifluoroacetoxy group the former ion was more intense than the latter. The base peak h_3 in the direct inlet spectrum was observed neither by Sjövall et al. [11] nor by us in other spectra recorded with the GC—MS combination. An impurity enhancing its intensity to 100% cannot be excluded. As in the case of dihydroxy bile acids, the elimination of the trifluoroacetoxy group could also occur without a ring hydrogen (ions a_1a_4, $2a_1a_4$).

Ketonic bile acids. The usual fragmentation pattern of the 3α-hydroxy-7-keto-cholanoic acid could be easily recognized (Table V). The molecular ion at *m/e* 636 was pronounced in both direct and GC inlet records. Major fragmentation pathways were loss of water (a), of a trifluoroacetyl group (a_1, a_4), and their combination (aa_1), of the hexafluoroisopropyl ester group (ac) and the side-chain (f, af, aa_1f). The fission of rings C (i) and D (aa_1h) was also pronounced.

Sjövall et al. [11] emphasized the directing effect of a 7-keto group for fragmentation reactions. In the spectrum of the silyl ether of 3α-hydroxy-7-keto-5β-methyl-cholanoate a diagnostically important peak at *m/e* 292 was noted (fission of the 5,6 and 9,10 carbon bonds). The corresponding peak of HFIP ester at *m/e* 428 was a dominant signal. The cleavage of the 8,14 and 12,13 bonds of the methyl ester trifluoroacetate of 3α-hydroxy-7-keto-cholanoate gave rise to the peak at *m/e* 303. This ion was seen also in the spectrum of the HFIP ester. Last, ketonic bile acids with 7-keto groups lost water more readily than other ketocholanoates. The intensity of the "a" ion in the HFIP ester was also in accordance with this finding.

The simultaneous presence of two hydroxyl groups in 3α,12α-dihydroxy-7-keto-5β-cholanoic acid changed the directing effect of the 7-keto group (Table VI). The spectrum was characterized by the usual fragments due to loss of trifluoroacetyl groups, water, and side-chain. Loss of water occurred, however, less readily than in 3α-hydroxy-7-keto-5β-cholanoic acid and the

ion at *m/e* 303 was missing. The intense fragment at *m/e* 426 might have been formed by fission of the 9,10 and 5,6 carbon bonds and additional loss of the 12-trifluoroacetyl group (analogous to the typical peak at *m/e* 428 in Table V).

TABLE V

MASS SPECTRUM OF THE TRIFLUOROACETATE OF HEXAFLUOROISOPROPYL 3α-HYDROXY-7-KETO-5β-CHOLANOATE

m/e	Ion	Intensity (%)	
		Direct inlet	GC inlet
636	M^+	100.0	19.4
618	a	40.6	4.9
603	ab	4.9	13.9
523	a_4	11.9	13.9
522	a_1	4.6	35.9
504	aa_1	6.4	15.8
489	aa_1b	17.2	15.6
451	ac	32.4	13.1
428		87.9	43.2
385	f	16.4	23.1
367	af	34.4	18.3
303		27.1	12.2
290	i	18.6	8.4
253	aa_1f	13.3	22.7
211	aa_1h	17.6	17.7

TABLE VI

MASS SPECTRA OF THE TRIFLUOROACETATES OF HEXAFLUOROISOPROPYL 3α,12α(3α,7α-DIHYDROXY-7(12)-KETO-CHOLANOATES

m/e	Ion	Intensity (%)		
		3α,12α-7-keto (direct inlet)	3α,12α-7-keto (GC inlet)	3α,7α-12-keto (GC inlet)
748	M^+	18.3	—	20.5
730	a	6.3	—	—
635	a_4	56.4	21.0	5.6
634	a_1	98.9	41.3	11.3
616	aa_1	46.6	16.1	—
521	$2a_4$	17.3	14.3	—
520	$2a_1$	7.1	19.3	5.5
487	$2a_1ab$	6.8	7.2	—
457	h_2	—	—	26.2
426		71.3	41.4	—
343	a_1h_2	56.3	28.6	38.9
383	a_1f	100.0	92.4	—
365	aa_1f	24.1	17.0	—
269	$2a_1f$	13.7	64.1	6.5
251	$2a_1af$	14.8	33.8	—
229	$2a_1h_2$	9.3	8.4	100.0

The spectrum of 3α,7α-dihydroxy-12-keto-5β-cholanoic acid (Table VI) was recorded with the GC inlet only and originated from a biological sample. The number and the kind of substituents could be easily recognized (molecular ions and fragments a_1, $2a_1$). The position of the keto group was clearly indicated, analogous to the TMS derivative [11], by the intense fragment h_2 and the base peak $2a_1h_2$.

DISCUSSION

Gas chromatography

Derivatives of bile acids suitable for GC analysis should be easily and quantitatively prepared without by-products, and should be stable during storage and chromatographic analysis. TFA-HFIP derivatives meet these criteria very well. Preparation is fast and simple and no by-products arise during derivatization. This is true even in complex mixtures of extraneous compounds. During GC, all compounds tested, except hyocholic acid, gave a single peak. TFA-HFIP derivatives were found to be stable for at least five days (ref. 9 and our own experience) when stored in the refrigerator, provided moisture was excluded.

Methyl esters are mostly prepared with diazomethane, which is, however, unstable and therefore not a suitable reagent for routine assays. Moreover, diazomethane may convert hydroxyl groups of the steroid nucleus to methoxy groups [12, 13]. Besides TFA derivatives methyl esters are also converted into TMS derivatives for GC analysis, but side reactions have been observed with silylating agents too [14, 15]. TFA-methyl derivatives are stable for at least two days, when they are stored in the refrigerator and moisture is excluded. They may, however, undergo thermal decomposition in the chromatograph. TFA derivatives of 3,6,7-trihydroxy bile acids are reported to be particularly sensitive compounds [6]. This should be true for TFA-HFIP derivatives too, although our experimental experience is limited to hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic acid). These types of derivatives, therefore, are probably not suited for the analysis of bile acids in mice, rats, and pigs.

Preparation of HFB derivatives is more laborious and time-consuming. By-products of the derivatization procedure were not observed with hydroxy bile acids, but thermal decomposition during GC was occasionally seen. With ketonic bile acids we often saw two peaks during GC, but we do not know if these artifacts originate from derivatization or from thermal decomposition. This represents a serious disadvantage in the use of HFB derivatives, which otherwise enable the analysis of conjugated bile acids without prior deconjugation [10]. Musial and Williams [10] report HFB derivatives to be stable for one year.

In spite of their higher molecular weights, bile acid hexafluoroisopropyl esters and heptafluorobutyrates (except the cholic acid HFB derivative) are eluted earlier than the corresponding methyl esters. This is probably due to interference of the fluoro groups in the compound with the fluoro groups of QF-1. Besides the nature of the carboxyl derivative, the retention behaviour depends on the number, nature and position of substituents. From the RRTs

of hydroxy bile acids it is concluded that introduction of an hydroxyl group brings about an effect opposite to the fluorinated ester or anhydride groups. The RRTs of monohydroxy bile acid TFA-HFIP and HFB derivatives are all less than those of TFA-methyl derivatives. With dihydroxy bile acids there is little variation, whereas the TFA-HFIP and HFB derivatives of trihydroxy acids show longer RRTs than the corresponding methyl ester trifluoroacetates. Similar effects can be seen with hydroxyketo acids. So TFA-HFIP derivatives of most bile acids are more effectively resolved than the TFA-methyl derivatives considering the number of overlapping peaks. The resolution of heptafluorobutyrates on QF-1 is in general less efficient, but sufficient for the separation of many bile acids.

On the fluorosilicone QF-1, selective for carbonyl groups [5], keto bile acids are effectively separated and resolution is again best for TFA-HFIP derivatives. In this respect QF-1 is superior to non-polar phases such as OV-1, SE-30, and to the less polar OV-17. Separation on OV-210, another fluorosilicone, is similar to that on QF-1 [16]. Data for OV-225, a cyanopropylphenylsilicone, are available for methyl ester acetates only [7]. The results are generally comparable with our results, but the separation of ketonic bile acids is less efficient.

Capillary GC [17—19] and high-performance liquid chromatography (HPLC) of bile acids [20—22] can not yet substitute for the well-established packed column GC methodology for routine assays. Capillary GC is hampered mainly by insufficient resolution on many commercial stationary phases, limited life of columns and adsorptive effects of bile acid derivatives [18], whereas HPLC of bile acids is still in an early developmental stage.

Mass spectrometry

Substitution of the methyl ester group by the more polar hexafluoroisopropyl ester group did not result in any disadvantages for mass spectrometric identification. General fragmentation pathways, indicating number and kind of substituents, were retained. Neither did the presence of an HFIP group influence fragmentation rules characterizing positional isomers. In dihydroxy bile acids the intensity ratio of a_1f:$2a_1$ fragments indicates the presence of the 12-hydroxyl group; similarly the ratio of $2a_1f$:$3a_1$ fragments in trihydroxy bile acids shows the presence of the 12-hydroxyl group. The directing effect of the 7-keto group is also distinct (typical ions *m/e* 303, 428, and 618 in 3α-hydroxy-7-keto-cholanoic acid and probably the ion *m/e* 426 in 3α, 12α-dihydroxy-7-keto-cholanoic acid). The fission of ring D, typical of 12-keto acids, is also seen (ions h_2, $2a_1h_2$ in 3α,7α-dihydroxy-12-keto-cholanoic acid).

HFIP esters show a more intense a_1 fragment in hydroxy bile acids than the corresponding methyl esters. In ketohydroxy bile acids this tendency was not observed. For both types of substituents a more intense cleavage of the CF_3COO group instead of the CF_3COOH group is typical.

HFIP esters are suitable for combined GC—MS analysis with packed columns. In spite of their higher polarity they were not retained in the GC—MS interface and only a minor tailing of their GC peaks was observed. Due to their higher molecular weights more fragments in the higher mass range are found. A continuous ion registration in this mass range shows some drawbacks,

for example, a decline of sensitivity due to the decreased resolution, but the enhanced specificity of these ions is more convenient for selective ion monitoring.

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FULLY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A NEW CHROMATOGRAPH FOR PHARMACOKINETIC DRUG MONITORING BY DIRECT INJECTION OF BODY FLUIDS*

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SUMMARY

A new fully automated high-performance liquid chromatograph is described which detects drugs from directly injected plasma (urine, saliva) without sample pretreatment. The apparatus consists of a programmable automatic sampling unit, which is connected via two alternating working pre-columns to an analytical column ("alternating pre-column sample enrichment"). The new device is able to operate with directly injected body fluids like an auto-analyzer and is especially useful for pharmacokinetic and clinical studies, where drug concentrations have to be determined from plasma, urine or saliva.

INTRODUCTION

In pharmacokinetics, which deal with the description and interpretation of time-dependent levels of drugs in the body, non-radioactive analytical methods are gaining in prominence because of their selectivity in the specific detection of drugs or metabolites and their sensitivity in the nanogram range [1—5]. In this field, high-performance liquid chromatography (HPLC) is one of the most important techniques for the separation and the determination of drugs and their metabolites in body fluids [5, 6].

The use of an automatic sampler and the application of auto-analyzer reactor systems in connection with HPLC was a great step forward in the partial automatization of liquid chromatography [7—16]. But a still unsolved problem, especially for pharmacokinetic investigations, was the question how to avoid the classical sample pretreatment steps for biological samples prior to HPLC analysis.

*Dedicated to Prof. Dr. Leopold Horner on the occasion of his 70th birthday.

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The application of a pre-column in its property as a protecting unit to extend the lifetime of the analytical column is a very common technique in liquid chromatography [2, 14]. Moreover, the employment of small columns with hydrophobic packing materials yields good results in the preconcentration of xenobiotics [4, 7, 17, 18].

Concerning the development of HPLC methods for biological samples (e.g. plasma, serum, saliva, urine), one of the most time-consuming steps, involving considerable sources of error, is sample pretreatment and enrichment prior to injection into the chromatograph. An approach towards the on-column sample-enrichment technique using a gradient elution system and microbore columns has been described [19].

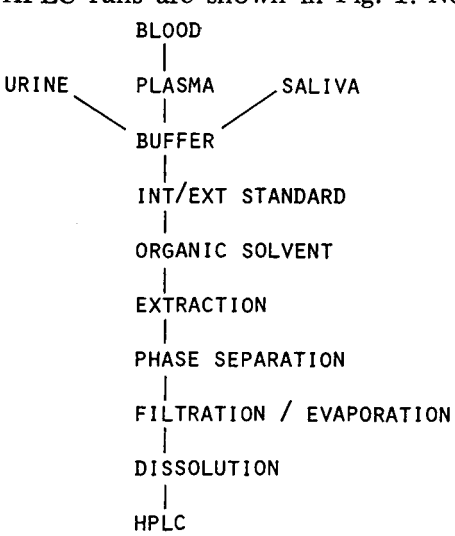
So far it has not been possible to determine drugs by repeated injection of native body fluids into a liquid chromatograph. Thus, we adapted the idea of the pre-column as a protecting device for the analytical column and, at the same time, the possibility to adsorb and preconcentrate drugs on small reversed-phase columns, for the development of a new technique.

The following report describes a novel automatic high-performance liquid chromatograph, with alternating pre-column sample enrichment, for direct plasma (urine, saliva) injection without classical sample pretreatment steps. Our automated system uses the "on-column" enrichment technique on small pre-columns and the pre-column backflushing technique in addition with pre-column re-equilibration.

EXPERIMENTAL

Sample pretreatment

Steps in "classical" sample pretreatment of biological fluids for subsequent HPLC runs are shown in Fig. 1. Nearly all steps are susceptible to errors and



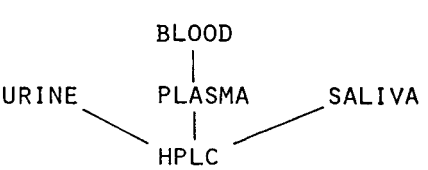


Fig. 1. "Classical" steps in the pretreatment of body fluids prior to high-performance liquid chromatography.

Fig. 2. Handling of body fluids prior to automated HPLC with alternating pre-column sample enrichment.

waste time and laboratory capacity. The application of the recently developed automatic HPLC device, with alternating pre-columns, reduces these steps significantly (Fig. 2).

Fully automatic HPLC

The apparatus, illustrated schematically in Figs. 3 and 4, consists of an injection system (autosampler) and two pneumatically driven valves, which alternatingly connect two pre-columns with the injection system and pump A. The columns can also be connected in the "backflush mode" with the analytical column and pump B. The programmable autosampler, affiliated to a time relay, controls the whole analytical procedure.

Columns

The pre-columns (typical dimensions 25 mm × 4.6 mm I.D.) are made by dividing commercially available Knauer columns (Dr. Knauer, West Berlin, G.F.R.) into smaller columns 25 mm long. They are dry packed. In the examples described in this paper we used C_{18}-Corasil (Waters Assoc., Königstein/Ts, G.F.R.) with a particle size of 37—50 μm (see legends to Figs. 6 and 9).

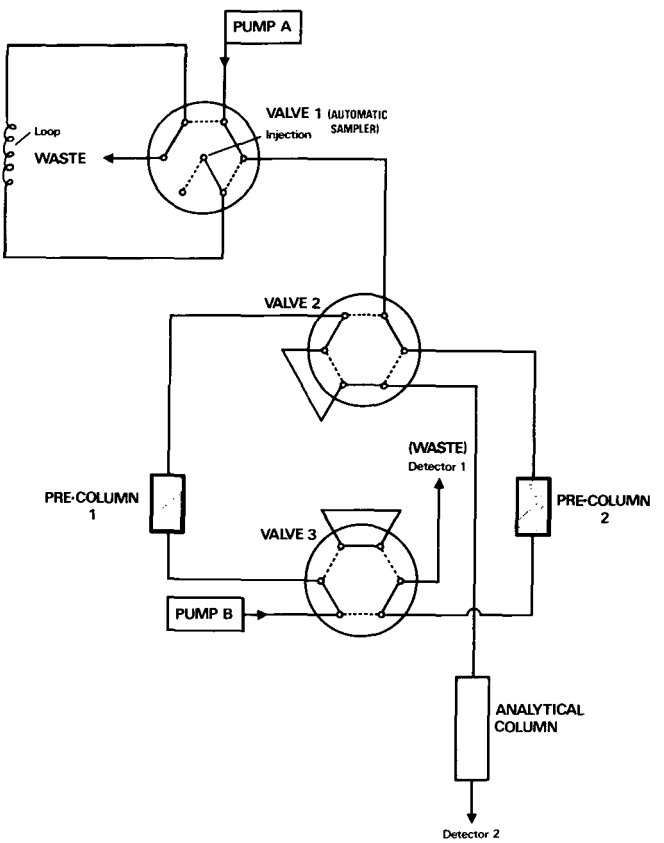


Fig. 3. Alternating pre-column switching technique for sample enrichment, demonstrated with three 6-way valves.

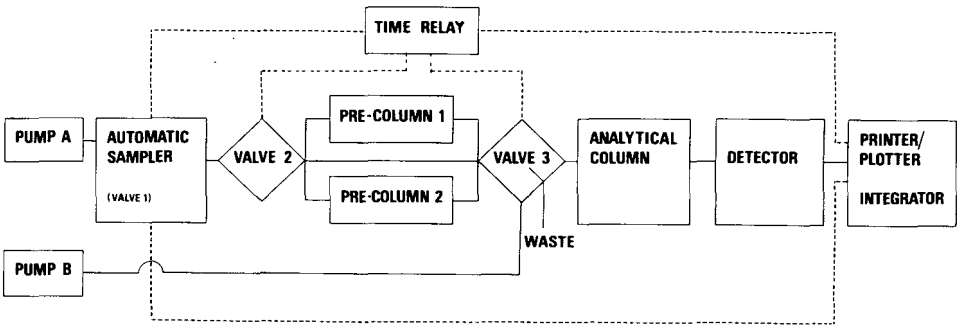


Fig. 4. Flow-chart of the automated HPLC system with alternating pre-column sample enrichment. (— — —), electronic connections.

The analytical columns (typical dimensions 120 mm × 4.6 mm I.D.) are filled by the usual slurry technique with LiChrosorb RP-18 (5 μm) from Merck (Darmstadt, G.F.R.) for analysis of R-A 233 (Dr. K. Thomae GmbH, Biberach/Riss, G.F.R.) (Fig. 6), and with ODS-Hypersil (5 μm) from Shandon Southern Products (Cheshire, Great Britain) in the analysis of AR-L 115 BS (Dr. K. Thomae GmbH).

Detailed description of technique

Samples of body fluids (maximum 48 samples) are pipetted into the sample holder of the automatic sampling device (e.g. WISP, Waters). Volumes of between 10 and 2000 μl of plasma, urine or saliva, preferentially between 10 and 150 μl can be automatically injected. It should be checked that the samples contain no solid particles which might block the injection needle. Pre-column 1 (PC1) onto which the first sample is injected, has been conditioned with the purge phase (water or buffer solution) delivered by pump A (Fig. 3). After the injection to PC1 (typical dimensions 25 mm × 4.6 mm I.D.) the pre-column is washed for a further 5 min with water (buffer).

The adsorption material for the pre-columns is reversed-phase or ion-exchange material with a particle size of about 20—50 μm. Here the substances to be detected are selectively adsorbed on C_{18}-Corasil (37—50 μm) and thus enriched. At the same time all accompanying water-soluble co-products are eliminated with the purge phase (pump A). A second pre-column (PC2) has been added to save time. While PC1 is reconditioned, PC2 is eluted in the backflush mode onto the analytical column and vice versa (= alternating pre-column sample enrichment). Simultaneously to the application—injection step, the autosampler (WISP) activates an electronic controller (time relay) which controls the purge-phase period, after which it switches the pneumatic valves and starts the printer/plotter integrator. This switching process causes two subsequent steps: pre-column 1 (PC1) where the injected drug has been absorbed is switched to the solvent stream of pump B. Pump B delivers the eluent cocktail, necessary for separation and chromatography, in the backflush mode from PC1 to the analytical column (typical dimensions 120 mm × 4.6 mm I.D.), filled, for example, with reversed-phase material (particle size 5—10 μm). Parallel to this process, PC2 is switched to the eluent stream of pump A (purge phase) which removes the rest of the organic solvent from

the pre-column. The whole working cycle thus consists of an equilibration phase, an adsorption phase and a purge phase on the pre-columns and a chromatographic phase on the analytical column. The automatic sample device (WISP) has been computed for one working cycle and the printer/plotter is stopped by an internal equilibration delay device. During the print-out of the results (Hewlett-Packard, Model 3370) the second pre-column (PC2) is switched to the purge stream of pump A for re-equilibration (equilibration phase). After the next injection step, the electronic timer restarts. It then switches again to the pneumatically working valves and activates the printer/plotter, and so on (Fig. 5).

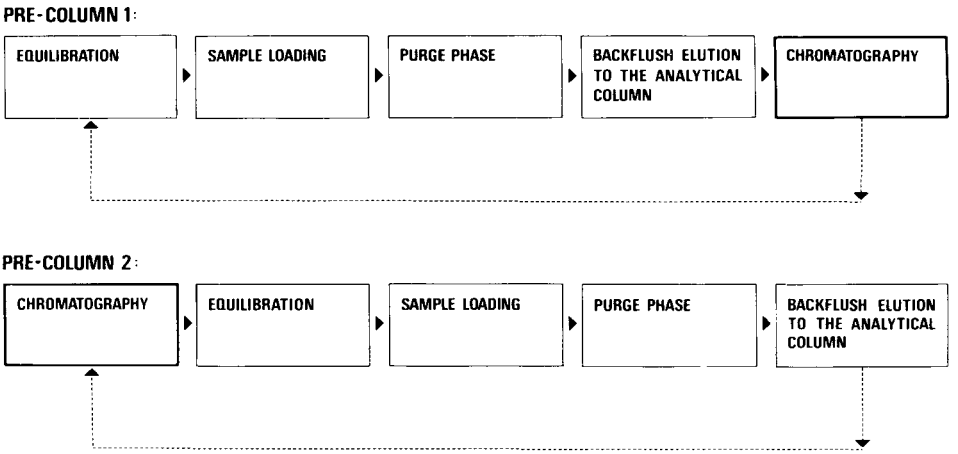


Fig. 5. Parallel working steps of pre-column 1 and pre-column 2, in the automated high-performance liquid chromatograph with alterning pre-column switching.

RESULTS

Chromatography

Drugs which can be separated and detected by reversed-phase or ion-exchange chromatography should also be detectable using automated HPLC with direct injection of body fluids. So far the new method has been used for the determination of AR-L 115 (a new cardiotonic agent) from urine, saliva and plasma, and for Rapenton^{R} (R-A 233 BS), an anti-platelet drug, and dipyridamole (Persantine^{R}), from both plasma and urine.

Typical chromatograms for automatic HPLC (direct sample injection) with alternating pre-column sample enrichment from an overnight run are shown in Figs. 6—8. It can be seen that the example demonstrated in Fig. 6 shows an HPLC determination with an extremely high precision [coefficient of variation (C.V.) = 1.4%].

As the printer is immediately started by the time relay (Fig. 4) with the backflush elution from the pre-column to the analytical column, we get only the main cut-out of the chromatogram with the relevant peaks and the calculated areas. Because of the peculiarity of the novel pre-column switching technique, the peaks from the first, third, fifth, etc., runs derive from pre-

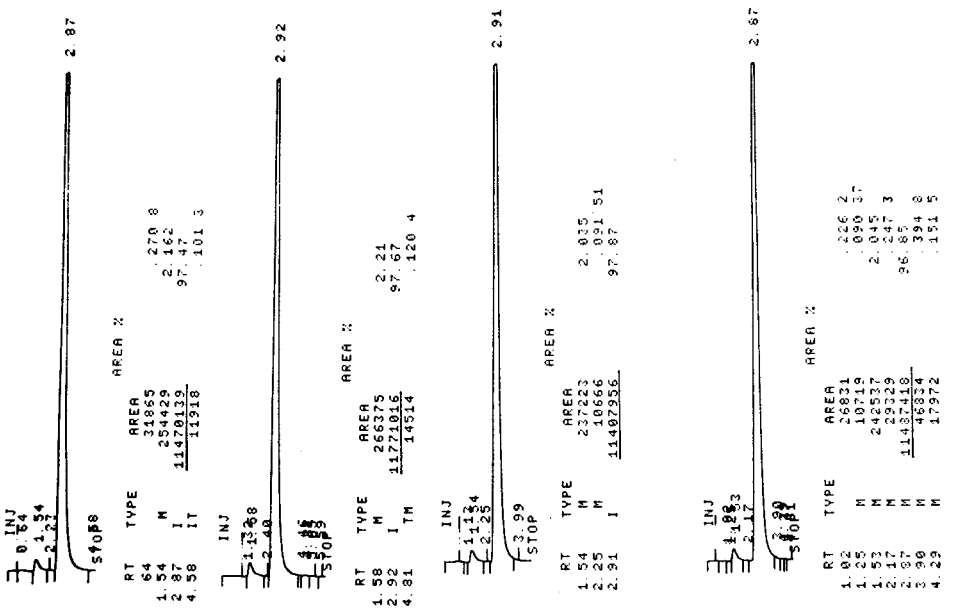


Fig. 6. Typical chromatograms of directly injected human plasma (injection volume = 150 μl), spiked with 100 ng/ml R-A 233, a new anti-platelet drug. C.V. = 1.4%. HPLC conditions: reversed-phase material (Corasil^R, 37—50 μm) in the pre-columns and RP-18 (LiChrosorb^R, 5 μm) in the analytical column. Column dimensions: pre-column 25 mm × 4.6 mm I.D.; analytical column 120 mm × 4.6 mm I.D. Pump A, water; pump B, methanol—0.2 *M* Tris buffer (pH 8.6) (80:20). Flow-rate, 1 ml/min. Fluorimetric detection: 465/510 nm.

column 1 and those from the second, fourth, sixth, etc., runs come from pre-column 2.

Backflush—elution

Immediately after the injection of a drug—plasma solution to the pre-column, the drug is adsorbed and enriched in the very first area on top of the pre-column. This effect could be demonstrated by the application of drugs which show a strong fluorescence in visible light. It is therefore important that the adsorbed material is eluted from the pre-column in the backflush mode to prevent zone-spreading effects. The effect of the 25-mm pre-column to peak width and tailing was negligible in all cases tested.

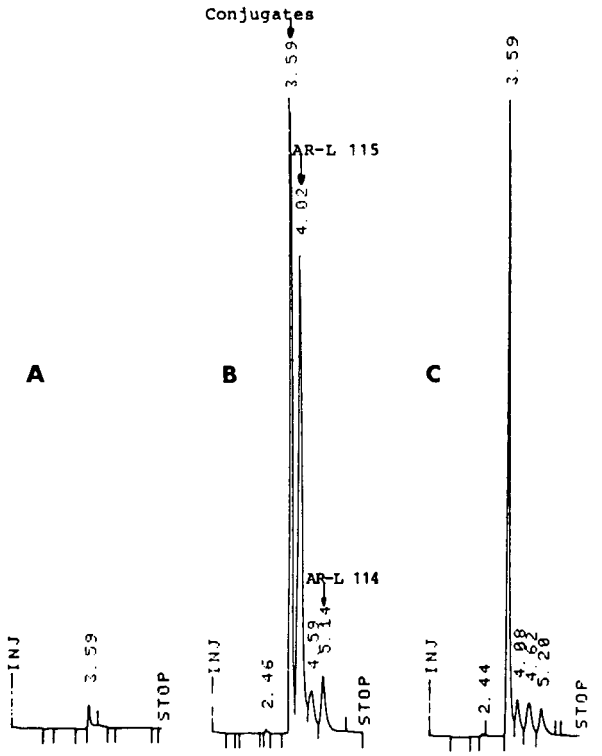


Fig. 7. HPLC chromatogram from native urine (injected volume = 100 μl) after oral administration of 100 mg of AR-L 115. (A) Blank, (B) sampling period 0—4 h, (C) sampling period 4—8 h. For HPLC conditions see Fig. 9; analytical column is 120 mm long.

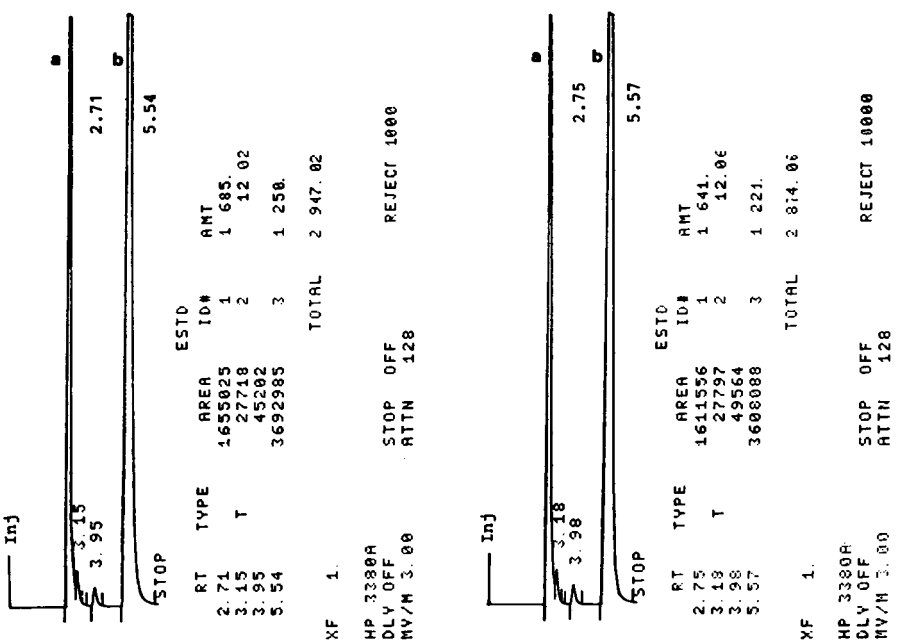


Fig. 8. HPLC chromatogram from native saliva (injected volume = 50 μl) 0.5 h after oral administration of 75 mg of AR-L 115 BS (for HPLC conditions see Fig. 9). (A) AR-L 115; (B) metabolite AR-L 113. Fluorimetric detection: 330/370 nm.

Stability of the automated system

The stability of the system in the time range of 24 h and also between days is extremely high. Fig. 9 shows a chromatogram injected at zero time and 24 h later. The C.V. between day-to-day analyses was in the range of 3—5%.

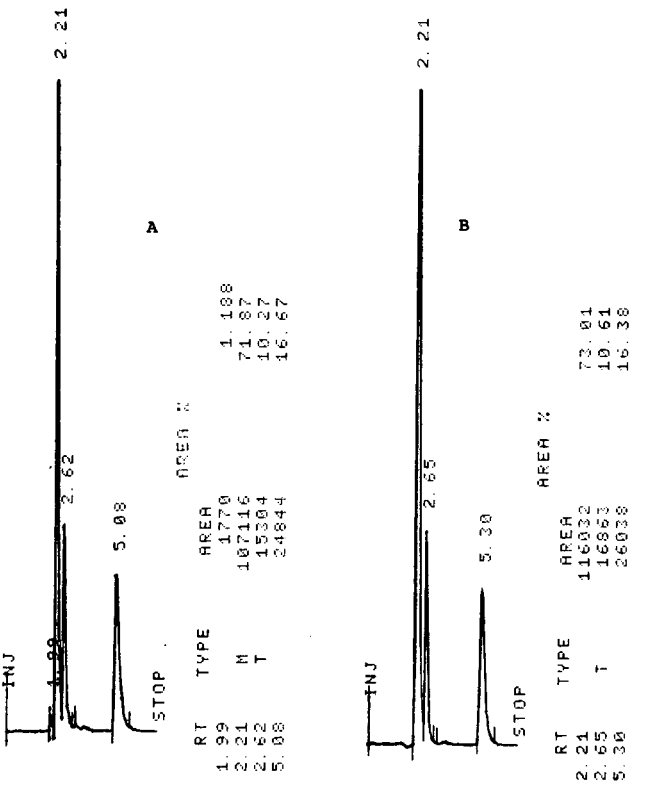


Fig. 9. HPLC chromatograms of AR-L 115, a new cardiotonic drug (t_R = 2.21 min) and two metabolites from a pharmacokinetic run at 0 time (A) and the same injection 24 h later (B). HPLC conditions: reversed-phase material (Corasil^R,37—50 μm)in the pre-columns and ODS-Hypersil^R (5 μm) in the analytical column. Column dimensions: pre-column 25 mm × 4.6 mm I.D.; and analytical columns, 120 mm × 4.6 mm I.D. Pump A: water pump B: acetonitrile—0.03 *M* phosphate buffer (pH 6.8) (1:2). Flow-rate, 1 ml/min. Fluorimetric detection: 330/370 nm.

Lifetime of the columns

In a series of about 1000 analyses of AR-L 115 BS, with an injection volume of about 10—150 μl of plasma, where normal tap water was used for washing the pre-columns and with acetonitrile—phosphate buffer (pH 6.8), there was no need for a column exchange. The results showed constant quality as regards selectivity and resolution.

Protein binding

We tested the influence of protein binding by comparing the recovery

after injection of spiked water and spiked plasma solutions with different concentrations of Rapenton^R. No effect of protein binding on recovery could be found in this case. The affinity of the protein-bound drug to the reversed-phase packing material in the pre-column seems to be stronger than the interaction with the protein. It seems to be possible that differences in recovery may result with strongly protein-bound drugs and "weak" reversed-phase packings in the enrichment column.

Memory effects

Memory effects from the pre-column ("substance bleeding") may be easily identified by injecting blank solutions after a run with definite amounts of drug. No memory effect could be observed.

Precision and accuracy

The fully automated system reaches a high level of precision (Table I). For the calibration curve of Rapenton^R (R-A 233 BS) (HPLC conditions as in Fig. 6), and anti-platelet drug, we obtained coefficients of variation of 1.3% and 1.2% (within day) and of 2.6% (day-to-day) for concentrations of 50 ng/ml and 1000 ng (n = 5). In all cases tested we obtained identical results with the automated technique compared to the normal HPLC using organic extracts of plasma samples. In the case of ^{14}C-labelled R-A 233 BS, we obtained nearly identical results for injected and eluted radioactivity.

TABLE I

PRECISION AFTER AUTOMATICALLY REPEATED INJECTIONS IN A CALIBRATION RUN FOR RAPENTON^R, AN ANTI-PLATELET DRUG

See Fig. 6.

Injected volume (μl plasma)	Concentration (ng/ml)	Coefficient of variation (CV %); n = 5
150	50	1.3
150	100	1.5
150	200	3.5
100	400	2.5
50	600	2.3
50	800	1.9
10	2000	0.8

Internal/external standard

The commonly used internal standard method with an additional compound, which needs to show chromatographic and spectroscopic properties similar to the compound to be detected, is of great importance in HPLC with classical sample pretreatment (Fig. 1). As in the case of fluorimetric determinations, it is sometimes difficult to find a suitable standard compound. With the recently developed HPLC technique, the drug which is to be detected may be used simultaneously as an external standard. There is no need for an additional standard compound. Vials containing plasma samples spiked

with the pure compound give us information about the stability of the whole automated system and about the range of linearity of the method.

CONCLUSION

The fully automated high-performance liquid chromatograph with an "alternating pre-column sample enrichment" device is able to sample body fluids such as plasma, urine or saliva directly on pre-columns, which act simultaneously as enrichment columns. From a theoretical point of view the new technique should be applicable to all classes of compounds that are detectable by reversed-phase and ion-exchange liquid chromatography. The system carries out overnight runs and thus increases laboratory capacity for routine analysis. The HPLC method reported is a useful and precise analytical tool, particularly in pharmacokinetic investigations, where drug concentrations have to be determined from body fluids.

Characteristic features of the device are: no sample pretreatment, direct injection of body fluids (plasma, urine, saliva), only one pipetting step per sample, no further standard compound required, peak width independent of the injected volume, fully automated chromatography, high precision, overnight runs, and low costs.

The new fully automated HPLC system may find application in the fields of drug development, drug monitoring (clinical and outdoor), clinical chemistry, control of chemical syntheses, stability testing, and forensic chemistry.

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THE APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN ENZYMATIC ASSAYS OF CHONDROITIN SULFATE ISOMERS IN NORMAL HUMAN URINE

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SUMMARY

A study of the urinary excretion of isomeric chondroitin sulfates in normal individuals by high-performance liquid chromatographic (HPLC) determinations of the unsaturated disaccharides produced by digestion with chondroitinases is described. The composition of the HPLC mobile phase was systematically varied in order to select the optimal conditions for separation.

The data show that chondroitin 4-sulfate is the major component of the chondroitin sulfate isomers in normal urine, and that chondroitin 6-sulfate is a lesser component. It is also evident that dermatan sulfate is present in small quantities in normal urine.

INTRODUCTION

Chondroitin 4-sulfate (C-4S) and chondroitin 6-sulfate (C-6S) have been reported to be the principal urinary glycosaminoglycans (GAG) in normal subjects [1—4]. However, the assays for chondroitin sulfate isomers vary according to the method used [5—9]. An enzyme method based on the quantitative determination of the unsaturated disaccharides produced by the digestion of urinary isomeric chondroitin sulfates with chondroitinase ABC and AC is specific for the quantitative measurement of each of the isomeric chondroitin sulfates [10, 11]. A high-performance liquid chromatographic (HPLC) method for the rapid and sensitive quantification of these unsaturated disaccharides has been developed [12] which is applied to elucidate the isomeric chondroitin sulfate distribution in normal urine in the present study.

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EXPERIMENTAL

Materials

The C-4S, C-6S, dermatan sulfate (DS), the unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-OS), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S), chondroitinase ABC and chondroitinase AC were products of Seikagaku Kogyo (Tokyo, Japan) and were purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Methanol and acetonitrile, distilled in glass, were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Instruments

The apparatus used was a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system equipped with a Waters Assoc. Model 440 UV detector (254 nm) and Model U6K injector. Peak heights, peak areas and retention times were measured by an on-line Columbia Model Supergrator-1 integrator (Columbia Scientific Industries, Austin, TX, U.S.A.). A Partisil-10 PAC (10 μm, 25 cm × 4.6 mm I.D.) column (Whatman, Clifton, NJ, U.S.A.) was employed.

Isolation of urinary GAG

The procedure used was essentially the method described earlier [13]. The urine specimens were filtered through Whatman No. 2 paper. Ten milliliters of the filtered urine were adjusted to pH 5.0 with 0.5 *M* acetic acid and mixed with 150 μl of 5% cetylpyridinium chloride (CPC) in 0.9% sodium chloride. The GAG were precipitated as CPC—polysaccharide complex after standing at 4°C overnight. The resulting precipitate was washed three times with 0.1% CPC and dissolved in 0.5 ml 2.0 *M* sodium chloride. The insoluble material was centrifuged and discarded. Four volumes of absolute ethanol were added to the supernatant and the GAG were precipitated at 0°C for 4 h. The precipitate was collected by centrifugation and washed successively with 80% ethanol, absolute ethanol and diethyl ether. After drying in air the GAG obtained were dissolved in 0.3 ml distilled water and precipitated at 0°C for 4 h in four volumes of absolute ethanol saturated with sodium acetate. The last step was repeated once. The precipitate was washed with absolute ethanol and diethyl ether and dried at reduced pressure over phosphorus pentoxide. The GAG isolated from 10 ml urine were dissolved in 180 μl distilled water and separated into two tubes for digestion with chondroitinase ABC and AC, respectively.

The total uronic acid in CPC fraction was determined using glucuronolactone as a standard by the borate—carbazole method [14]. Creatinine was determined by the method of Tausskey [15].

Enzymatic procedure

The digestion mixture contained 90 μl of a solution of urinary GAG in water (0.015—0.22 μmole of GAG as uronic acid), 30 μl of enriched Tris buffer (pH 8.0) and 30 μl of an aqueous solution of either chondroitinase ABC or chon-

droitinase AC (10 units/ml). The exhaustive digestion was carried out at 37°C for 5 h. Standard chondroitin sulfates were also digested in the same manner to check the enzyme activity. Four volumes of absolute ethanol were added after incubations and the mixture left overnight at 4°C. The clear supernatant obtained by centrifugation was dried under a stream of nitrogen and the residue, dissolved in 90% methanol, was applied to the chromatograph for analysis.

Recovery of added isomeric chondroitin sulfates

Solutions were prepared which contained 1 mg/ml of standard C-4S, C-6S and DS in distilled water. Aliquots of 50 μl of each of these solutions were added separately to 2 ml of normal urine. The GAG in these augmented urines were precipitated with 100 μl of 5% CPC in 0.9% sodium chloride and isolated as described above. Reproducibility of recovery was measured by duplicate isolations of GAG of each spiked normal urine sample. Enzyme reaction conditions and sample treatment for HPLC analysis were the same as described above.

HPLC

In the HPLC separations, the eluent used was acetonitrile—methanol—ammonium formate buffer. A Whatman Partisil-10 PAC, a bonded cyano-amino-type column was used at a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Ratio of acetonitrile to methanol concentration in the eluent

The capacity ratios (k') of the disaccharides (ΔDi-0S, ΔDi-6S and ΔDi-4S) were determined as a function of the acetonitrile and methanol content of the mobile phase, while keeping the content of ammonium formate buffer constant at 20% (Fig. 1). Benzene was used as the inert peak in the calculations of the k' values. For sulfated disaccharides the k' values first decrease with increasing acetonitrile content and decreasing methanol content, pass through a minimum at an acetonitrile—methanol ratio of about 3.0 and then increase sharply. The k' value of nonsulfated disaccharide increases slightly in the region in which k' values of sulfated disaccharides decrease and then increases sharply as did the sulfated disaccharides. Fig. 1 demonstrates that the acetonitrile and methanol content is a valuable parameter for adjusting the retention. It is found that at a ratio of acetonitrile to methanol of 3.0, effective separation and good resolution of the three disaccharide isomers in the enzyme digest of urinary GAG is achieved. Thus, in further investigations of the influence of parameters in this aqueous buffer, this ratio for the two organic solvents was used.

Effect of pH, concentration and content of aqueous ammonium formate

The dependence of the k' values on the pH of ammonium formate buffer for the three unsaturated disaccharides was studied. For sulfated disaccharides the k' values decrease with an increasing pH of the buffer. However, the k' value of ΔDi-0S only changes slightly when the pH was varied from 3.5 to 5.75. Peak shapes of the three compounds become more symmetrical as the pH is

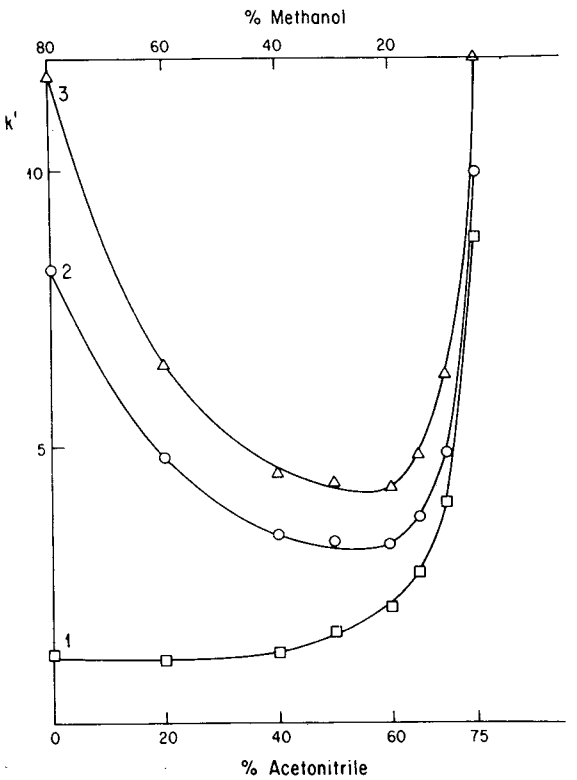

Fig. 1. Capacity ratios (k') of the three unsaturated disaccharides as a function of the acetonitrile and methanol content of the mobile phase. 1 (□), ΔDi-0S; 2 (○), ΔDi-6S; 3 (△), ΔDi-4S; solvent system, acetonitrile—methanol—0.5 *M* ammonium formate pH 4.5 (X:Y:20, v/v/v) (X, % acetonitrile; Y, % methanol; $X + Y = 80\%$). Column, Whatman Partisil-10 PAC; flow-rate, 2.0 ml/min.

increased. Although the degree of retention can be affected by the pH of aqueous buffer, good resolution (selectivity factors for any two of the disaccharides are at least 1.22) is observed in the pH range tested. The three disaccharides are more retained as the ionic strength decreases. The baseline separation for the three compounds can be obtained when the concentration of ammonium formate is below 0.6 *M*. The effect of buffer content in the mobile phase on the k' values of the three disaccharides is shown in Fig. 2. As expected, the k' values decrease as the amount of aqueous buffer is increased. This phenomenon is probably due to the increase in the overall ionic strength of the mobile phase and the increased solvation of the solutes as the solvent becomes more polar.

All of the parameters in the mobile phase described above have a significant effect on separation. In particular, the ammonium formate buffer content in combination with variation of pH can be used to adjust the retention and selectivity of these unsaturated disaccharides. These studies demonstrate the flexibility of the HPLC system and serve as a guide for the selection of the best conditions for chromatography.

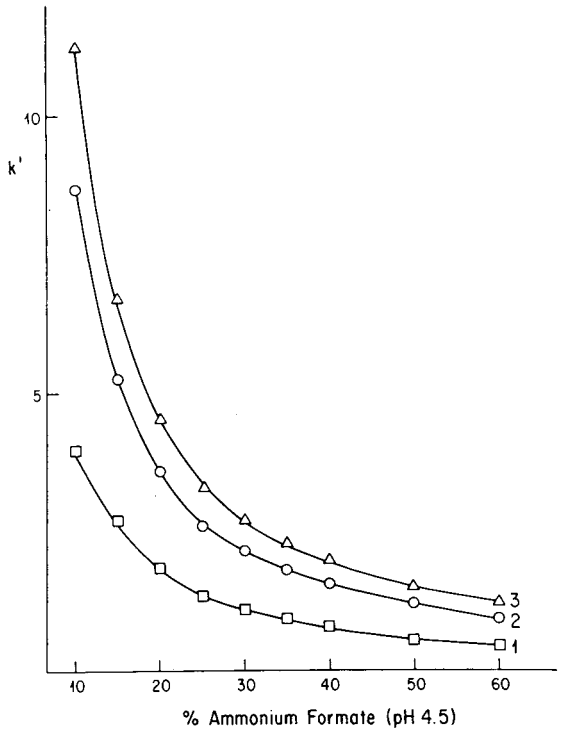

Fig. 2. Capacity ratios (k') of the three unsaturated disaccharides as a function of the ammonium formate content of the mobile phase. 1 (□), ΔDi-0S; 2 (○), ΔDi-6S; 3 (△), ΔDi-4S; solvent system, acetonitrile—methanol (3:1, v/v) with different amounts of 0.5 *M* ammonium formate (pH 4.5) added. Column, Whatman Partisil-10 PAC; flow-rate, 2.0 ml/min.

Linearity

The linearity of the HPLC response to the unsaturated disaccharides was evaluated by analyzing enzymatic digestion products derived from both fixed and varying amounts of normal urinary GAG. Duplicate urinary GAG (equivalent to a 8-ml normal urine in each) was subjected to enzyme reaction with chondroitinase ABC and AC respectively. The resulting disaccharide products, dissolved in 200 μl 90% methanol, were injected onto the HPLC column in increasing amounts (from 5 μl to 20 μl). It was established that for the range of 100 ng to 10 μg a linear relationship existed between the peak heights of disaccharides and amounts of digestion products injected. This wide linear range is more than sufficient for the determination of disaccharide isomers in enzymatic digests of normal urinary GAG.

Fig. 3 illustrates the linear relationship between the amounts of isomeric chondroitin sulfates in mixtures with other urinary GAG and the peak heights of disaccharide products. The GAG isolated from 200 ml normal urine was dissolved in 2 ml distilled water. Aliquots of different volumes were subjected to enzymatic degradation with chondroitinase ABC (Fig. 3A) and AC (Fig. 3B) and products were determined by HPLC. The difference in the slopes of ΔDi-4S lines between Fig. 3A and 3B is insignificant which indicates that little DS is present.

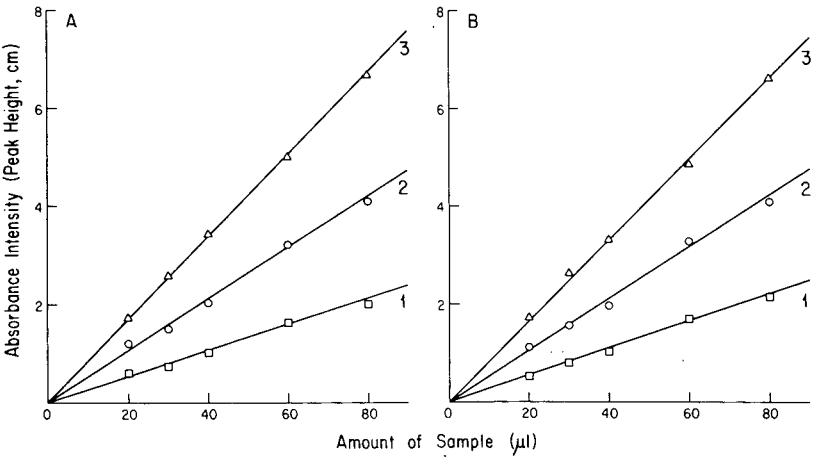

Fig. 3. Linear relationship between the amounts of chondroitin sulfate isomers in a normal urine and the peak heights of disaccharide products by HPLC. The amounts of sample are described in text. Ten percent of the degradation products from each sample were injected on the HPLC column. (A) Digestion with chondroitinase ABC; (B) digestion with chondroitinase AC. Compounds: 1 (□), ΔDi-0S; 2 (○), ΔDi-6S; 3 (△), ΔDi-4S.

Recoveries

Recovery studies were made by adding known quantities of standard C-4S, C-6S and DS to normal urine as described in the Experimental section. Chromatograms in Fig. 4 present a graphical illustration of recovery studies. All chromatograms are from independently prepared aliquots of enzymatic digestion products of GAG from normal urine with and without added standards. HPLC separations of disaccharides from normal urinary GAG after digestion with chondroitinase ABC and AC are shown in Fig. 4A and 4B, respectively. These blanks determine the amount of chondroitin sulfates in normal urine. The ΔDi-4S and ΔDi-6S are produced in similar amounts by treatment with either enzyme. Non-sulfated disaccharides from chondroitin and hyaluronic acid which are present in trace amounts appear at 4.2 min and are not completely separated. The recovery of added C-4S standard as its disaccharide fragments is illustrated in Fig. 4C and 4D, the effect of added C-6S standard is shown in Fig. 4G and 4H. The major peaks in these chromatograms correspond to the expected disaccharides, that is, ΔDi-4S in C-4S spiked urine and ΔDi-6S in C-6S spiked urine. The height increase of the ΔDi-6S peak in the enzyme digests of C-4S spiked urine and of the ΔDi-4S peak in the digests of C-6S spiked urine compared to the blanks (Fig. 4A and 4B), may be due to the presence of copolymers [16] and/or contaminants in the standard chondroitin sulfates. The added DS gave a large increase in amount of ΔDi-4S when digested with chondroitinase ABC (Fig. 4E), but did not when digested with chondroitinase AC (Fig. 4F).

Table I shows the recovery of the standard chondroitin sulfates added to normal urine, which represents the recoveries following three consecutive procedures: urinary GAG isolation, enzyme reaction and HPLC quantitation. The apparent lower recovery of DS in the chondroitinase ABC digest may be

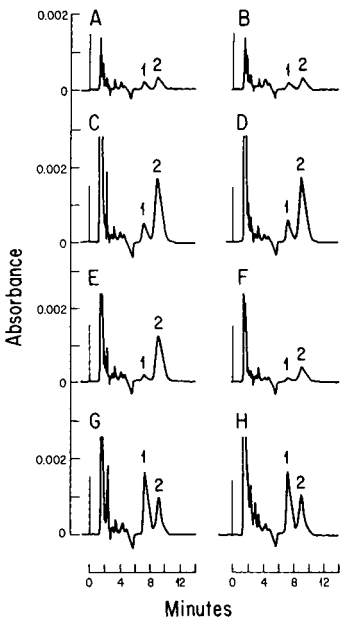

Fig. 4. Chromatograms of enzymatic degradation products of GAG from normal urine with and without standard isomeric chondroitin sulfates added. GAG from a 2-ml normal urine (A) degraded by chondroitinase ABC; (B) degraded by chondroitinase AC; (C) with standard C-4S spike degraded by chondroitinase ABC; (D) with standard C-4S spike degraded by chondroitinase AC; (E) with standard DS spike degraded by chondroitinase ABC; (F) with standard DS spike degraded by chondroitinase AC; (G) with standard C-6S spike degraded by chondroitinase ABC; (H) with standard C-6S spike degraded by chondroitinase AC. Peaks: 1, ΔDi-6S; 2, ΔDi-4S. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Solvent system, acetonitrile—methanol—0.5 *M* ammonium formate (pH 4.5) (60:20:20, v/v/v); flow-rate 2.0 ml/min; pressure, 700 p.s.i. Injection amount, 10% of the degradation products for (A) and (B), 7.5% of the degradation products for others. UV detection at 254 nm, 0.01 a.u.f.s.

due to oversulfated disaccharide products [17] which were not measured in this study. About 4% recovery of DS is found in the chondroitinase AC digest which could be due to contamination of the DS preparations by other chondroitin sulfates. Duplicate isolation of GAG and enzyme treatment from urines containing chondroitin sulfates as internal standards indicated that recovery had an experimental error of less than 7%.

Determination of isomeric chondroitin sulfates in normal urine samples

Table II represents the data for C-4S, C-6S and DS in twelve normal urine samples. The amount of C-6S was calculated from the mean values of ΔDi-6S by digestion of urinary GAG with chondroitinase ABC and AC. The C-4S was determined from the value of ΔDi-4S by digestion of urinary GAG with chondroitinase AC. The DS was calculated by subtracting the ΔDi-4S obtained by digesting GAG with chondroitinase AC from that with chondroitinase ABC. The data show that C-4S is the major component of normal urinary chondroitin sulfate isomers and comprises at least 72% of total urinary chondroitin sulfates in the cases examined. The C-6S is present in a lesser amount

TABLE I

RECOVERY OF ISOMERIC CHONDROITIN SULFATES ADDED TO NORMAL URINE

Fifty micrograms of standard C-4S, C-6S and DS were added separately to 2-ml samples of normal urine. The urinary GAG were isolated from these augmented urines and incubated separately with chondroitinase ABC and AC as described in Materials and methods. The resulting disaccharide products were analyzed by HPLC.

Enzyme	Sources of GAG	Products detected (μg)			Recovery	
		Δ Di-6S	Δ Di-4S	Total	μg	%
Chondroitinase ABC	urine alone	2.91	6.72	9.63		
	urine + C-4S	9.05	39.88	48.93	39.3	78.6
	urine + DS	2.91	37.2	40.11	30.48	60.96
	urine + C-6S	29.09	18.05	47.14	37.51	75.02
Chondroitinase AC	urine alone	2.76	6.55	9.31		
	urine + C-4S	9.03	38.97	48	38.69	77.38
	urine + DS	2.6	8.85	11.45	2.14	4.28
	urine + C-6S	29.03	20.12	49.15	39.84	79.68

TABLE II

RELATIVE PROPORTIONS OF THE THREE CHONDROITIN SULFATES IN NORMAL URINE SAMPLES BY ENZYMATIC AND HPLC ANALYSES

Case No.	Initials	Age (years)	Sex	Isomeric chondroitin sulfates (by HPLC)				Total uronic acid in CPC fraction, by carbazole (nmole/mg creatinine)	Molar ratio*
				Total amount (nmole/mg creatinine)	Percent				
					C-4S	DS	C-6S		
1	L.S.	2	M	34.36	87.5	1.4	11.1	44.62	77
2	W.L.	3	F	42.33	86.8	2.7	10.5	57.99	73
3	T.C.	3	F	41.22	83.4	3.8	12.8	55.03	74.9
4	M.J.	4	M	26.71	91.7	—	8.3	40.84	65.4
5	J.C.	5	M	33.9	81.5	1.5	17	50.22	67.5
6	A.W.	11	M	21.33	91.8	—	8.2	30.87	69.1
7	H.L.	25	F	5.29	72.8	6.4	20.8	6.09	86.8
8	J.G.	29	M	3.91	69.4	7.1	23.4	4.75	82.4
9	L.L.	32	F	3.85	79.7	3.7	16.6	5.13	75.1
10	H.K.	34	M	3.82	74.9	12.5	12.6	5.21	73.3
11	J.Y.	38	M	3.82	75.6	4.2	20.2	4.85	78.8
12	M.K.	65	F	5.21	78.6	6.7	14.7	8.26	63.1

*Total amount of sulfated disaccharide produced from urinary chondroitin sulfate detected by HPLC was divided by the uronic acid content in CPC fraction.

whereas DS is found to be only a minor component and negligible in certain cases. Several reports have indicated that C-6S is excreted in the largest amounts among normal urinary chondroitin sulfate isomers [7—9]. However, our values of C-4S in normal urine are essentially in agreement with that reported by Linker and Terry [5]. The presence of a small amount of DS is consistent with most reported values [4, 7, 18]. The total amounts of chondroitin sulfate isomers per mg of excreted creatinine determined by the combined enzymatic and HPLC methods indicate that with an advancing age

there is a trend to decreased excretion of urinary chondroitin sulfates. It is also evident that the amounts of urinary GAG precipitated by CPC and measured as uronic acid by the carbazole method exhibit a similar trend. It has been reported that in the measurement of normal urinary GAG the uronic acid: creatinine ratio is high in children, falling to a low level in adults [19—21]. The present results are in accord with these data. The molar ratio of total chondroitin sulfate isomers to total uronic acid indicates that normal urinary GAG consists mainly of chondroitin sulfate isomers. Analysis of the relative urinary chondroitin sulfate levels may reflect the metabolic states of connective tissues in the human body. The HPLC separation used with these specific enzyme reactions is convenient and reliable for these studies.

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CHROMBIO. 701

GEL CHROMATOGRAPHIC SEPARATION OF HUMAN C-PEPTIDE AND PROINSULIN

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SUMMARY

The immunoreactivity of circulating C-peptide is separated into two main peaks on a Bio-Gel column; the faster peak should not be proinsulin but an associated C-peptide without a covalent bond. Proinsulin is in fact eluted in the fraction prior to the faster eluting peak of C-peptide immunoreactivity with 1 *M* acetic acid as the eluting buffer. Therefore the use of gel chromatography to study C-peptide and proinsulin needs to be carefully re-evaluated, although the method has been established as one of the standard methods.

INTRODUCTION

It is now well established that proinsulin is synthesized as a precursor molecule and is converted within the pancreatic beta cell to insulin with release of the connecting peptide and several basic amino acids [1]. C-peptide is released from beta cells in equimolar amounts with insulin [2] and can be detected in the peripheral circulation by radioimmunoassay [3]. The gel chromatographic separation of circulating C-peptide in the peripheral circulation was performed by Block et al. [4] using 3 *M* acetic acid as eluent after acid—alcohol extraction

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[5, 6]. C-peptide immunoreactivity (CPR) in human plasma separates into two peaks on a Bio-Gel column; the faster eluting peak elutes in the region corresponding to proinsulin, and the other peak elutes in the region corresponding to insulin [3, 4]. The first peak should contain in part the immunoreactivity of proinsulin, but the proportion of proinsulin and associated C-peptide in this peak has not been entirely elucidated. The present study was undertaken to determine the nature of circulating CPR after acid—alcohol extraction in man, using the Bio-Gel column by which two analogues of insulin were separated [7, 8].

EXPERIMENTAL

Materials

Reagents. Bio-Gel P-30 (100—200 mesh) was obtained from Bio-Rad, Richmond, CA., U.S.A. $Na^{125}I$ (carrier free) was obtained from The Radiochemical Centre, Amersham, Great Britain. Anti-pig insulin guinea-pig serum M 8309 and porcine monocomponent insulin were generous gifts from Dr. J. Lindholm (Novo Research Institute). Single component insulin was obtained from Drs. R.E. Chance and M.A. Root (Lilly Research Laboratories). The C-peptide assay kit was obtained from Daiichi Radioisotope Laboratories, Tokyo, Japan. All other chemicals were reagent grade.

Subjects. Approximately 1 g of insulinoma tissue was obtained from a patient (aged 53 years, female, fasting blood sugar level = 20—70 mg/dl) as a surgical specimen. Four normal subjects (aged 20—23 years, fasting blood sugar level = 84—95 mg/dl) and four diabetic subjects on diet therapy (aged 58—71 years, fasting blood sugar level = 108—149 mg/dl) were studied. There was no significant difference in obesity between normal and diabetic subjects.

Methods

Tests. All normal and diabetic subjects were given oral glucose tolerance tests (O-GTT). The serum samples taken at 60 min after the O-GTT were frozen at −20°C until used.

Extraction and gel chromatography. A piece of insulinoma tissue was immediately placed in Krebs—Henseleit bicarbonate buffer solution equilibrated with O_2—CO_2 (95 : 5). Within 20 minutes after removal from the patient, the tissue was homogenized in a siliconized glass tube with acid—ethanol (350 ml of 99.5% ethanol, 7 ml of conc. HCl, 153 ml of distilled water) using a Polytron PT 10-35 (Kinematica, Steinhofhalde, Switzerland) for 1 min at setting 7. Subsequent extraction and gel filtration of the samples were the same as the previously reported methods [7].

The extracted sample was dissolved in 3 ml of 1 *M* acetic acid, and centrifuged at 600 *g* for 5 min. The clear supernatant was applied to a Bio-Gel P-30 (100—200 mesh) column (90 × 1.6 cm) equilibrated with 1 *M* acetic acid and eluted with the same elution buffer at 4°C with a flow-rate of 0.14 ml/min. The column was calibrated with porcine [^{125}I] insulin, porcine [^{125}I] proinsulin and synthetic [^{125}I] Arg-Arg-human C-peptide-Lys-Arg. The fraction size was 4.05 ml. Porcine [^{125}I] proinsulin elutes at a V_e/V_0 of 2.4, porcine [^{125}I] insulin at 4.7 and synthetic [^{125}I] Arg-Arg-human C-peptide-Lys-Arg at 4.8.

Assay for immunoreactive insulin and C-peptide immunoreactivity. Assay for immunoreactive insulin (IRI) and CPR was performed according to previously reported methods [10, 11].

Dilution tests for peaks I and II of CPR. Dilution tests for pooled peak I and peak II of CPR from normal subjects were performed using the C-peptide assay system.

Gel chromatography of CPR peak I obtained from normal subjects after treatment with 7 M urea. The CPR peak I fractions were pooled and lyophilized. The lyophilized sample was dissolved with 1 *M* acetic acid and urea was added to the solution to make a 7 *M* urea solution (total volume = 3 ml). The solution was applied to the same column as mentioned above with the same elution buffer. Each fraction was assayed with the same assay system used for CPR.

Dilution tests for proinsulin. The immunoreactivity that eluted in fraction 24 and which possessed both CPR and IRI activity was tested using the dilution technique with the same assay systems for CPR and IRI.

RESULTS AND DISCUSSION

The two derivatives of insulin were separated on the Bio-Gel column as described in earlier reports [7, 8]. Using the same column with the same elution buffer, the extracted CPR from human serum was also separated into two peaks as shown in Fig. 1. The immunoreactivities of peak I (V_e/V_0 = 3.0) and that of peak II (V_e/V_0 = 4.2) were studied by the dilution method using the C-peptide assay system. It was demonstrated that both peaks had the same immunoreactivity as that of synthesized human C-peptide as shown in Fig. 2. Peak I of CPR (V_e/V_0 = 3.0) was pooled and, after treatment with 7 *M* urea was gel chromatographed using the same column. Most of peak I of CPR (V_e/V_0 = 3.0) was transferred to the position of peak II of CPR (V_e/V_0 = 4.2 and 4.4) as shown in Fig. 3.

The values of peak I (V_e/V_0 = 3.0) and peak II (V_e/V_0 = 4.2) of CPR in the human peripheral circulation were studied by the same system (Table I). In normal subjects the total CPR level was 4.2 ± 1.5 ng/ml (mean ± S.D.). This

TABLE I

LEVELS OF PEAK I AND PEAK II OF CPR AT 60 min AFTER O-GTT

50-g oral glucose tolerance tests (O-GTT) were given to all normal and diabetic subjects. The serum samples taken at 60 min after O-GTT were frozen at −20° C until used. Extraction and gel filtration of the sera were the same as the reported methods [7]. All values were corrected with the reported recovery rate of extraction of C-peptide (0.607) [12] and the calculated recovery rate of gel filtration of labeled C-peptide (0.717). The results are expressed as mean ± S.D. All *P* values were obtained by a paired Student's *t*-test.

Case	Maximum BS* level (mg/dl) after 50-g O-GTT	Peaks of C-peptide (ng/ml)	
		Peak I	Peak II
Normals (n=4)	131.8 ± 24.6	2.10 ± 0.54	0.33 ± 0.21
	$P < 0.05$	n.s.**	n.s.
Diabetics (n=4)	251.8 ± 47.4	3.21 ± 1.02	0.63 ± 0.39

*BS = blood glucose.

**n.s. = not significant.

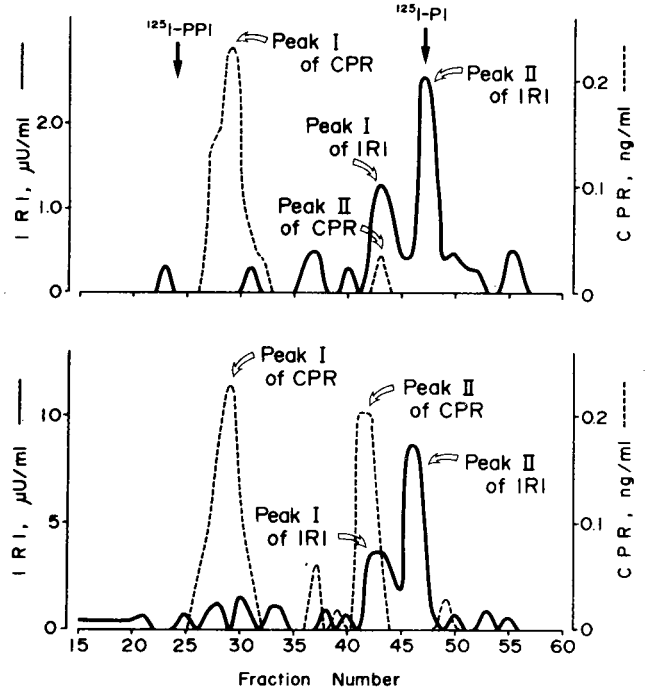

Fig. 1. Elution profiles of extracted human serum C-peptide (CPR) and insulin (IRI) on the Bio-Gel column (4.05 ml fraction size) obtained from a normal young adult and an adult onset diabetic patient on diet therapy. Upper panel: a normal adult (N.M., 23 years old, male). Lower panel: an adult onset diabetic patient (M.W., 68 years old, female). Detection level for CPR is 0.02 ng/ml and that for IRI is 0.2 μU/ml. PPI = porcine proinsulin, PI = porcine insulin.

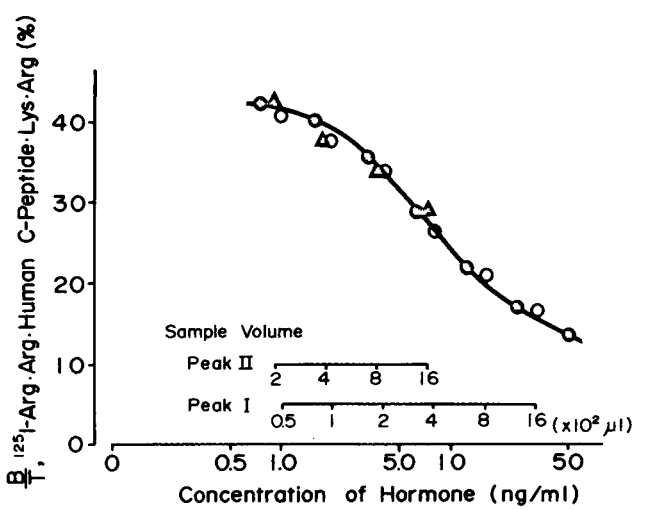

Fig. 2. Comparison of the immunoreactivities of peak I, peak II obtained from normal subjects, and synthesized human C-peptide in the CPR assay. Closed circle (•) represents synthesized human C-peptide. Open circle (○) represents peak I of CPR, and closed triangle (▲) represents peak II of CPR. All values are the mean from paired determinations. B/T = percent bound.

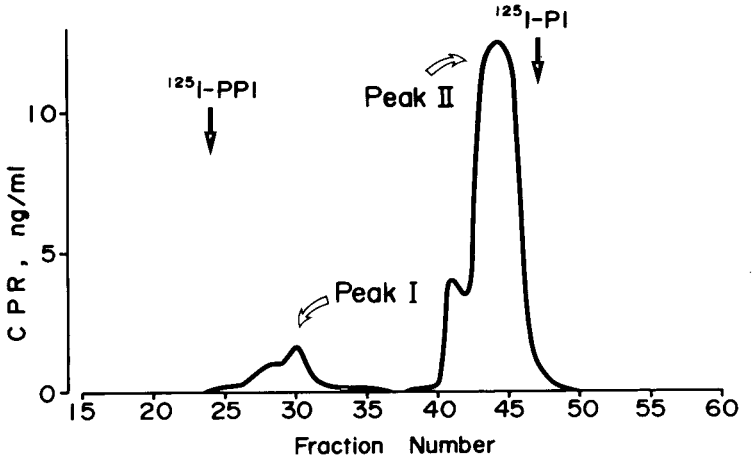

Fig. 3. Elution profile of pooled peak I of CPR obtained from normal subjects, after treatment with 7 *M* urea on the Bio-Gel column (4.05 ml fraction size). Detection level for CPR is 0.05 ng/ml. PPI = porcine proinsulin, PI = porcine insulin.

level is compatible with the previously reported result [4]. The value of CPR peak I was 2.1 ± 0.54 ng/ml (mean ± S.D.), and that of peak II was 0.33 ± 0.21 ng/ml (mean ± S.D.) at 60 min after a 50-g O-GTT. In diabetics, the total CPR value was 5.2 ± 2.4 ng/ml (mean ± S.D.). This level is compatible with the previously reported result [4]. The value of peak I for diabetics was 3.21 ± 1.02 ng/ml (mean ± S.D.), and that of peak II was 0.63 ± 0.39 ng/ml (mean ± S.D.) at 60 min after the 50-g O-GTT. There was no significant difference between the value for normal subjects and that for diabetics, although the absolute value of CPR in diabetics was more than that of normals. In the earlier reports [7, 8]

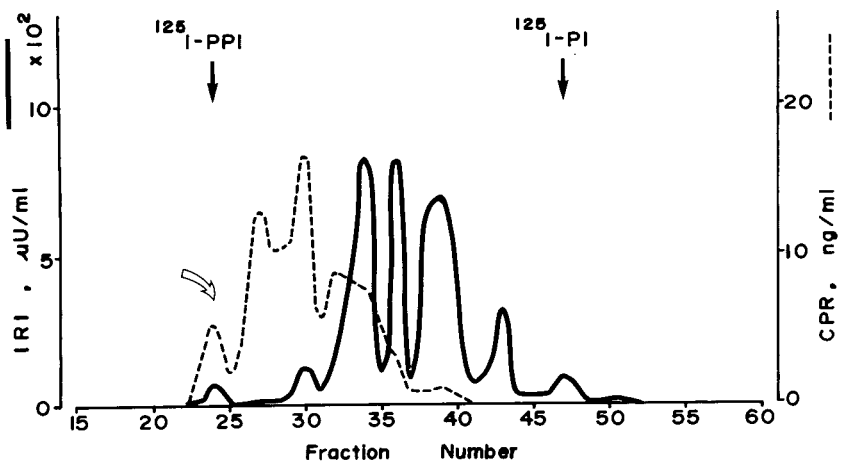

Fig. 4. Elution profile of extracted human C-peptide (CPR) and insulin (IRI) on the Bio-Gel column (4.05 ml fraction size) obtained from human insulinoma tissue (T.O., 53 years old, female). Open arrow shows the peak which has both CPR and IRI. Detection level for CPR is 0.4 ng/ml and that for IRI is 4 μU/ml. PPI = porcine proinsulin, PI = porcine insulin.

there was a significant difference between normals and diabetics in the values for insulin derivatives. Therefore, the involvement of the insulin secretion system in glucose stimulation could precede that of the C-peptide secretion system in the course of this disease.

Gel chromatographic separation of IRI and CPR was performed using the same methods after extraction from the insulinoma tissue (Fig. 4). There were possibly intermediates of insulin molecules, besides peak I (V_e/V_0 = 4.2) and

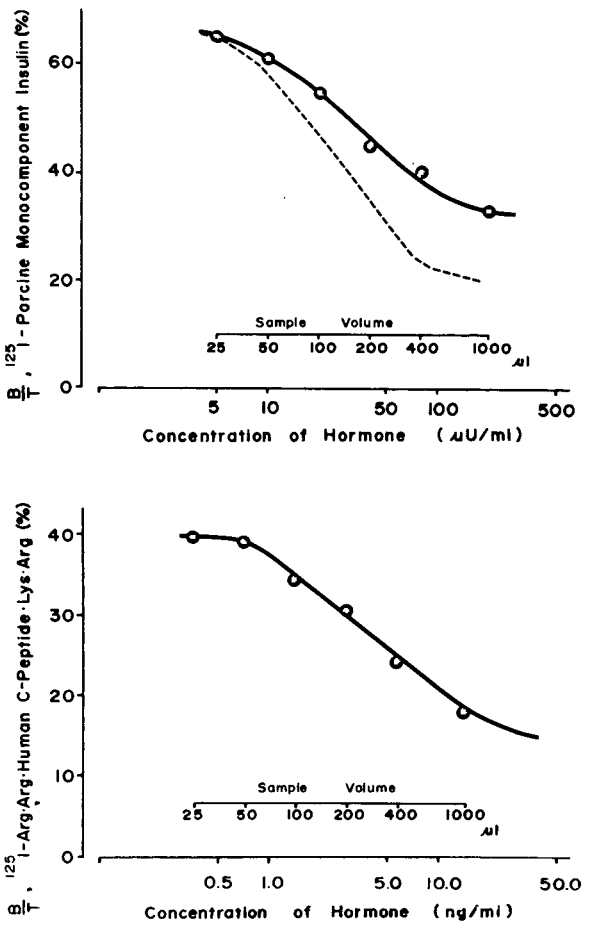

Fig. 5. Comparisons of the immunoreactivities of the peak which has the immunoreactivities of CPR and IRI obtained from human insulinoma tissue, porcine insulin and synthesized human C-peptide in the IRI (upper panel) and CPR (lower panel) assay systems. Upper panel: antiserum is anti-pig insulin guinea-pig serum M 8309. Standard hormone is porcine monocomponent insulin (lot No. 834098). Single component porcine insulin (lot No. 615-1082B-108-I) was used as labeled hormone after iodination with ^{125}I. Closed circles (•) represent the peak substance obtained from human insulinoma, which was eluted at the position of porcine [^{125}I]proinsulin. The dotted line (— — —) represents the immunoreactivity of standard hormone. Lower panel: C-peptide assay kit obtained from Daiichi Radioisotope Laboratories was used. Closed circles (•) represent the same peak substance as mentioned above. The solid line (—) represents the immunoreactivity of standard hormone. All values are the mean from paired determinations. B/T = percent bound.

peak II (V_e/V_0 = 4.8) of IRI [7, 8] and peak I (V_e/V_0 = 3.0) of CPR. Also a peak (V_e/V_0 = 2.4) which had both IRI and CPR activities and whose eluting position (V_e/V_0 = 2.4) corresponded to that of porcine [^{125}I] proinsulin, was detected (Fig. 4, open arrow). The immunoreactivity of this peak was studied by the dilution method using the C-peptide and insulin assay systems (Fig. 5). The immunoreactivity of this peak substance, corresponding to that of porcine [^{125}I] proinsulin, showed almost the same reactivity as that of human proinsulin [9] (Fig. 5, upper panel), and the same peak showed the same immunoreactivity as that of synthesizes human C-peptide [4, 11] (Fig. 5., lower panel). In their reported study [9, 11], natural human proinsulin was obtained from Drs. Steiner and Rubenstein. In the report of Block et al. [4], who carried out their work in the same place as Drs. Steiner and Rubenstein, the immunogenicity of human proinsulin is also almost the same as that of human C-peptide in their assay system. These results are compatible with our results (Fig. 5). Therefore the peak substance should be human proinsulin itself.

From the above-mentioned results, the following conclusions can be made: (1) There are two groups of C-peptide immunoreactivity in human peripheral circulation: one elutes rapidly (V_e/V_0 = 3.0) and is the associated form of C-peptide, the other is a slower peak (V_e/V_0 = 4.2). And the association of C-peptide is not by means of a covalent bond. (2) The involvement of the insulin secretion system could precede that of the C-peptide secretion in the course of diabetes under the load of glucose stimulation. (3) Proinsulin should be eluted at the position (V_e/V_0 = 2.4) which precedes peak I of CPR on the column. Therefore the gel chromatographic study for proinsulin and C-peptide needs to be re-evaluated carefully, although this method has been established as one of the standard methods.

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CHROMBIO. 706

ISOTACHOPHORESIS FOR THE DETERMINATION OF OXALATE IN UNPROCESSED URINE

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SUMMARY

The principle of isotachophoresis has been used to develop a simple, specific and sensitive analytical procedure for the determination of oxalate in unprocessed urine. Analytical conditions were optimized. The accuracy and precision of the method were estimated. The specificity was checked with oxalate decarboxylase. Separation of oxalate from a number of organic acids was achieved. The influence of factors such as storage, calcium concentration, pH or ionic strength was examined.

The 24-h urine excretion rates for healthy children, healthy adults and for patients with idiopathic stone formation were established. Lower absolute excretion rates were found in children and females. Urinary oxalate/creatinine ratios were higher in children than in adults. The mean oxalate excretion in 24-h urines of adult healthy individuals was 413 ± 150 μmol per 24 h per 1.73 m^2 (range 195—732). The mean oxalate/creatinine ratio was 0.033 ± 0.011 (range 0.018—0.065).

INTRODUCTION

In clinical chemistry there is a need for a simple, rapid and specific method for the determination of oxalate in urine. None of the currently existing methods meets all these requirements and none of the existing methods allows the determination of oxalate in unprocessed urine.

Previously, Everaerts et al. [1] demonstrated that carboxylic acids can be quantitated in aqueous solutions by isotachophoresis. In a preliminary communication [2] it has been demonstrated that the isotachophoretic measurement of oxalate in aqueous samples and urines is possible without pretreatment procedures.

It is the aim of the present investigation to give a complete and detailed

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description of this new method and to present some results of its application to human urines in health and disease.

PATIENTS AND METHODS

Experimental

The isotachophoretic separations and determinations were performed in an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with the 23-cm Teflon capillary (I.D. 0.5 mm).

The starting voltage was 2 kV with the final voltage varying between 4 and 8 kV at 150 μA and 12°C. UV absorption at 254 nm was used for quantitation. Reliable identification of oxalate by the specific step height of the thermal signal was possible when the injected oxalate content exceeded 2 nmol. The time for analysis depended on the electrolyte content of the sample and varied between 20 and 60 min. The chemicals used were generally commercially available and of analytical grade. The water used was twice-distilled. Oxalate decarboxylase (EC 4.1.1.2) was purchased from Sigma (Munich, G.F.R.; No. 0-3500).

Electrolytes

Leading electrolyte: HCl Suprapur (E. Merck, Darmstadt, G.F.R.) 5×10^{-3} *M*, NaCl 1×10^{-3} *M*, 0.4% HPMC (Methocel 90 HG, 15,000 cps; Dow Chemical, Midland, MI, U.S.A.). No adjustment of pH (2.2) was carried out.

Terminating electrolyte: acetic acid 5×10^{-3} *M* (p.a. grade; Merck).

The electrolyte solutions were replaced after each run. In addition, the purification procedure for the terminating electrolyte solution was performed as proposed by Everaerts et al. [1].

Injection

The samples were injected using 10-μl Hamilton microsyringes (for some experiments Model 700 with stainless-steel cannula and plunger, and for routine purposes Model AA 701 SN equipped with a platinum needle and tungsten plunger, were used). The syringes had been siliconized twice before use. The samples were injected through the septumless syringe injector, as described by Fredriksson [3], into the leading electrolyte, the injection volume being adjusted to give at least 1 nmol of oxalate. The injection volume varied between 2 and 10 μl (leading electrolyte: 10×10^{-3} *M* NaCl).

Evaluation

The quantity of oxalate was determined by measuring the zone length with a graticule or a ruler. The usual chart speed was 0.5—2 mm/sec. For the reasons given below, calculation of the oxalate content of the samples was performed using the combined zone lengths of both the UV-absorbing and UV-non-absorbing zone of oxalate present in each sample examined (Fig. 1). Though oxalate shows a weak UV absorption at 254 nm, for better discrimination the term "UV-non-absorbing zone" is used in the following.

Treatment with oxalate decarboxylase

The effect of oxalate decarboxylase treatment on urinary or aqueous solutions of oxalate was tested at a final enzyme concentration of 0.2 U/ml at pH 3 and 37°C. The pH was kept constant by using either the citrate buffer as proposed by Hallson and Rose [4], or a 20 mmol phthalate—HCl buffer. Because of its pronounced UV absorption phthalic acid can be easily detected by isotachophoresis. The incubation time varied between 2 min and 16 h.

Urine samples

Urines were collected under outpatient conditions without dietary restrictions from 17 healthy children, 27 healthy adults, 18 adult idiopathic renal stone formers and two individuals with congenital hyperoxaluria. Urines were collected in plastic bottles kept at 4°C during the collection period. Concentrated $ZnCl_2$ (final concentration 10 mmol/l) was then added and urinary oxalate measured within 12 h after collection of the above urines.

RESULTS

Measurement of oxalate in aqueous solutions and urine samples

Aqueous solutions of oxalate were tested with concentrations in the range 0.1—10 mmol/l. The injected volume was adjusted so that the amount injected was 1—10 nmol of oxalate. As shown for urine in Fig. 1, a small UV-absorbing zone was recorded which was followed by a UV-non-absorbing zone. Both zones had high isotachophoretic mobilities. The lengths of the UV-absorbing and the UV-non-absorbing zones increased in parallel with increasing oxalate concentrations. In all aqueous or urinary solutions, these two zones were the first zones that could be detected by the UV detector.

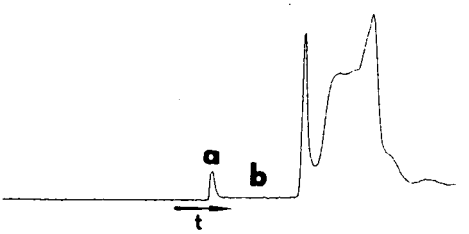

Fig. 1. Isotachopherogram (UV detection) of a 24-h urine sample. Only the initial zones are shown. Injected volume 10 µl; calculated oxalate concentration 470 µmol/l; injection with Hamilton microsyringe Model 700. (a) UV-absorbing zone of the assumed ferrioxalate complex; (b) UV-non-absorbing zone of oxalate.

When Model 700 Hamilton microsyringes (steel needle, steel plunger) were used, considerable problems were encountered, which could be avoided when Model AA 701 SN (see below) was used. The relative contribution of the UV-absorbing zone increased from 5—10% to 10—30% when Model 700 syringes had been in use for more than one week. This observation suggested that the first UV-absorbing zone might represent oxalate in complex form, probably due to interaction of oxalate with cations released from the surfaces of the syringe. Evidence for this assumption was provided by treating aqueous solu-

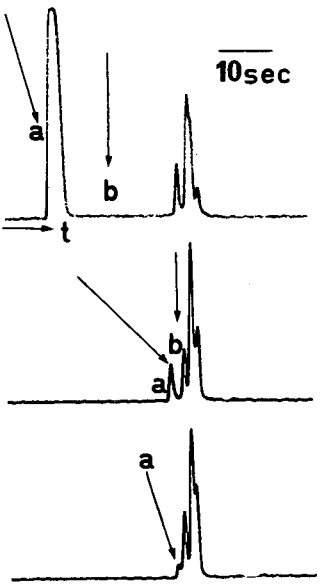

Fig. 2. Isotachophoretic monitoring of enzymatic decarboxylation of oxalate in aqueous solution by oxalate decarboxylase (EC 4.1.1.2). Conditions: oxalate 1 mmol/l, oxalate decarboxylase 0.2 U/ml, pH 3, citrate buffer after the method of Hallson and Rose [4]. The samples were injected with Hamilton microsyringe Model 700. Oxalate was measured isotachophoretically immediately, after 10 min and after 30 min of addition of the enzyme. After 30 min only a small shoulder of the initial UV-positive zone can be detected. For details see text. (a) UV-absorbing zone of oxalate (ferrioxalate complex); (b) UV-non-absorbing zone of oxalate.

tions of oxalate with oxalate decarboxylase, which decarboxylates oxalic acid specifically; under the influence of the enzyme both zones were markedly diminished and disappeared completely after 30 min (Fig. 2). It was further observed that the UV-absorbing zone of oxalate could be greatly increased by adding ferric ions as chlorides [2] or nitrates [5]. As all steel surfaces in contact with air are covered by a layer of ferri oxide, the first UV-absorbing zone of oxalate is probably a ferrioxalate complex. In aqueous solutions of oxalate, Fredriksson [5] observed that the UV-absorbing zone disappeared after addition of EDTA. In urine, however, addition of EDTA (up to 50 mmol/l), EGTA (50 mmol/l), desferrioxamine (10 mmol/l) or oxaloacetate (50 mmol/l) did not significantly influence the UV-absorbing zone, though treatment with oxalate decarboxylase affected both zones in a similar manner as in aqueous solutions (Fig. 3). The difference between aqueous and urine samples remains unexplained. Addition of spermine, spermidine or putrescine, which are known to form salts or complexes with oxalate [6], did not affect the relative length of the UV-positive or UV-negative zone of oxalate.

A significant reduction in the UV-absorbing zone of oxalate in urine samples was observed when Model 701 syringes with platinum needles and tungsten plungers were used, which had been siliconized twice before use (Hamilton microsyringe Model AA 701 SN). Any possible interaction of urine with the septum and septum bleed was avoided by using the septumless injector as described by Fredriksson [3].

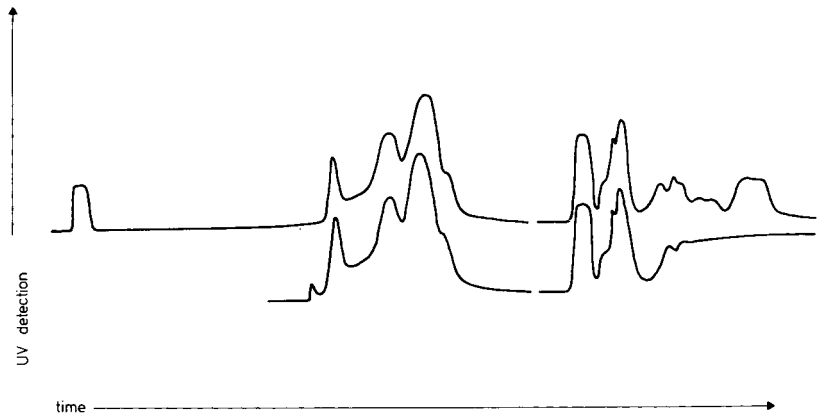

Fig. 3. Isotachophoretic run (UV detection) of a 24-h urine sample of a 11-year-old female child with congenital hyperoxaluria (oxalosis type I). The sample was analyzed by isotachophoresis both before and after treatment with oxalate decarboxylase (0.2 U/ml, incubation time 12 h, pH 3, 20 mmol phthalate—HCl buffer). Treatment with oxalate decarboxylase resulted in a significant, but not complete, disappearance of both zones of oxalate (lower curve). Injected volume 10 μl; time of analysis 40 min; calculated oxalate concentration 1150 μmol/l; injection with Hamilton microsyringe Model 700, which had been in use for two weeks.

It follows from the above experiments that both the UV-positive and the UV-negative zones contain oxalate. Consequently, for quantitation both zones have to be measured. Oxalate concentrations in unknown samples were calculated from the combined zone length of both zones. The lengths of the UV-negative and UV-positive zones were added and the oxalate concentration in the sample was calculated by comparing the sum of the zone lengths with the sum of the zone lengths of standard solutions. There was a linear relation between the zone lengths (UV-positive plus UV-negative zone) and oxalate concentration in aqueous solutions between 0.1 and 10 mmol/l (r=0.998) and in urine between 0.1 and 2 mmol/l (r=0.995).

Reproducibility

Aqueous solutions of oxalic acid or sodium oxalate (1—4 nmol injected) gave excellent reproducibility (C.V. in ten repetitive determinations was 1.2%). In 24-h urine samples, ten repetitive measurements of five samples gave a C.V. of 3.2%, when the amount of injected oxalate was adjusted to at least 1—2 nmol.

Recovery

In 24-h urines the recovery of added oxalate (1 mmol/l) varied between 70 and 100%, probably due to crystal or complex formation. While the addition of EDTA, EGTA, magnesium, citrate, or the use of citrate as terminating electrolyte were all ineffective, rates of recovery could be markedly improved by the addition of $ZnCl_2$ (final concentration 10 mmol/l). Using $ZnCl_2$ as an additive to urine, 102% of added oxalate (range 80—111) was recovered when the total amount of oxalate injected was at least 1 nmol. The effect of the addition of $ZnCl_2$ to ten different urines to which oxalate and/or calcium chloride had

TABLE I

EFFECT OF ZINC IONS ON ISOTACHOPHORETIC RECOVERY OF URINARY OXALATE IN URINES, TO WHICH OXALATE, CALCIUM, OR BOTH HAD BEEN ADDED

Full recovery of 1 mmol/l added oxalate can be achieved even in the presence of additional 10 mmol/l $CaCl_2$. Without zinc, the addition of $CaCl_2$ results in significantly lower recovery rates of added oxalate.

No.	Added to urine samples:			
	$ZnCl_2$ 10 m*M*	$ZnCl_2$ 10 m*M* Oxalate 1 m*M*	Oxalate 1 m*M* $CaCl_2$ 10 m*M*	$ZnCl_2$ 10 m*M* Oxalate 1 m*M* $CaCl_2$ 10 m*M*
1	321	1366	1151	1348
2	303	1276	910	1285
3	116	1214	1000	1160
4	411	1392	1285	1392
5	821	1803	1285	1785
6	232	1178	553	947
7	419	1482	1017	1428
8	232	1312	1089	1160
9	249	1249	1026	1249
10	276	1339	741	1267
Oxalate (μmol/l) $\bar{x}$	338	1361	1005	1302
S.D.	191	179	228	218

been added, is depicted in Table I. In the presence of $ZnCl_2$, the recovery of exogenous oxalate was still complete in the presence of high concentrations of calcium (up to 15 mmol/l). In urine samples, optimal recovery was not observed before 2 h after addition of $ZnCl_2$. Therefore, in all experiments urines were examined at least 5 h after the addition of $ZnCl_2$.

Specificity

Since organic acids other than oxalic acid are present in urine, a variety of acids with comparable mobilities [1] were examined. Specimens containing the respective organic acid and oxalic acid in aqueous solutions and/or urine samples were analyzed by isotachophoresis. No evidence of interference was found. Furthermore, the addition of derivatives of oxalic acid, i.e. parabanic acid or oxaluric acid (Fig. 4), resulted in zones that were clearly separable from oxalic acid by either the thermal signal or the UV signal. Some compounds may cause interference under strong conditions by releasing or forming oxalic acid. Oxalic acid could be detected in significant amounts after hydrolysis (120—150°C, pH 3, aqueous solution) of oxalacetic acid, parabanic acid and oxaluric acid. This is in accordance with previous observations by Hodgkinson and Zarembski [7]. The oxalic acid released under these conditions could be completely decarboxylated by oxalate decarboxylase. Oxalate decarboxylase did not attack native oxaluric acid (Fig. 5).

Influence of pH, ionic strength, and storage

Changing the pH of the urine samples from 4 to 8 did not influence the results. Changing ionic strength by increasing the sodium chloride concentra-

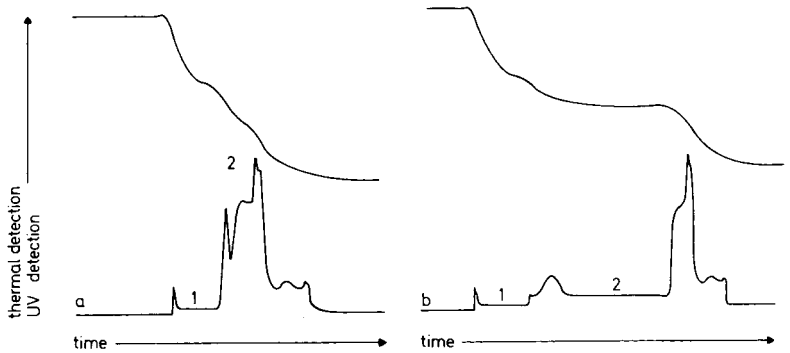

Fig. 4. Isotachopherogram (thermal detection and UV detection) of a 24-h urine sample, both before (a) and after (b) addition of oxaluric acid (1 mmol/l). Oxaluric acid has a high isotachophoretic mobility and is recorded with weak UV absorption. In higher concentrations it can be reliably separated from oxalic acid by its significantly different thermal step height. In all urine samples, oxaluric acid is separated from oxalate by yet unidentified substances, which precede oxaluric acid and show pronounced UV absorption. The traces of the thermal signal have been adapted to the UV traces for better comparison. 1 = Oxalic acid; 2 = oxaluric acid.

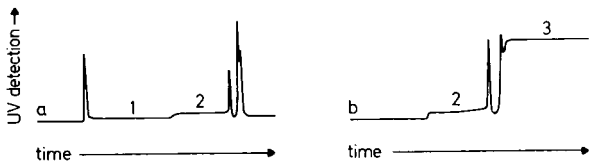

Fig. 5. The effect of oxalate decarboxylase treatment on an aqueous sample containing oxalic acid and oxaluric acid. (a) Aqueous mixture of oxalic acid and oxaluric acid (10 nmol of each substance injected). (b) Isotachophoretic record of the same sample, which has been incubated with oxalate decarboxylase in a 20 mmol phthalate—HCl buffer (pH 3). Both zones of oxalate completely disappeared from the isotachopherogram after 30 min incubation. 1 = Oxalic acid; 2 = oxaluric acid; 3 = phthalic acid.

tion in urine samples did not markedly influence recovery rates (± 5%) but increased the time of analysis considerably (up to 90 min). Significant changes of measured oxalate concentration were seen after storing. At both 4°C and −25°C, up to 20% of all urine samples showed deviations in an unpredictable way, i.e. decreases or increases of up to 20%. This has also been observed by other investigators [8].

Effect of oxalate decarboxylase treatment on isotachophoretic measurement of oxalate in aqueous and urine samples

In aqueous samples of oxalate, addition of oxalate decarboxylase (final concentration 0.2 U/ml, incubation time 2—30 min, pH 3, citrate buffer) caused complete disappearance of both the UV-positive and the UV-negative zones (Fig. 2). In contrast, in urine samples neither the UV-positive nor the UV-negative zone disappeared completely after treatment with decarboxylase for up to 16 h, although there was a parallel decrease in both zones (Fig. 3). Such a failure of oxalate decarboxylase to remove all the oxalate in urine may be related to the presence of substances in urine which are inhibitory to the enzyme.

It could be shown that both phosphate and sulfate up to 20 mmol/l were strong inhibitors of decarboxylase in aqueous solutions. While, in the absence of phosphate or sulfate, no oxalate was demonstrable after 30-min incubation under the conditions mentioned above, about 70% of the initial amount of oxalate was still present after 30 min in the presence of 20 mmol/l phosphate or sulfate. However, with both inhibitors prolonged incubation for 2.5 h still led to complete disappearance of oxalate in aqueous solutions. This was not the case in urinary solutions of oxalate, suggesting that additional inhibitory substances might be present or that other physico-chemical factors such as crystal formation or protein binding might be operative. Distinction between these possibilities for inhibition of oxalate decarboxylase in urine requires more detailed kinetic analysis.

Co-determination of other substances

A variety of substances that are important in renal stone disease (phosphate, citrate, glycolate) could be easily separated in aqueous solutions using the operational system given above. In an attempt to detect these and other acids in urine, the following substances could be detected by "spiking" (UV-positive substances) or "spacing" (UV-negative substances): oxalate, oxaluric acid, orotic acid, phosphate, citrate, glycolate, formate (Fig. 6). Quantitative co-determination of urinary citrate was possible with high accuracy and precision [9].

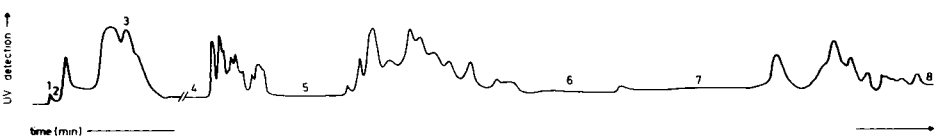

Fig. 6. Complete isotachopherogram (UV detection) of a 24-h urine sample with low oxalate content, to which glycolic acid (1 mmol/l) has been added. A number of anionic substances could be identified by "spiking" for UV-absorbing substances and "spacing" for non-UV-absorbing substances: 1 = ferrioxalate complex of oxalate; 2 = UV-non-absorbing zone of oxalate; 3 = orotate; 4 = phosphate; 5 = citrate; 6 = glycolate; 7 = formate; 8 = acetate (terminator). For quantitation of glycolate, other operational systems might be preferable, since at low concentrations of glycolate no clear-cut separation is possible from other anions either by the UV signal or by the thermal signal. Glycolate can be recognized without difficulty, however, in urine of patients with primary hyperoxaluria type I, who usually excrete high amounts of glycolate.

Urinary oxalate in healthy individuals and in renal-stone patients

As shown in Table II, urinary oxalate was lower in children than in adults. Urinary oxalate excretion was higher in adult males than in adult females [495 ± 192 (S.D.) μmol per 24 h vs. 336 ± 81 (S.D.) μmol per 24 h]. However, if oxalate excretion was corrected for urinary excretion of creatinine (thus accounting for differences in muscle mass and/or incomplete urine collection) no difference was present between male and female adults [34 ± 13 (S.D.) μmol/mmol creatinine vs. 31 ± 6 (S.D.) μmol/mmol creatinine].

The frequency distribution of the oxalate/creatinine ratio in 44 healthy individuals (age 3—48 years, weight 14—120 kg, 17 female, 27 male) is given in Fig. 7. Within the age bracket studied, i.e. if children were included, a signifi-

TABLE 2

OXALATE EXCRETION, MEASURED BY ISOTACHOPHORESIS, IN HEALTHY CHILDREN, HEALTHY ADULTS AND IDIOPATHIC STONE FORMERS

	Healthy children (6 F, 11 M)		Healthy adults (10 F, 17 M)		Idiopathic stone formers (8 F, 10 M)	
	$\bar{x}$ ± S.D.	Range	$\bar{x}$ ± S.D.	Range	$\bar{x}$ ± S.D.	Range
Age (years)	8.4 ± 3.6	3—14	28 ± 8.5	17—48	37 ± 14	18—70
Weight (kg)	28.1 ± 10.9	14—48	68.4 ± 17.5	45—120	73 ± 11.2	57—93
UVO_x (μmol per 24 h)	292 ± 162	87—650	436 ± 176	190—815	471 ± 195	207—871
Oxalate per 1.73 m^2 body surface (μmol)	488 ± 203	148—801	413 ± 150	195—732	445 ± 186	190—878
Oxalate/ creatinine (μmol/ mmol)	0.066 ± 0.021	0.034—0.095	0.033 ± 0.011	0.018—0.065	0.034 ± 0.011	0.015—0.057

cant correlation could be found between UVO_x on the one hand and age, weight, body surface or UVC_r on the other hand (Fig. 8). However, within the groups of adult individuals, no such correlations existed. Normalization of urinary oxalate for urinary creatinine seemed to be the best approach to express urinary oxalate excretion (Fig. 8, Table II). A correlation between the urinary excretion rate of calcium and urinary excretion rate of oxalate was found, which was modest (r=0.3) in females and more marked (r=0.6) in males. The day-to-day variability of urinary excretion of oxalate was examined in five subjects on three consecutive days; the C.V. was 15%.

Urinary oxalate was also measured in two female children with congenital hyperoxaluria and recurrent stone formation (patient 1: 11 years, 3 operations,

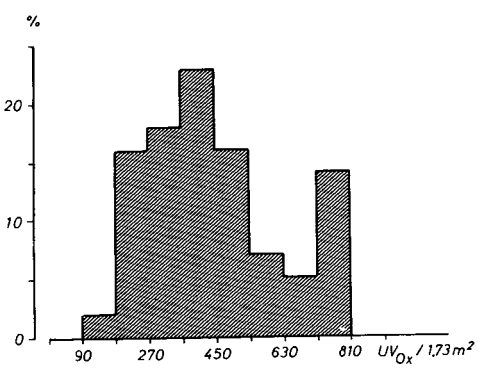

Fig. 7. Distribution of urinary oxalate/creatinine ratio in 44 healthy individuals [17 children (6 F, 11 M), 27 adults (10 F, 17 M), age 3—48 years, weight 14—120 kg].

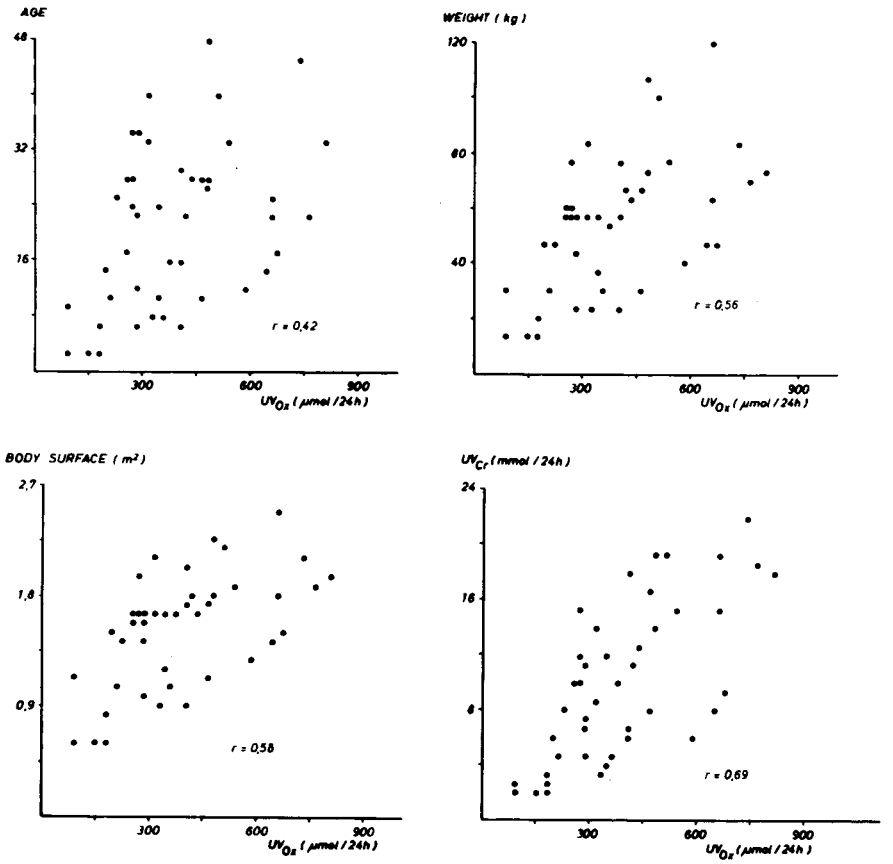

Fig. 8. Correlation of oxalate excretion rates with age, weight, body surface and urinary excretion of creatinine in 44 healthy individuals (children and adults).

GFR 80 ml/min; patient 2: 17 years, 9 operations, GFR 60 ml/min). Urinary oxalate in patient 1 was 1170 µmol per 24 h, in patient 2, 1890 µmol per 24 h. The isotachopherogram of a 24-h urine sample of patient 1 is given in Fig. 5b, before and after treatment with oxalate decarboxylase.

DISCUSSION

Isotachophoresis is based on the principle that the net mobility of each of the participating ions is constant under defined conditions. At equilibrium, therefore, the ion concentration in each unit length of a given zone is constant. Quantitation can then simply be achieved by measuring the length of each sample zone.

Oxalic acid is characterised by two dissociation constants; pK_{A^1} 1.19 and $pK_{A^2} = 4.21$. The low pH of the leading electrolyte solution was chosen for two reasons: first, oxalate is most soluble at low pH; second, at pH 2.2 nearly all oxalic acid exists as the monoprotonated species, $HC_2O_4^-$. The use of a pH at which oxalate is present almost exclusively as one molecular species offers

considerable advantages since, at other pH levels, determination of oxalate could be difficult because of its unique properties as a complexing agent [10, 11].

Isotachophoretic measurement of urinary oxalate has the advantage of permitting examination of urine without pretreatment such as precipitation steps, extraction steps, ion-exchange chromatography. Furthermore, the technique is rapid, simple and easy to perform. Finally, co-determination of other substances involved in lithogenesis (e.g. citrate, glycolate) can easily be carried out.

Previous investigations [1, 2, 5] demonstrated that oxalate can be measured by isotachophoresis in aqueous solutions. The measurement is complicated by the fact that a UV-positive zone, presumably consisting of Fe—oxalate complexes, precedes oxalate which is registered as a UV-negative zone. The present study demonstrates that the technique used to measure oxalate in aqueous solutions is unsatisfactory for urine samples. However, formation of the putative Fe—oxalate complex can be avoided with proper modifications (reduction of the Fe-containing surfaces by special syringes). Furthermore, it could be demonstrated that addition of zinc chloride considerably improved reproducibility and recovery of isotachophoretic measurements of oxalate in urine. The mechanism by which zinc chloride improves recovery (virtually 100%) has not been completely clarified, but it is suggestive to assume that complex formation takes place [10, 11]. With these modifications (syringes with low Fe content, addition of Zn) oxalate can be measured satisfactorily in urine samples.

Quantitation of oxalate must take into consideration the fact that oxalate is contained in both the UV-positive and the UV-negative zones, as demonstrated by the virtual disappearance of both zones after incubation with oxalate decarboxylase. It has not been strictly demonstrated that the molar concentration of oxalate in both zones is identical. However, comparison of standard curves in aqueous solution and recovery curves in urine samples demonstrate that the resulting error, if any, must be very small.

It is difficult to compare the normal range, as found in the present study, with data in the literature, since no absolute reference method exists. Furthermore, the marked dependence of urinary oxalate excretion rate on body surface, as confirmed in the present study, makes it impossible to evaluate data in the literature which are not normalized for body surface area. In sixteen adult subjects, Gibbs and Watts [12] found a range of 24—49.3 mg of anhydrous oxalic acid per 24 h per 1.73 m^2, corresponding to 266—548 μmol per 24 h per 1.73 m^2. Hodgkinson and Williams [13] reported a range of 17.2—46.8 mg of anhydrous oxalic acid per 24 h per 1.73 m^2, corresponding to 191—520 μmol per 24 h per 1.73 m^2, in 24 adults. These values are somewhat lower than those reported in the present study, but this may be a result of the small sample size in previous studies which may be particularly conducive to error because of the non-normal distribution of urinary oxalate excretion rates. With isotopic dilution methods, the problem of incomplete recovery may arise, since Gibbs and Watts [12] noted that radioactive oxalate equilibrates with some fraction of urinary oxalate which does not extract into organic solvents. Whether this fraction is identical with the oxalate still present in urine even after prolonged incubation with oxalate decarboxylase, remains conjectural. Although a number

of investigators reported differences in absolute oxalate excretion rate between males and females, the present study clearly demonstrates that urinary oxalate excretion rates, normalized for body surface, are identical in males and females. This finding is in agreement with previous studies of Gibbs and Watts [12] and Hodgkinson and Williams [13]. In contrast, urinary oxalate excretion rates, normalized for body surface area, were higher in children than in adults. This result is also in agreement with previous observations [14]. The reason for higher urinary oxalate in children is unknown, but it may be due to more effective intestinal calcium absorption in children, which is known to lead to a higher fractional intestinal absorption of oxalate [15]. Urinary oxalate, when studied under conditions of no dietary restriction, showed little day-to-day variability. However, more detailed studies with the isotachophoretic technique are required to define the dependence of urinary oxalate on dietary oxalate.

Progress in oxalate research has been hampered to a large extent by the lack of a simple, rapid and accurate method of measuring oxalate in body fluids. Isotachophoretic measurement of oxalate provides a valuable investigational tool for studies of urinary oxalate in metabolic disease, e.g. oxalosis and nephrolithiasis, as demonstrated by the present study. Urinary oxalate was found to be elevated in recurrent renal-stone formers by some [14] but not all [15] investigators and higher urinary oxalate excretion rates were also noted in the present study.

The present study provides clear evidence that isotachophoretic separation of unprocessed urine permits satisfactory measurements of urinary oxalate when the technical details described above are used. It is hoped that isotachophoresis will facilitate further research in this area.

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CHROMBIO. 702

QUANTITATIVE DETERMINATION OF TULOBUTEROL AND ITS METABOLITES IN HUMAN URINE BY MASS FRAGMENTOGRAPHY

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SUMMARY

A method is described for the simple and simultaneous determination of tulobuterol and its metabolites in human urine by gas chromatography—mass spectrometry. Quantification was achieved by single-ion monitoring at *m/e* 86 derived from trimethylsilyl-tulobuterol and its metabolites using a column packed with a mixed phase, 2% OV-1—2% QF-1 (1 : 1, w/w). The detection limits were estimated to be 2 ng/ml in urine for tulobuterol and 5 ng/ml for metabolites, respectively.

INTRODUCTION

Tulobuterol (Fig. 1) is one of the sympathomimetic amines synthesized by Koshinaka et al. [1], which has intensive bronchodilatory activity.

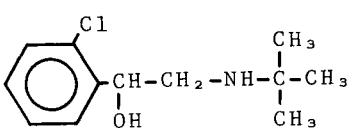

Fig. 1. Structure of tulobuterol.

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Determination of these sympathomimetic amines in biological fluids has mostly been undertaken using radio-labelled compounds [2—4], because of the relatively low concentration of these drugs in the body. Recently, quantitative analysis of salbutamol and terbutaline in plasma using gas chromatography (GC)—mass fragmentography has been reported by Martin and co-workers [5, 6] and Leferink et al. [7].

In the present work, we have described the simple and simultaneous determination of tulobuterol and its possible metabolites, predicted from metabolism in the rat [8], in human urine after the therapeutic dosage of tulobuterol.

EXPERIMENTAL

Materials

All reference samples of tulobuterol·HCl (I), its metabolites [4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV), 5-hydroxytulobuterol (V)], and 4-methoxytulobuterol (internal standard, IS) were prepared in this laboratory according to the published method [1]. N,O-Bis(trimethylsilyl)acetamide (BSA) was obtained from Nakarai Chemical (Kyoto, Japan) and glusulase from Endo Labs. (Garden City, NY, U.S.A.). Other reagents and solvents were of analytical grade and were used without further purification.

Gas chromatography

A Hitachi Model 063 gas chromatograph with a flame ionization detector was used. The glass columns were packed with 1.5% SE-30 (Chromosorb G AW DMCS, 60—80 mesh), 5% SE-52 (Chromosorb W AW DMCS, 60—80 mesh), 2% OV-1 (Chromosorb W AW DMCS, 100—120 mesh), 1.5% OV-17 (Chromosorb G AW DMCS, 60—80 mesh), 2% OV-225 (Gas-Chrom Q, 80—100 mesh), 2% F-50 (Chromosorb W AW DMCS, 80—100 mesh), 2% XF-1150 (Chromosorb W AW DMCS, 80—100 mesh), 2% Dexil 300GC (Chromosorb W AW DMCS, 80—100 mesh), 2% QF-1 (Gas-Chrom Q, 80—100 mesh), mixed phase of 2% OV-1 (Chromosorb W AW DMCS, 100—120 mesh) and 2% QF-1 (Gas-Chrom Q, 80—100 mesh) (1 : 1, w/w).

The flow-rates for the carrier gas (nitrogen), hydrogen, and air were 40, 30, and 580 ml/min, respectively. The temperature for both the injector and detector was 190°C.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out on a system of JMS D-300 consisting of a JGC-20KD gas chromatograph and JMA-2000 data system (JEOL, Tokyo, Japan).

GC separation was carried out by using a glass column (3 m × 2 mm I.D.) packed with mixed phase, 2% OV-1—2% QF-1 (1 : 1, w/w). Helium was used as the carrier gas (flow-rate 33 ml/min) and ammonia at a pressure of ca. 1.0 Torr was used as the chemical ionization (CI) reagent gas. The temperature of the column oven was 165°C for the trimethylsilylation and 190°C for the O-TMS, N-TFA derivatization. In addition, the temperatures of the injection port, separator, and ion source were 200°C, 290°C and 150°C, respectively. The ionization energy was 70 eV for the electron impact (EI) mode and 210 eV for the CI mode, and the trap current was 300 μA for both modes.

Drug administration and sample collection

Three healthy male volunteers received an oral dose of 1 mg tulobuterol·HCl (as a tablet) and the urine samples were collected separately for 0—4, 4—8, 8—12, 12—24 and 24—32 h after dosing and stored at −20°C until analysed.

Extraction and derivatization

Conjugated metabolites. To 4 ml of urine in a 15-ml glass-stoppered tube which contained 2.5 μg of 4-methoxytulobuterol as internal standard, was added 0.75 ml of 1 *M* acetate buffer and the pH was adjusted to 5.1—5.3. After the addition of 0.25 ml of enzymatic solution (glusulase), the sample was incubated overnight at 37°C. After cooling, 2 g of NaCl and 1 ml of NH_4Cl—NH_4-OH buffer (1.0 *M*, pH 10.0) were added and the pH was adjusted with 1 *N* NaOH to 9.5. The sample was shaken with ethyl acetate—acetone (3:1, v/v) for 10 min and centrifuged for 5 min at 2500 *g*; 3 ml of the organic layer was transferred to another tube and evaporated to dryness in a water-bath (40—50°C) under reduced pressure. The residue was dissolved in 250 μl of ethyl acetate, and 250 μl of BSA was then added. The contents of the tube were allowed to react at room temperature for at least 1 h. This final solution was injected into GC—MS system.

Unconjugated metabolites. Extraction and derivatization of unconjugated metabolites were carried out in the same way, omitting the glusulase incubation.

RESULTS AND DISCUSSION

In order to evaluate the GC properties and separation of compounds I—V, an aliquot of reference compounds was derivatized and analysed using various columns (Table I). As a result, symmetrical peaks and good separation could be obtained for the TMS derivatives of I—V when a glass column packed with mixed phase, 2% OV-1—2% QF-1, was used (Fig. 2.).

Fig. 3 shows the CI and EI spectra of the TMS derivatives (I—V). The quasi molecular ion $(M+1)^+$ and the fragment ion $(M-OTMS)^+$ were present in the CI spectra, while in the EI spectra of these compounds a base peak appeared at

TABLE I

RETENTION TIMES OF TMS DERIVATIVES

II= 4-Hydroxytulobuterol; III= 3-hydroxytulobuterol; V= 5-hydroxytulobuterol.

Column system				Retention time (min)		
Packing support	Mesh	length (m) × mm I.D.	Temperature (°C)	II	III	V
1.5% SE-30 Chromosorb G AW DMCS	60—80	2 × 3	165	12.0	12.0	10.7
5% SE-52 Chromosorb W AW DMCS	60—80	2 × 3	190	15.8	15.8	14.3
2% OV-1 Chromosorb W AW DMCS	100—120	2 × 3	170	18.1	17.3	15.7
1.5% OV-17 Chromosorb G AW DMCS	60—80	2 × 3	165	12.3	12.3	11.3
2% OV-225 Gas-Chrom Q	80—100	3 × 3	170	6.7	6.7	6.0
2% F-50 Chromosorb W AW DMCS	80—100	3 × 3	180	16.8	15.8	14.3
2% XF-1150 Chromosorb W AW DMCS	80—100	0.5 × 3	150	4.9	4.9	4.3
2% Dexil 300GC Chromosorb W AW DMCS	80—100	1 × 3	160	7.6	7.4	6.3
2% QF-1 Gas-Chrom Q	80—100	2 × 3	130	14.3	12.8	12.3
2% OV-1 − 2% QF-1 (1:1, w/w)		3 × 3	140	22.0	20.1	18.7

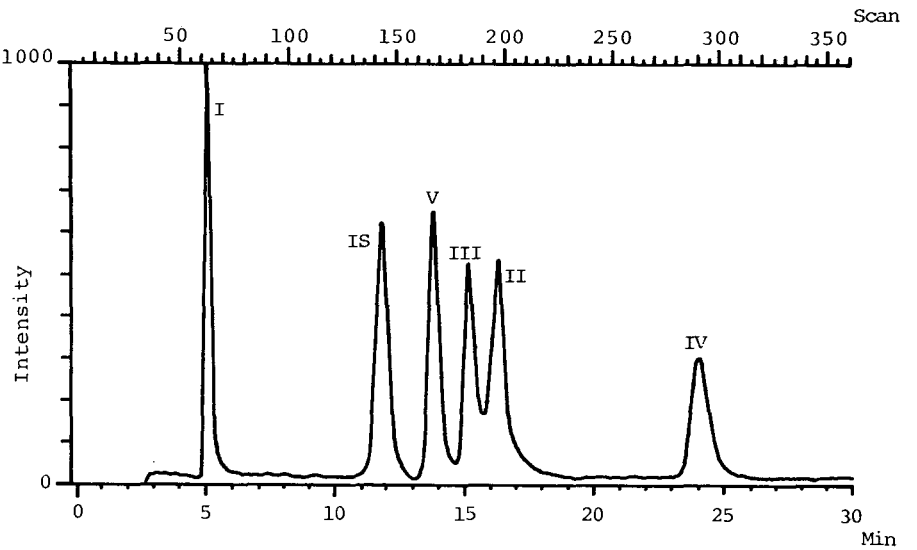

Fig. 2. Total ion current chromatogram of tulobuterol (I), 4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV), 5-hydroxytulobuterol (V) and 4-methoxytulobuterol (internal standard, IS) as their TMS derivatives.

m/e 86 which had resulted from fragmentation by β-cleavage. We chose the single-ion monitoring method at *m/e* 86 for the quantitative analysis of compounds I—V, because we could not achieve reproducible ionization in the CI measurement.

4-Methoxytulobuterol was evaluated for use as the internal standard, because the TMS derivative of this compound gave mass spectra very similar to that of the corresponding compounds I—V and the GC retention time was different from those of I—V.

We also examined other derivatizing methods such as acylation by trifluoroacetic anhydride (TFA) or pentafluoropropionic anhydride [9] and a two-step derivatization by bis(trimethylsilyl)trifluoroacetamide and TFA [10], but these methods were unstable and/or time-consuming compared to TMS derivatization by BSA.

Calibration graphs of compounds I—V in human urine are shown in Fig. 4. The plots of concentration vs. peak area ratio relative to internal standard added to the sample were found to be linear over the range 5—300 ng/ml urine.

The analytical recoveries and standard deviations for compounds I—V were reasonable, as summarized in Table II. This also indicates that the method is very precise and accurate. Futhermore there was no influence due to non-specific interference at *m/e* 86, which is a relatively low mass, from endogenous material of urine extracts.

Fig. 5 illustrates single-ion monitoring from urine after an oral dose of a tulobuterol tablet (1 mg). The major peaks of I, II, V, and two unidentified metabolites were detected in urine before enzymatic hydrolysis, although a trace peak of III less than 5 ng/ml appeared in the chromatogram. On the other hand, from the urine sample after treatment with glusulase, metabolites III and IV were apparently detected in addition to I, II and V.

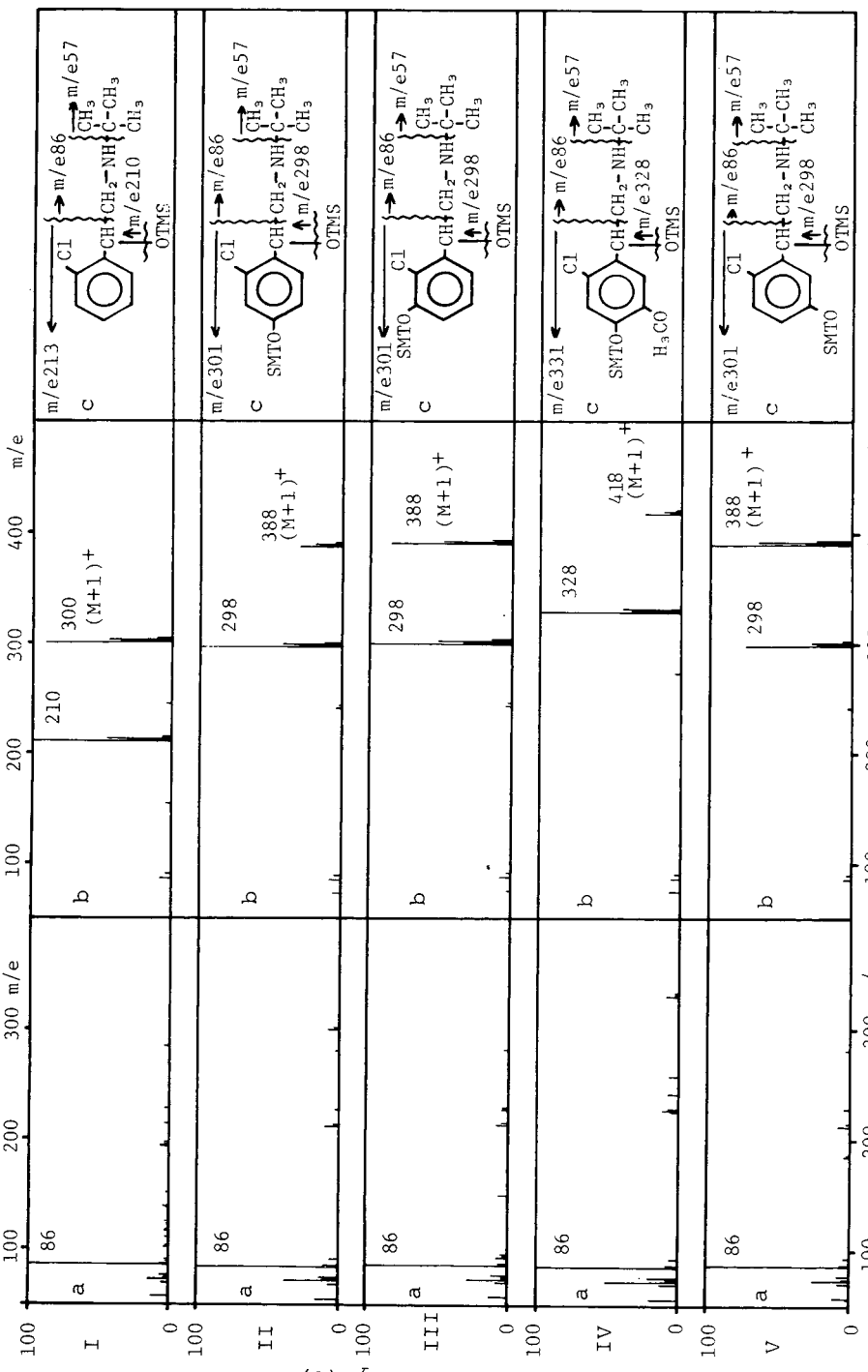

Fig. 3. Mass spectra and fragmentation of tulobuterol (I), 4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV) and 5-hydroxytulobuterol (V) as their TMS derivatives. (a) EI mass spectra; (b) CI mass spectra; (c) fragmentation.

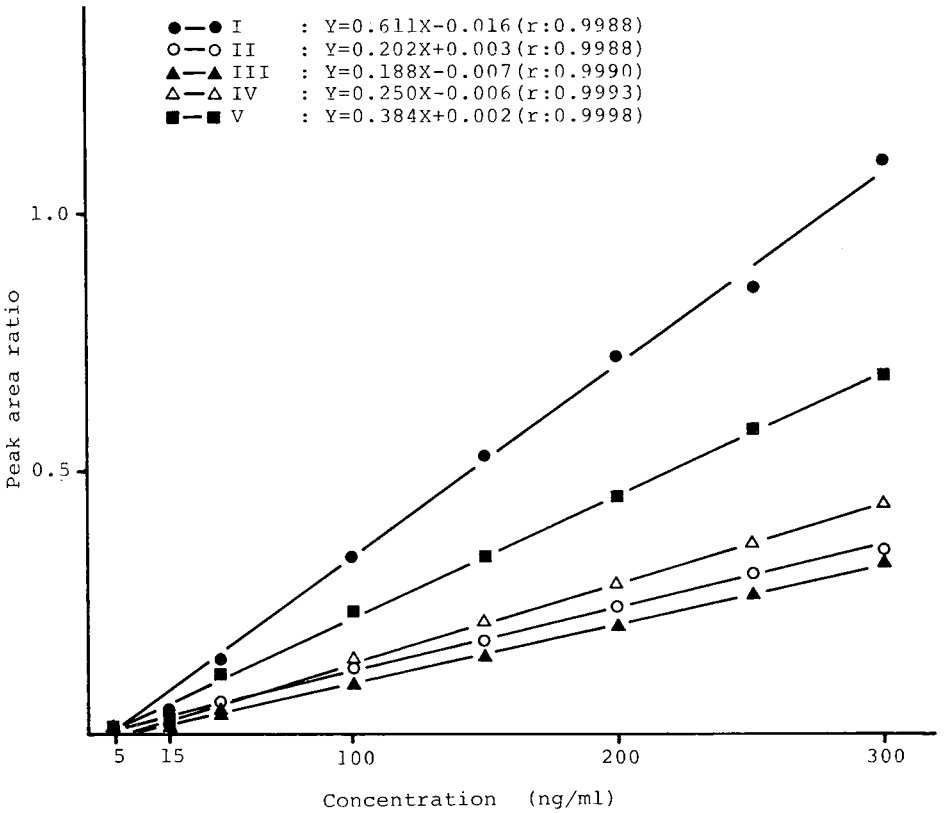

Fig. 4. Calibration curves for tulobuterol (I), 4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV) and 5-hydroxytulobuterol (V) in human urine.

TABLE II

RECOVERIES OF TULOBUTEROL (I), 4-HYDROXY- (II), 3-HYDROXY- (III), 4-HYDROXY-5-METHOXY- (IV) AND 5-HYDROXYTULOBUTEROL (V) IN HUMAN URINE

$n = 6$.

Metabolite	Added (ng/ml)	Found (ng/ml)	Recovery (% ± S.D.)
I	100	95	94.7±6.64
	250	273	109.2±6.76
II	100	95	95.0±3.52
	250	230	92.1±3.90
III	100	96	95.7±5.31
	250	255	101.9±3.30
IV	100	94	93.5±7.15
	250	246	98.2±6.30
V	100	100	100.0±3.24
	250	249	99.7±2.57

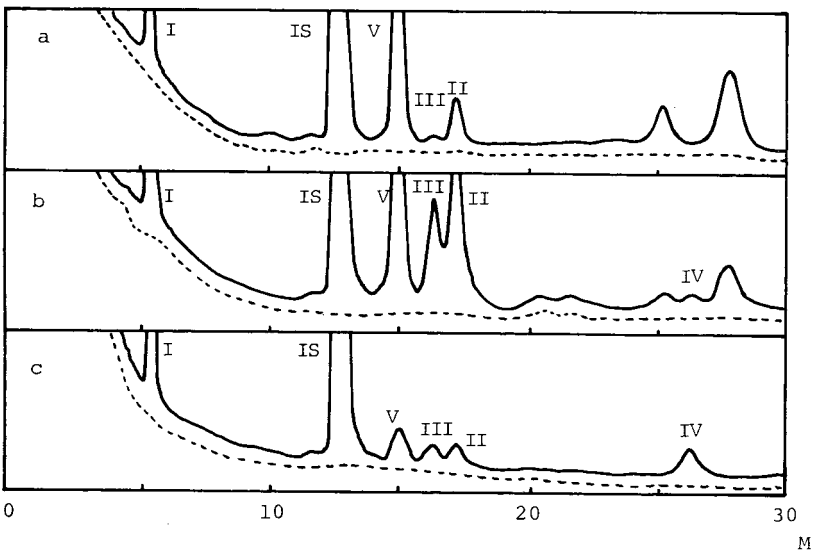

Fig. 5. Single-ion (*m/e* 86) monitoring of urine extracts. (a) Untreated urine (subject O.K., 0—4 h after dose); (b) treated urine (subject O.K., 0—4 h after dose) with glusulase; (c) blank urine spiked with compounds I—V (50 ng/ml urine) and 4-methoxytulobuterol [internal standard (IS), 500 ng/ml urine]. Dotted lines, tracings for blank urine.

TABLE III

URINARY EXCRETION OF TULOBUTEROL AND ITS METABOLITES AFTER ORAL ADMINISTRATION OF 1 mg OF TULOBUTEROL TO HUMAN SUBJECTS

I = Tulobuterol; II = 4-hydroxytulobuterol; III = 3-hydroxytulobuterol; IV = 4-hydroxy-5-methoxytulobuterol; V= 5-hydroxytulobuterol. N.D.= Not detected.

Subject	Time(h)	Dose in urine (%)									
		I		II		III		IV		V	
		Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
M.O.	0—4	7.4	18.0	N.D.	0.2	N.D.	0.1	N.D.	0.1	0.2	0.2
	4—8	5.5	11.1	N.D.	0.2	N.D.	0.0	N.D.	0.1	0.2	0.1
	8—24	9.6	20.2	N.D.	0.7	N.D.	0.4	N.D.	0.3	0.3	1.0
	24—32	1.0	1.2	N.D.	0.2	N.D.	N.D.	N.D.	0.1	0.2	0.1
	Total	23.5	50.5	–	1.3	–	0.5	–	0.6	0.9	1.4
I.T.	0—4	5.0	8.1	1.2	1.9	N.D.	3.4	N.D.	N.D.	0.8	1.0
	4—8	2.6	4.2	0.6	1.7	N.D.	2.4	N.D.	N.D.	0.9	1.9
	8—24	3.8	3.0	1.1	0.8	N.D.	2.0	N.D.	0.4	1.2	1.2
	24—32	0.6	N.D.	N.D.	0.2	N.D.	1.1	N.D.	N.D.	0.2	0.5
	Total	12.0	15.3	2.9	4.6	–	8.9	–	0.4	3.1	4.5
O.K.	0—4	2.5	5.7	1.0	2.8	N.D.	2.8	N.D.	0.2	1.2	2.3
	4—8	1.7	3.0	0.5	1.9	N.D.	2.0	N.D.	0.3	1.1	2.8
	8—24	2.0	2.9	0.9	1.7	N.D.	2.0	N.D.	0.6	1.4	4.0
	24—32	0.8	N.D.	N.D.	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	0.3
	Total	7.0	11.6	2.4	6.6	–	6.8	–	1.1	3.7	9.4

The overall analytical results are given in Table III, and indicate that the main metabolic pathway of tulobuterol in man is the ring-hydroxylation, but a considerable amount of unchanged drug is excreted in urine.

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IMPROVED GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NICOTINE AND COTININE IN BIOLOGIC FLUIDS

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SUMMARY

Improved methods have been developed for the determination of nicotine and its major metabolite, cotinine, in blood, plasma, and urine samples. These methods utilize gas chromatography with alkali flame ionization (nitrogen—phosphorus) detection and structural analogs of nicotine and cotinine as internal standards.

INTRODUCTION

A variety of methods for the determination of nicotine in biologic specimens have been reported in the literature, including methods based on radioimmunoassay [1, 2], liquid chromatography [3, 4], and gas chromatography using electron-capture [5], flame ionization [6—8], or alkali flame ionization [9—12] detectors or combined gas chromatography—mass spectrometry [13, 14]. In attempting to use published gas chromatographic methods, we encountered difficulty with reproducibility. This paper describes improved gas chromatographic methods for nicotine and cotinine determination with structural analogs of both substances utilized as internal standards. Using these methods, concentrations of nicotine as low as 1 ng/ml, and concentrations of cotinine as low as 5 ng/ml may be reliably measured in 1-ml samples of blood, plasma, or urine.

MATERIALS AND METHODS

Reagents and chemicals

Commercial nicotine base was converted to the bitartrate with (+)-tartaric

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acid, and purified by three recrystallizations from 80% aqueous ethanol. Cotinine was synthesized by the method of Bowman and McKennis [15] and converted to the crystalline fumarate (2:1) salt. Myosmene was synthesized by the procedure of Brandänge and Lindblom [16], and γ-3-pyridyl-γ-oxobutyric acid was synthesized by the method of McKennis et al. [17]. Other chemicals were obtained from commercial sources. All solvents were reagent grade.

Synthesis of the internal standard for nicotine, N-ethylnornicotine

Sodium cyanoborohydride (1 g) was added to a solution of myosmene [16] (3.0 g) in 50 ml of 50% aqueous ethanol. The pH of the vigorously stirred solution was adjusted to 2.2 by the dropwise addition of concentrated hydrochloric acid. For a period of 1.5 h, the solution was stirred and the pH was maintained in the range of 1.8—2.2 by periodic addition of concentrated hydrochloric acid. Analysis of an aliquot by gas chromatography indicated that the reduction to nornicotine was largely complete. The pH was adjusted to 6.0 by the addition of concentrated aqueous sodium acetate, then acetaldehyde (4 g) and fresh sodium cyanoborohydride (0.2 g) were added. The solution was stirred for 30 min, made alkaline (pH 12) with sodium hydroxide, and extracted with two 50-ml portions of methylene chloride. Concentration of the extract on a rotary evaporator followed by distillation provided 2.6 g of a colorless liquid, boiling point 96—99°C (2.5 mmHg). The 60 MHz NMR spectrum was consistent with the structure of N-ethylnornicotine. The free base (2.6 g) was converted to the bis-oxalate by addition of a solution of oxalic acid dihydrate (3.7 g) in 50 ml of methanol. The methanol was evaporated, and the residue was recrystallized twice from absolute ethanol to give, after vacuum drying, 4.0 g (64% yield from myosmene) of white crystalline powder, melting point 130.5—132°C. Microanalytical data for carbon, hydrogen, and nitrogen were within accepted limits.

Synthesis of the internal standard for cotinine, N-(2-methoxyethyl)-norcotinine

Sodium hydroxide (0.3 g) was added to a solution of γ-(3-pyridyl)-γ-oxobutyric acid [17] (1 g) in 5 ml of 2-methoxyethylamine, and the solution was stirred and warmed until the solid had dissolved. Toluene (25 ml) was added, and the solution was heated to about 60°C while the solvent was removed under reduced pressure using a rotary evaporator. The residue was taken up in a solution of anhydrous methanol (15 ml), and 2-methoxyethylamine (5 ml) was added followed by sodium borohydride (0.5 g). An exothermic reaction with gas evolution resulted. After stirring overnight, excess hydride was decomposed by addition of methanolic HCl (to pH 1.0), and solvent was removed using a rotary evaporator. Triethylamine (5 ml) was added to the residue, which was then distilled bulb to bulb (130—150°C at 0.1 mmHg) using a Kugelrohr distillation oven. The distillate was dissolved in dilute aqueous HCl (10 ml) and extracted with two 10-ml portions of methylene chloride. The aqueous layer was made basic with sodium hydroxide, and the product was extracted with methylene chloride (10 ml twice). The extract was concentrated in a rotary evaporator, and then distilled bulb to bulb (150°C oven temperature at 0.1 mmHg) to give 0.43 g of a yellow oil. The 80 MHz NMR spectrum was consistent with the structure of N-(2-methoxyethyl)-norcotinine. The succinate salt was prepared from 102 mg (0.44 mmol) of free base by combining with a solu-

tion of succinic acid (47 mg, 0.40 mmol) in methanol. Evaporation of the solvent under reduced pressure gave a light brown oil that solidified on standing. The product was recrystallized twice from ethyl acetate to give a white crystalline solid, melting point 92—93°C. Microanalytical data for carbon, hydrogen, and nitrogen were within accepted limits.

Gas chromatography

Gas chromatographic analyses were performed using a Hewlett-Packard Model 5711A instrument equipped with dual alkali flame (nitrogen—phosphorus) detectors and a Varian Model 9176 strip-chart recorder. The nitrogen (carrier gas), air, and hydrogen flow-rates were 30, 50, and 5 ml/min, respectively. Columns (1.8 m for nicotine, 1.2 m for cotinine) were 2 mm I.D. glass, configured for on-column injection, packed with 2% Carbowax 20M + 2% KOH on Gas-Chrom P (100—120 mesh) (Applied Science Labs., State College, PA, U.S.A.) or 3% SP-2250 DB on Supelcoport (100—120 mesh) (Supelco, Bellefonte, PA, U.S.A.). The injection port and detector temperatures were 250°C and 300°C, respectively.

Assay of nicotine

Preparation of reagents and tubes for blood collection. Diethyl ether (anhydrous, reagent grade) was distilled from sodium benzophenone ketyl before use. Solutions of sodium hydroxide and hydrochloric acid were prepared from reagent grade chemicals using tap distilled water that had been redistilled from dilute chromic acid. All manipulations of samples in open tubes were carried out under an Edgegard^{R} Model EGB-4252 laminar flow hood (Baker, Sanford, ME, U.S.A.) in a laboratory in which smoking was prohibited. Nicotine bitartrate and the internal standard, N-ethyl-nornicotine oxalate, were stored as aqueous solutions. These solutions were found to be stable for at least one month when refrigerated. Meticulous preparation and storage of tubes was found to be necessary to avoid contamination with environmental nicotine. Screw-top glass tubes were soaked overnight in 30% nitric acid, rinsed thoroughly and dried at 80°C. Tubes were stored in the oven until just before use when they were transferred to the laminar flow hood, where anticoagulant was added. The PTFE-lined tube caps were soaked overnight in dilute HCl, rinsed, dried in the oven, and stored in tightly closed containers.

Assay procedure

A flow diagram of the extraction procedure is shown in Fig. 1. To 1-ml aliquots of blood or plasma were added 20 ng of N-ethylnornicotine [and N-(2-methoxyethyl)-norcotinine as well, if cotinine was to be assayed] followed by 0.5 ml 2 *N* sodium hydroxide. Freshly distilled diethyl ether (2 ml) was added to each tube, and the tubes were agitated for 1 min using a vortex mixer. After centrifuging to break up emulsions, the ether layers were transferred to tubes containing 0.5 ml 1 *N* HCl. The tubes were vortex mixed for 1 min and then the ether layers were removed and discarded. Sodium hydroxide (0.5 ml of 2 *M*) and 0.5 ml of ether were added to the aqueous layer; the tubes were again agitated on the vortex mixer for 1 min. The ether layers were separated and dried over anhydrous potassium carbonate in small vials. The extracts (1—5-μl

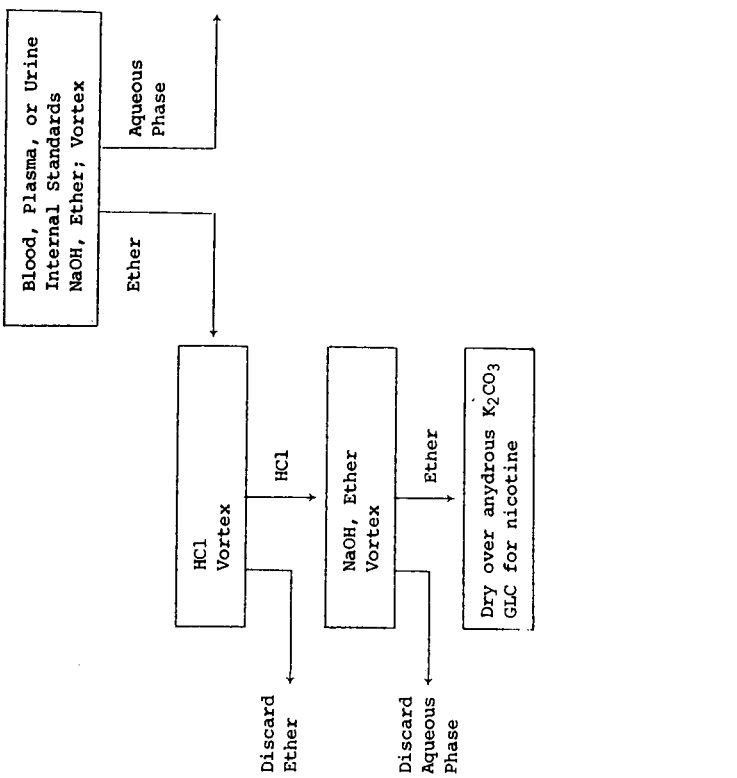


Fig. 1. Flow diagram of nicotine and cotinine extraction procedure.

aliquots) were analyzed by gas chromatography on 1.8 m × 2 mm I.D. Carbowax—KOH or SP-2250 DB columns at 145°C. Examples of chromatograms from plasma extracts are presented in Fig. 2. Standard curves, prepared from blank plasma samples to which nicotine and internal standard were added, were linear over the entire range studied, 0—100 ng/ml. Quantitation was achieved by calculating peak height ratios of nicotine to internal standard and referring to the standard curve. The assay for urine samples was identical, with the exception that larger amounts of internal standard (200 ng) were added.

Assay of cotinine

To 1 ml of blood or plasma were added internal standard, N-(2-methoxyethyl)-norcotinine (20 ng or 100 ng, depending on the anticipated cotinine levels), and 0.5 ml 2 *N* NaOH. (If the samples were to be assayed for nicotine as well, internal standards for both nicotine and cotinine were added. The

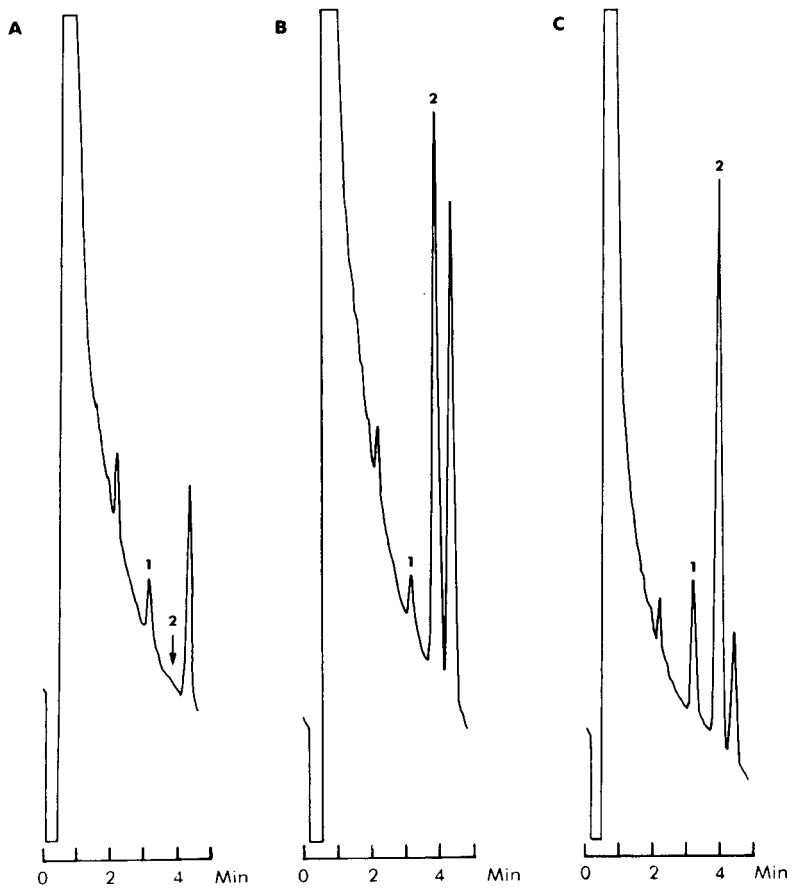


Fig. 2. Gas chromatograms of plasma extracts containing nicotine (1) and internal standard (2). (A) Plasma extract of nonsmoker; (B) extract of nonsmoker's plasma containing 20 ng/ml internal standard; (C) extract of plasma containing 5 ng/ml nicotine and 20 ng/ml internal standard.

cotinine analysis was carried out following the extraction of nicotine.) The samples were extracted with 2.5 ml 1-butanol by vortex mixing for 2 min, then cooled in a dry ice—acetone bath and centrifuged to break up emulsions. The butanol layers were transferred to tubes containing 0.5 ml 1 *N* sulfuric acid and vortex mixed for 1 min. After centrifuging to facilitate separation of layers, the butanol layers were separated and discarded. Sodium hydroxide (0.5 ml of 2 *N*) was added to the aqueous layers, which were then extracted with 1-ml aliquots of methylene chloride by vortex mixing for 1 min. The methylene chloride layers were transferred to small conical vials and evaporated under a current of nitrogen. Ethyl acetate (50 μl) and a small amount of anhydrous potassium carbonate were added; the tube was agitated for a few seconds on the vortex mixer, and an aliquot (2—5 μl) of the extract was analyzed by gas chromatography on a 1.2 m × 2 mm I.D. Carbowax—KOH column at 210°C. Standard curves were linear over the entire concentration range studied, 0—1000 ng/ml. Typical chromatograms are reproduced in Fig. 3. Calculations were performed as described for nicotine.

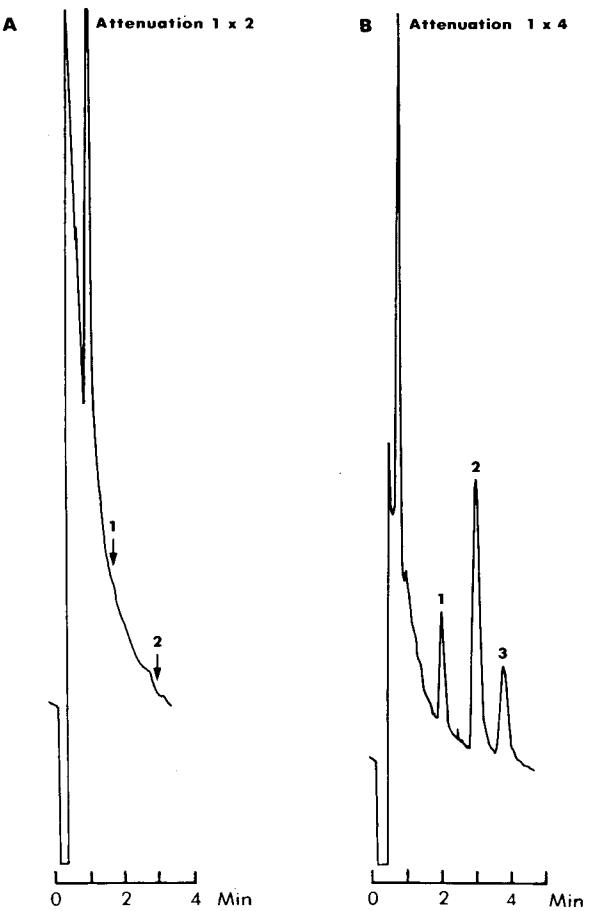


Fig. 3. Gas chromatograms of extracts containing cotinine (1) and internal standard (2). (A) Extract of control monkey blood; (B) extract of nonsmoker's plasma spiked with 5 ng/ml cotinine and 20 ng/ml internal standard. Peak 3 is caffeine.

Evaluation of extraction solvents for cotinine and internal standards

Stock solutions for all compounds listed in Table I were prepared in 1 *N* NaOH at concentrations for measuring UV absorbance [25 μg/ml and 261 nm for cotinine, N-(1-propyl)-norcotinine, and N-(2-methoxyethyl)-norcotinine; 200 μg/ml and 254 nm for lidocaine]. Aliquots of stock solutions (3 ml) were extracted with 3 ml of the appropriate solvent by vortex mixing for 1 min. The aqueous phase was separated, and the absorbance was measured using a Zeiss Model PMQ-II spectrophotometer. Concentrations were determined using a standard curve of each substance in 1 *N* NaOH, and were used to calculate the percent extraction into the organic solvents listed in Table I.

TABLE I

PERCENT EXTRACTION OF COTININE AND INTERNAL STANDARDS FROM AQUEOUS SOLUTION USING VARIOUS SOLVENTS

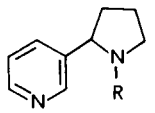
Solvent	Cotinine (%)	Lidocaine (%)	N-Propyl-norcotinine (%)	N-Methoxyethyl-norcotinine (%)
Heptane	4	93	7	3
Toluene	13			
Diethyl ether	8	>95	34	11
Ethyl acetate	33	>95	68	40
1-Octanol	80			
Methylene chloride	88	>95	92	93
1-Butanol	85		91	85
Chloroform	92			

RESULTS AND DISCUSSION

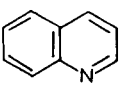
Nicotine analysis

Initially, we attempted to use previously described methods for analysis of nicotine using quinoline as an internal standard [7, 10, 12]. However, considerable variation occurred among identical samples, and occasionally we observed large variations in peak height ratios of nicotine to quinoline for the same ether extract injected repeatedly into the gas chromatograph. For example, repeated injection of one ether extract gave peak height ratios ranging from 1.6 to 5.5. It is likely that the variability was due to different relative losses of nicotine and quinoline during sample work-up and/or during gas chromatography. This problem might have been due to a poor choice of internal standard. Nicotine (pK_a 8.0) is considerably more basic than quinoline (pK_a 4.9). If an acidic site were present on glassware or in the gas chromatograph, a selective loss of nicotine would be expected, leading to variable results. (Subsequent to the development of our nicotine assay, a report appeared which described similar difficulties in the analysis of nicotine using quinoline as an internal standard [18].) Consequently, we have synthesized a new internal standard, N-ethylnornicotine (Fig. 4), a structural analog with chemical properties similar to nicotine.

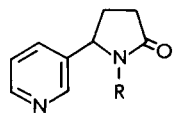
Experiments have demonstrated that N-ethylnornicotine is a highly satisfac-



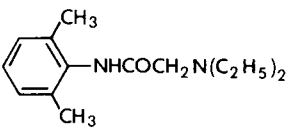
Nicotine, R=CH_3
N-Ethylnornicotine, R=C_2H_5



Quinoline



Cotinine, R=CH_3
N-n-Propylnorcotinine, R=n-C_3H_7
N-(2-Methoxyethyl)-norcotinine, R=$CH_2CH_2OCH_3$



Lidocaine

Fig. 4. Structures of nicotine, cotinine, and internal standards.

TABLE II

DAY-TO-DAY VARIATION OF IDENTICAL PLASMA SAMPLES

Results are mean of determinations performed on 6 different days.

Concentration (ng/ml)	Nicotine				Cotinine				
Given	5	10	20	50	5	20	50	150	600
Internal standard	20	20	20	20	20	20	20	150	150
Found, mean	5.3	9.9	19.3	52.2	4.78	19.4	49.4	147.5	598.7
Standard deviation	0.26	0.52	0.81	1.92	0.20	0.80	0.63	3.84	9.94
Coefficient of variation	4.9	5.3	4.2	3.7	4.2	4.1	1.3	2.6	1.7

tory internal standard for the gas chromatographic determination of nicotine. (The authors cited in ref. 18 found that a structural analog of nicotine, N-(1-propyl)-nornicotine, was a more satisfactory internal standard than quinoline, although they did not describe an assay procedure for biological samples.) Sharp peaks and baseline separation of nicotine from N-ethylnornicotine were obtained on both Carbowax—KOH (Fig. 2) and SP-2250 DB columns. Good reproducibility (Table II) was obtained under the same conditions that gave poor reproducibility using quinoline as an internal standard. A simple extraction scheme (Fig. 1) using diethyl ether as the extracting solvent was employed. To minimize contamination from environmental nicotine, extractions were carried out under a laminar flow hood located in a laboratory in which smoking was prohibited. All solvents had to be purified to minimize contamination by nicotine and other substances.

Cotinine analysis

Cotinine, the major metabolite of nicotine in humans, is of interest because of the relatively high concentrations found in the blood of smokers [19, 20] and its pharmacological activity [21—23]. Methods for cotinine determination

in plasma and urine specimens have been reported [6, 8, 11]. We have improved upon the published methods in two ways. Our extraction procedure (Fig. 1), which utilizes 1-butanol instead of methylene chloride [6, 11] to extract cotinine from blood or plasma, results in less emulsion formation and cleaner separation of layers. Furthermore, we have synthesized an internal standard, N-(2-methoxyethyl)-norcotinine (Fig. 4), which is a structural analog with solvent partitioning properties (Table I) and pK_a value similar to cotinine. Lidocaine, the internal standard utilized in published methods, is considerably more lipophilic and more basic than cotinine and, therefore, is a poor choice for an internal standard. Our internal standard may be added *prior* to the nicotine extraction procedure (unlike the published methods in which lidocaine must be added *after* extraction of nicotine) which corrects for losses of cotinine during nicotine extraction.

CONCLUSION

In summary, new methods have been developed for the determination of nicotine and cotinine concentrations in blood using gas chromatography with alkali flame ionization (nitrogen—phosphorus) detection. Simple solvent extraction procedures permit the use of the assays for large numbers of samples required for pharmacokinetic studies. Structural analogs were synthesized for use as internal standards and have resulted in assays that appear to be more reliable than those previously reported.

ACKNOWLEDGEMENTS

This study was supported by Grants No. DA01696 and DA02077 and Contract No. HSM-42-73-181 from the National Institute on Drug Abuse. We are grateful to Professor Neal Castagnoli, Jr., for helpful discussions and to Dr. Lang Gruenke for preliminary experiments in the development of the nicotine assay. NMR spectra were obtained by courtesy of the Magnetic Resource Laboratory, University of California, San Francisco.

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CHROMBIO. 698

DETERMINATION OF PLASMA AND URINE LEVELS OF A NEW ANTI-INFLAMMATORY AGENT, 4,5-BIS-(4-METHOXYPHENYL)-2-(2-HYDROXYMETHYLSULFINYL)-IMIDAZOLE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL OR ULTRAVIOLET DETECTION

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SUMMARY

The determination of a new anti-inflammatory substance, 4,5-bis-(4-methoxyphenyl)-2-(2-hydroxyethylsulfinyl)-imidazole, in plasma and urine by high-performance liquid chromatography is described. Ultraviolet and electrochemical detection modes are compared and special consideration is given to the mechanism of the electrochemical reaction. The site of oxidation in the molecule seems to be an aliphatic hydroxyl group yielding a carboxyl function in a four-electron transfer reaction. Plasma levels and urinary excretion after an oral dose of 250 mg to two male volunteers have been measured.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ECD) continues to gain in popularity for the sensitive and selective determination of trace components in complex biological samples. The sensitivity of this method of detection in the oxidative mode has been demonstrated by Kissinger et al. [1], who designed and built a system with a carbon-paste electrode, capable of measuring picograms of catecholamines. Other groups have constructed cells by using glassy carbon electrodes thus making this mode of detection suitable for non-aqueous eluents [2—4]. The selectivity of ECD is achieved by detecting only those compounds which are electrochemically active at the operating potential chosen. This is of particular interest in the analysis of biological samples. Many of the substances usually interfering which are co-extracted in the work-up of plasma or urine samples are electrochemically inactive and will therefore not interfere in ECD. Thus the suit-

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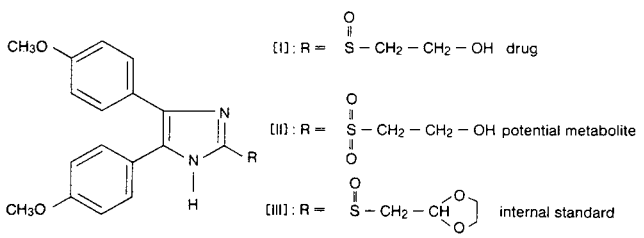
ability of the electrochemical detector to a given problem ultimately depends on the voltammetric characteristics of the compound to be detected in a suitable mobile phase and at a suitable electrode potential.

Since the electrooxidative determination of catecholamines many more classes of compounds have been investigated and found suitable for electrochemical detection, for example, tryptophan metabolites [5], alkaloids [6], β-blockers and their metabolites [7, 8], aromatic amine carcinogens [9], and ascorbic acid [10, 11].

The present report describes a sensitive and specific method of determination for a new substance with anti-inflammatory activity: 4,5-bis-(4-methoxyphenyl)-2-(2-hydroxyethylsulfinyl)-imidazole (compound I). ECD is compared to ultraviolet detection of the drug in plasma and urine extracts. Special interest was focussed on investigating the site of the electrochemical reaction in the molecule.



EXPERIMENTAL

Subjects and medication

Two healthy male volunteers (36 and 26 years of age and 65 and 96 kg body weight, respectively) were both given 250 mg of (I) orally in capsule form in the morning on an empty stomach. Breakfast was not allowed until 2 h after administration. Blood samples were taken at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24 and 48 h after the drug administration. The samples were immediately centrifuged and the plasma stored frozen until analysis. Urine was quantitatively collected at 2, 4, 6, 8, 10, 12, 24 and 48 h after administration and kept frozen until analysis.

Chemicals

Methanol, diethyl ether and disodium hydrogen phosphate were all of analytical-reagent grade (Merck, Darmstadt, G.F.R.) and used without further purification.

Compound (I), the internal standard (III) and the possible metabolite (II) of (I) were synthesized by Dr. Niedballa (Schering) and stored dissolved in methanol in concentrations of 100, 10 and 1 μg/ml, respectively.

Glassware

All glassware used in the extraction procedure was cleaned with chromic acid and washed with distilled water before use.

Extraction procedure

One millilitre of plasma or urine was pipetted into an 8-ml stoppered test-tube and 0.5 μg of internal standard and 3 ml of diethyl ether were added. After thorough mixing on a Vortex mixer for 1 min and centrifugation at 1200 *g* for 5 min, the organic phase was transferred to another test-tube and evaporated to dryness. The residue was dissolved in 200 μl of the HPLC mobile phase. After centrifugation (5 min, 1200 *g*) — only in the case of plasma extracts — 150 μl were injected for analysis.

The extraction efficiency was determined with 1-ml plasma and urine samples containing 0.5 μg of (I) or of internal standard (III) (n = 5). Peak heights were measured and correlated with peak heights of pure substances.

Chromatographic system

The HPLC system consisted of a solvent delivery pump (Waters, Königstein, G.F.R., type 6000A), a LiChrosorb RP-18 chromatographic column (10 μm particle size, 250 × 4.6 mm) with precolumn (40 × 4.6 mm; Knauer, Berlin, G.F.R.) and an electrochemical detector (E 611, cell 1096/2; Metrohm, Filderstadt, G.F.R.) using a glassy carbon working electrode and an Ag/AgCl/KCl reference electrode. Alternatively, a UV detector with variable wavelength (Schoeffel SF 770) was used. Injection was accomplished with a Rheodyne RH 7120 system or an automatic injector (WISP, Waters). The mobile phase consisted of methanol—water (1:1, v/v) with 0.01 *M* disodium hydrogen phosphate per litre. The eluent was degassed under reduced pressure before use. The chromatographic system was operated at ambient temperature with an eluent flow-rate of 2 ml/min.

The electrochemical potential of the working electrode was set at +1.0 V against the reference electrode. The current range used was 75—200 μA according to the concentration of the drug. The UV detector was set at 280 nm, the absorption maximum of (I). The detector signals were converted to chromatographic traces by a W + W recorder (Basle, Switzerland) at an input voltage of 1000 and 100 mV, respectively.

Calibration curve

Standard curves were constructed with 1-ml blank plasma and urine samples containing 0, 0.05, 0.1, 0.5, 0.75, 1, 1.5, 2, 2.5 and 5 μg of (I) and 0.5 μg of internal standard. These samples were extracted by the method described above. Peak heights of internal standard and drug were measured and the calibration curve [peak height ratio of (I):internal standard (III) versus the concentration of (I)] was constructed.

Unknown plasma and urine samples were processed together with five calibration points [5.0, 2.5, 1.0, 0.5 and 0.1 μg of (I)] which were used to correct for inter-assay variability.

The overall accuracy of the assay was calculated from five consecutive determinations of 0.5 μg of (I) in plasma and urine.

Electrochemical reaction

To specify the site of electrochemical reaction in the molecule, response (peak height) was measured as a function of the potential of the working

electrode for (I) and for two model substances in which only one structural element had been altered (see structural formulae). By this procedure contributions from background current and other residual currents which arise in scanning voltammetry are conveniently avoided [6].

RESULTS AND DISCUSSION

A highly sensitive and selective method for the determination of the new anti-inflammatory substance (I) in plasma and urine is described utilizing HPLC with electrochemical (plasma samples) or UV (urine samples) detection. Extraction from biological specimens is performed with diethyl ether. The recovery of this procedure was found to be about 40% for (I) and for the internal standard (III), in both plasma and urine (see Table I).

TABLE I

EXTRACTION RECOVERIES OF (I) AND INTERNAL STANDARD (III)

Recoveries were determined by extracting 1 ml of plasma or urine spiked with 0.5 μg of (I) or of internal standard, and comparing the ECD peak heights to those of non-extracted material.

Sample	Recovery (%)	
	(I)	Internal standard (III)
Plasma	41.2	40.8
	43.2	45.0
	38.4	41.6
	42.6	44.2
	40.4	42.2
Mean ± S.D.	41.2 ± 1.9	42.8 ± 1.8
Urine	33.6	46.0
	28.4	24.8
	41.2	40.0
	38.2	40.4
	41.8	45.6
Mean ± S.D.	36.6 ± 5.6	39.4 ± 8.6

Matrix constituents and possible co-extracted metabolites of (I) are then separated from the drug by HPLC using a reversed-phase system (see Fig. 1). For detection two types of systems were compared, electrochemical and UV detectors. As shown in Figs. 1—4, ECD is suitable for plasma and urine samples whereas UV detection can only be used for urine and not for plasma samples. The reason for this is the UV-absorbing plasma constituents which interfere in the optical mode but which are not oxidized and detected at the applied potential in the electrochemical mode.

Concentrations of (I) in plasma and urine were determined by comparing the peak heights of the drug and the internal standard added before extraction. Typical calibration curves of the assay for ECD and UV detection are described by the equations given in Table II.

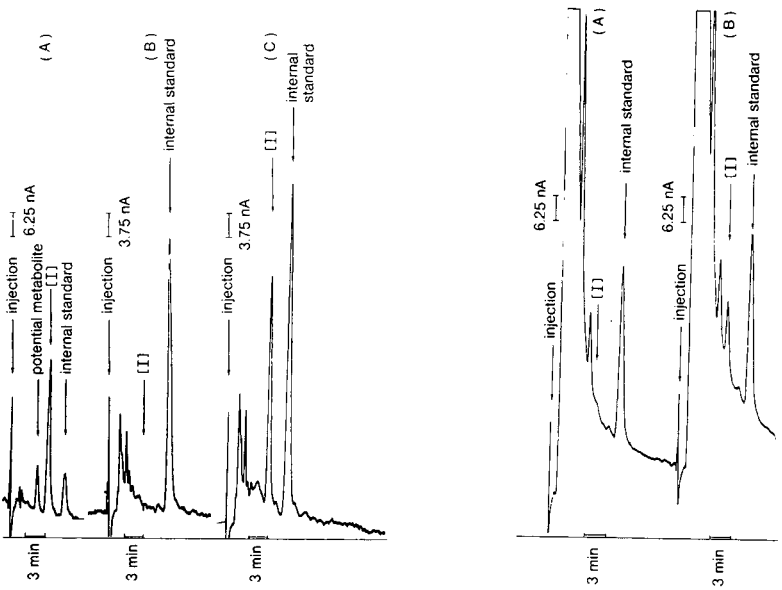


Fig. 1. HPLC chromatograms (electrochemical detector) of (A) 50 ng each of (I), its potential metabolite (II) and the internal standard (III), and of blank plasma samples spiked with (B) 500 ng of the internal standard and (C) 100 ng of (I) and 500 ng of the internal standard.

Fig. 2 HPLC chromatograms (electrochemical detector) of blank urine samples spiked with (A) 500 ng of internal standard, and (B) 50 ng of (I) and 500 ng of internal standard.

TABLE II

MATHEMATICAL EQUATIONS OF THE CALIBRATION CURVES FOR THE DETERMINATION OF UNKNOWN (I) CONCENTRATIONS

The curves were obtained by spiking 1 ml of plasma or urine with 0.5 μg of internal standard and various amounts of (I).

Specimen	Detection	Calbration curve*	Correlation coefficient
Plasma	EC	$y = 0.32 + 5.77x$**	0.9993
	UV	Not determined	
Urine	EC	$y = 0.16 + 6.48x$***	0.9993
	UV	$y = 0.05 + 2.69x$***	0.9992

*y = (peak height of (I))/(peak height of internal standard); x = concentration of (I).
**$n = 11$.
***$n = 10$.

The overall accuracy of the assay expressed as standard deviation of five consecutive determinations of 0.5 μg of (I) is shown in Table III. As can be seen from this table, UV detection is more suitable for urine samples than ECD because of better reproducibility.

The detection limit after extraction of 1 ml of plasma or urine is below

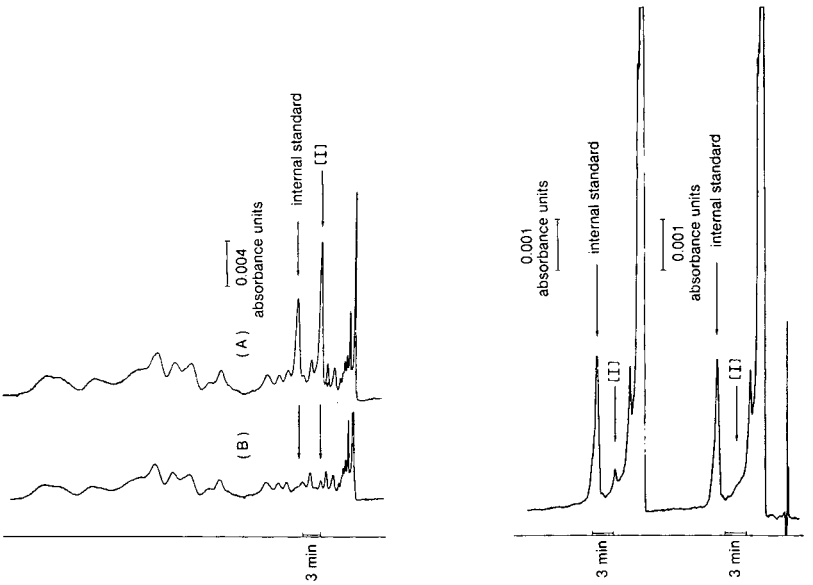


Fig. 3. HPLC chromatograms (UV detector) of (A) blank plasma sample spiked with 0.5 μg each of (I) and internal standard, and (B) blank plasma sample.

Fig. 4. HPLC chromatograms (UV detector) of blank urine samples spiked with (left) 0.5 μg of internal standard and 50 ng of (I) and (right) 0.5 μg of internal standard.

TABLE III

PRECISION OF THE ASSAY

Precision was calculated from five consecutive determinations of 0.5 μg of (I) in 1 ml of plasma or urine.

Specimen	Coefficient of variation	
	UV detection	EC detection
Plasma	Not determined	3.0
Urine	2.1	15.0

10 ng/ml for ECD and about 10 ng/ml for the UV mode. The limit may possibly be increased further by extracting larger sample volumes, by increasing the extraction efficiency (90% when extracting samples three times) and by lowering the applied current to about 5 nA.

Study of plasma and urine levels

(I) reached its maximum plasma level of 713 ± 163 ng/ml in the two test subjects 4 h after administration (Fig. 5). At 12, 24 and 48 h after dosing the concentration had fallen to 273 ± 47, 208 ± 32 and 36 ± 6 ng/ml, respectively. Renal excretion of unchanged drug amounted to 1.1 ± 0.2 mg in the first two days, which is equal to 0.4% of the dose (Fig. 6). Detailed pharmacokinetics of this drug including dose dependency will be reported elsewhere [12].

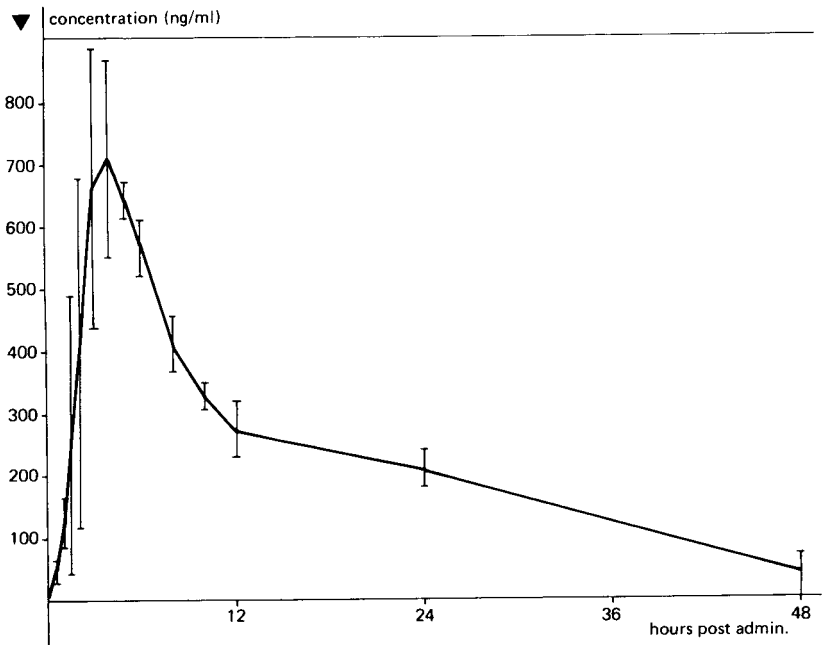


Fig. 5. Plasma level of (I) (mean ± S.D.) after oral administration of 250 mg to two healthy male volunteers.

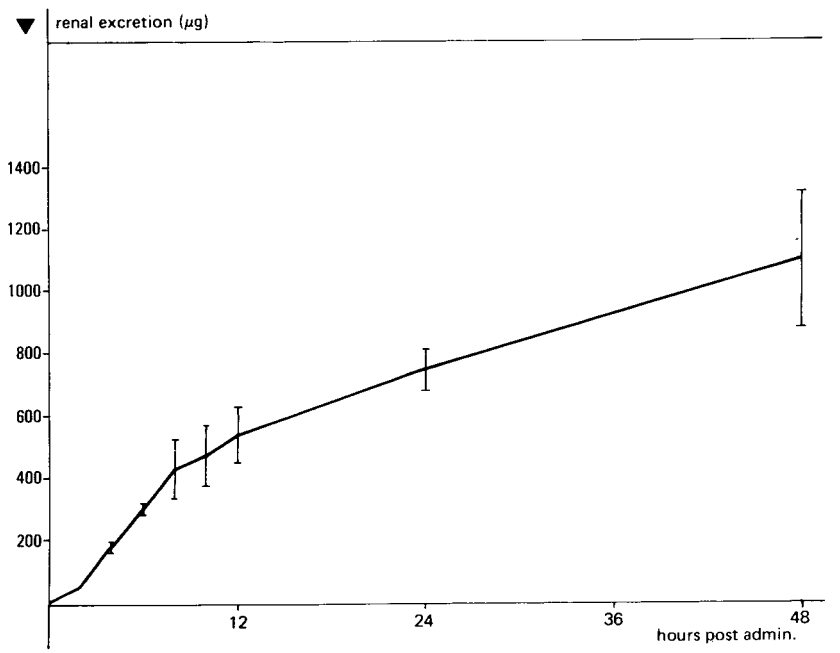


Fig. 6. Urinary excretion of (I) (mean ± S.D.) after oral administration of 250 mg to two healthy male volunteers.

Electrochemical reaction

At an electrode potential of +1.0 V used in the ECD mode substances eluted from the chromatographic column are changed by oxidation. (I) is prone to oxidation at different sites of the molecule. First, the primary alcohol group of the side-chain may be oxidized to the aldehyde or the carboxylic acid [13] and, second, the sulfinyl group may be converted to sulfonyl [14, 15], whereas the imidazole ring is quite stable to oxidation [16].

To clarify the site of oxidation two model compounds have been examined which differ from (I) only in one part of the molecule (see structural formulae). Current–potential curves of these three substances have been obtained by plotting peak heights of 0.5 μg of each vs. the potential applied (Fig. 7). From these curves the following conclusions can be drawn: (1) Compound (I) is more easily oxidized than (II). (2) (III) is more easily oxidized than (II), but less than (I). (3) In (I) and (II) the same amounts of electrons are exchanged (same peak heights in the end stage of oxidation). (4) In (III) only half of the electrons are transferred compared to (I) and (II).

If the sulfinyl group were oxidized, the peak heights of (I) and (III) would have to be the same and (II) should give no signal at all. With the primary alcohol group being the reaction centre, (I) and (II) have to yield the same response in the final stage of oxidation. If the reaction were to produce the aldehyde, (III) should give no signal, because this compound is already an aldehyde (in the form of its acetal). Therefore, the oxidation has to proceed from the alcohol via the aldehyde to the carboxylic acid. So in (I) and (II) there are four electrons transferred whereas in (III) there are only two.

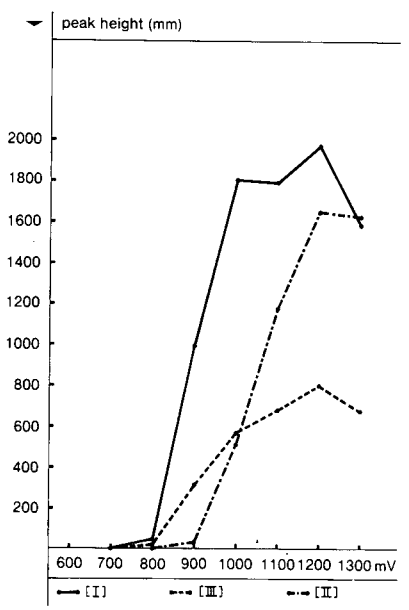
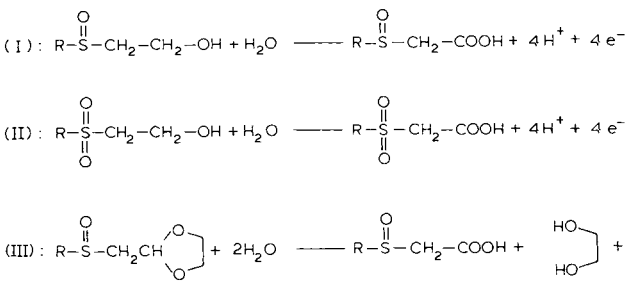


Fig. 7. Electrochemical response (peak height) of 0.5 μg each of drug (I), its potential metabolite (II), and internal standard (III) at various potential settings.



As (I) is more easily oxidized than (II), the reaction has to proceed under the influence of the sulfinyl group, possibly also under the mediation of the imidazole ring. One could think of a cyclic intermediate with the electron exchange starting from the $-\overset{\overset{O}{\|}}{S}-$ group.

In the literature little is known about the mechanism of electrochemical detection of most of the chemical classes studied. Catecholamines and substituted derivatives have been shown to yield benzoquinones with ring closure of the amine-containing side-chain [17—19]. Tryptophan and its metabolites seem to be oxidized at 5-hydroxy groups on the phenol ring or at higher voltages applied at the ring nitrogen atom to give N^+ [20]. Morphine is reported possibly to dimerize after a one-electron transfer to yield pseudomorphine, which is further oxidized in a second step [21]. The oxidation of aliphatic hydroxyl groups as described in the present report seems to be a new way of reaction in electrochemical detection.

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CHROMBIO. 713

DETERMINATION OF ACETYLMETHADOL AND METABOLITES BY USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received June 10th, 1980; revised manuscript received August 28th, 1980)

SUMMARY

A method is described for the simultaneous determination of *l*,α-acetylmethadol (LAAM) and five active metabolites — noracetylmethadol, dinoracetylmethadol, methadol, normethadol, and dinormethadol — in biofluids by high-performance liquid chromatography using a normal-phase column and a UV detector at 218 nm. The compounds are recovered from biofluids by a multistep liquid—liquid extraction. The mobile phase is methanol—acetonitrile (70:30, v/v) containing 0.015% ammonium hydroxide as the modifier. Retention times can be varied by adjusting the composition of the mobile phase to maximize peak height for quantitation using *l*-propranolol as the internal standard or peak separation for the collection of fractions. Using a UV detector the lower limit of sensitivity is 10 ng/ml of biofluid. Using fraction collection of radiolabeled drug and metabolites followed by liquid scintillation counting the lower limit of sensitivity is 1.0 ng/ml. Commonly used or abused narcotics including morphine, heroin, meperidine, methadone and propoxyphene do not interfere with the analysis. The method has been applied to plasma and urine samples from humans, sheep and rats. Extracts of urine from patients receiving maintenance treatment with LAAM contain LAAM and each of the five active metabolites.

INTRODUCTION

l,α-Acetylmethadol (LAAM) is a synthetic narcotic analgesic under development for the maintenance treatment of opiate dependence. LAAM has a relatively longer duration of action in the suppression of opiate withdrawal than methadone, the established maintenance drug [1]. Studies of the disposition of LAAM in maintenance patients indicate that this long duration of

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action is due predominantly to the formation of active and persistent metabolites [2]. These metabolites, all levo-alpha isomers, include noracetylmethadol (NAM), dinoracetylmethadol (DNAM), methadol (MOL), normethadol (NMOL) and dinormethadol (DNMOL) (Fig. 1). The clinical evaluation of LAAM has stimulated pharmacokinetic studies in man and laboratory animals. As might be expected for a drug with a complex metabolism, the disposition of LAAM has been studied using a number of analytical techniques including thin-layer chromatography [3—5], gas—liquid chromatography [6—8] and combined gas chromatography—mass spectrometry [9, 10]. None of these methods, as reported, fulfills both the selectivity and sensitivity requirements of a complete analytical method.

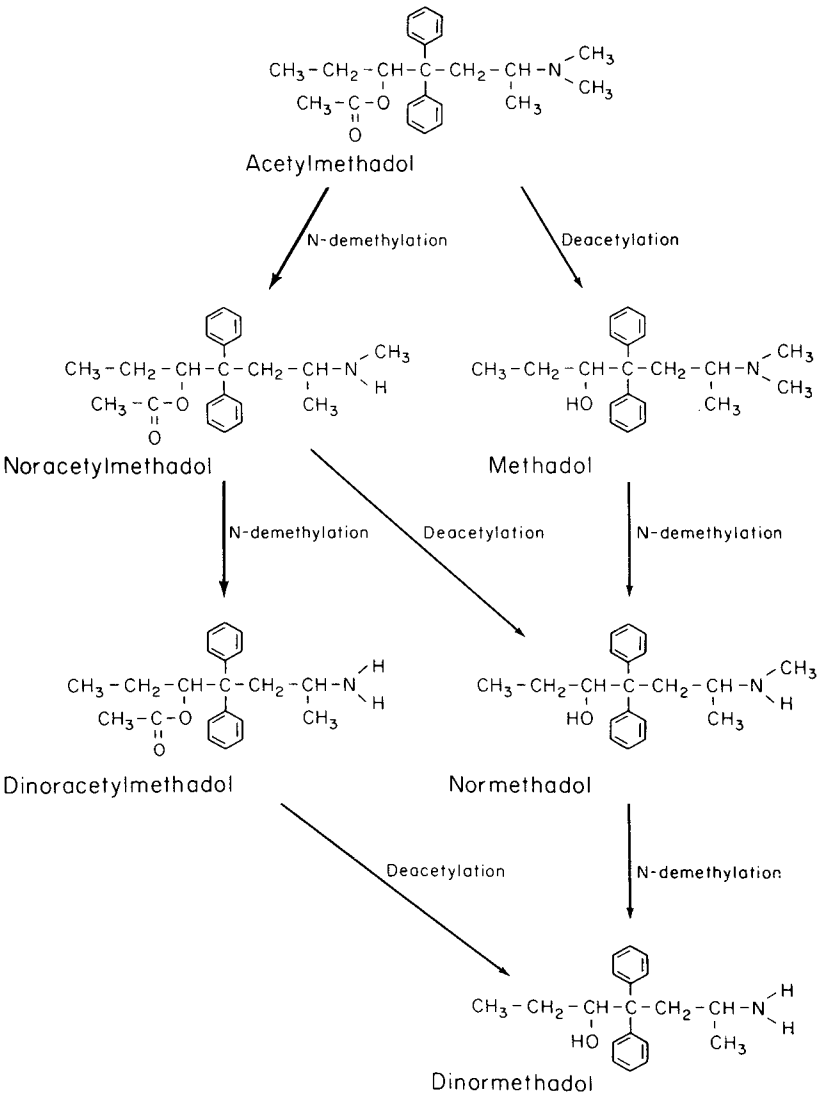

Fig. 1. Biotransformation pathways for acetylmethadol (LAAM), and structural formulae for LAAM, noracetylmethadol (NAM), dinoracetylmethadol (DNAM), methadol (MOL), normethadol (NMOL) and dinormethadol (DNMOL).

We will describe a method using high-performance liquid chromatography (HPLC) that can resolve the compounds of interest and demonstrate that this method has the requisite sensitivity to measure these compounds in biofluid samples from man and animals that have received single or multiple doses of LAAM.

MATERIALS AND METHODS

Chemicals and reagents

The following compounds were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the Medicinal Chemistry and Technology Section of the National Institute on Drug Abuse (NIDA), Rockville, MD, U.S.A.: *l*,α-acetylmethadol, *l*,α-noracetylmethadol, *l*,α-methadol and *l*,α-normethadol, all as the hydrochloride salt; *l*,α-dinoracetylmethadol and *l*,α-dinormethadol both as the maleate salt; and *l*,α-[O,O'-$^3H_2$]-acetylmethadol ([^{3}H] LAAM) at 1.4 Ci/mmole. The radiochemical purity of the [^{3}H] LAAM was found by HPLC to be greater than 98%. The *l*-propranolol was a gift of Dr. R. Levi, Cornell University Medical College (New York, NY, U.S.A.). Methanol, acetonitrile, *n*-butyl chloride, hexane and ethyl acetate were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ethyl acetate was redistilled before use. Ammonium hydroxide [58% NH_4OH in water (w/w)] was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). The percentage of ammonium hydroxide in the mobile phase refers to the final concentration of NH_4OH expressed as a percentage (v/v).

Stock solutions

LAAM, MOL, NAM, DNAM, NMOL and DNMOL stock solutions at a concentration of 1 mg/ml and the internal standard, *l*-propranolol, at 0.1 mg/ml are prepared in methanol and stored at −5°C. Calibration standards are prepared by diluting the stock solutions with a mixture of methanol—acetonitrile (20:80, v/v).

Sample preparation from biofluids

Into a 13-ml ground-glass centrifuge tube with a Teflon stopper cap, add 1 ml of biofluid (plasma or urine), 0.10 ml of a 1.0 μg/ml solution of the internal standard and 0.5 ml of Delory and King's (D & K) carbonate—bicarbonate buffer (1 *M*, pH 9.6) [11]. The sample is extracted with 5 ml of *n*-butyl chloride for 7 min in an automatic shaker and centrifuged for 6 min at 500 *g*. The *n*-butyl chloride phase (upper) is transferred to a clean tube and evaporated to dryness (Vortex Evaporator Model 3-2200, Buchler Corp., Fort Lee, NJ, U.S.A.). This process is repeated again and the combined residue of the *n*-butyl chloride extract is reconstituted in 5 ml of *n*-hexane. The *n*-hexane phase is extracted with 2.5 ml of 0.2 *N* HCl by shaking for 7 min. After centrifugation for 6 min the *n*-hexane layer (upper) is discarded. The acid phase is washed with 5 ml of *n*-hexane and the pH adjusted to 9.6 by titration with 5 *N* ammonium hydroxide and the addition of 0.5 ml of the D & K buffer.

This aqueous phase is extracted twice with 5 ml of ethyl acetate by shaking for 7 min. After centrifugation for 6 min the ethyl acetate is transferred to a

12-ml siliconized conical centrifuge tube and evaporated to dryness as above. The sample extract is reconstituted in 100 μl of methanol—acetonitrile (20:80, v/v), and an appropriate volume of up to 100 μl is injected into the HPLC system.

Procedure for radiolabeled samples

To biofluid samples collected from animals that had received [^{3}H] LAAM are added 100 μl of a 15 μg/ml solution of unlabeled LAAM and metabolites as carriers. The internal standard is added and the sample extracted as above.

Chromatographic conditions

The analysis is performed on a Varian Model 8500 liquid chromatograph (Varian Instrument Group, Sunnyvale, CA, U.S.A.) equipped with a displacement syringe pump, a UV—Vis variable-wavelength detector (Varichrom Model VUV-10) and a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). The column is a 30 cm × 4 mm I.D. Varian Micropak containing 5-μm LiChrosorb Si-60. Chromatograms are recorded on a Model A-25 dual-channel chart recorder set at 1 mV. The mobile phase is a mixture of methanol—acetonitrile (70:30, v/v) containing 0.015% ammonium hydroxide. The flow-rate is 1.5 ml/min and the column and detector temperatures are maintained at 30° C. The column effluent is monitored at 218 nm at recorder scale that varies from 8 to 32 mA at full scale, and the chart speed is 10 in./h.

Following the injection of radiolabeled samples the column effluent corresponding to the peak area of each compound of interest is collected directly into a glass scintillation vial. The column effluent is evaporated to dryness and the residue reconstituted in 0.5 ml of methanol. Ten milliliters of a liquid scintillator containing 1% Liquifluor (New England Nuclear, Boston, MA, U.S.A.) in toluene are added and the sample is counted in a Model LS3100 liquid scintillation counter (Beckman Instruments, Irvine, CA, U.S.A.). Counting efficiency is determined by the internal standard method using a tritiated toluene standard. The extraction recovery is estimated by comparing the peak height of each compound of interest to an absolute standard curve.

Calibration curves and quantitation

Standard calibration curves are established by adding LAAM, MOL, NAM, DNAM, NMOL, DNMOL and *l*-propranolol to drug-free plasma or urine and proceeding as described above. Quantitation is performed by drawing the baseline and measuring the peak height of the compounds of interest. The ratio peak height standard/*l*-propranolol is calculated. A standard curve is constructed by plotting the peak height ratio against the amount added. Each calibration curve is constructed from at least triplicate determinations of five points.

Human and animal studies

Biofluid samples were collected from humans, sheep and rats following LAAM administration. Plasma and urine were collected from former heroin addicts receiving 40—60 mg of LAAM, orally, 3—5 times per week for 3—7

weeks as part of a LAAM maintenance treatment program. A Dorset ewe weighing 50 kg and prepared with polyethylene catheter inserted into the hindlimb artery and vein received a LAAM infusion of 0.02 mg/kg per min into the vein for 6 h. Blood samples were collected prior to, during and at 18 h after the cessation of drug administration. Male Sprague-Dawley rats weighing 300—350 g were prepared by cannulation of the right jugular vein. Blood and urine samples were collected prior to and from 5 min to 40 h after the rapid intravenous injection of 2.5 mg/kg unlabeled LAAM plus 130 μCi of [^{3}H] LAAM per rat. Blood samples were centrifuged at 500 *g* for 10 min and the plasma recovered. Plasma and urine are frozen at —20°C prior to analysis.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

LAAM and its metabolites are weak bases, which partially ionize in aqueous solution. This property makes it difficult to achieve a good separation using a reversed-phase column unless ionization of the compounds of interest can be suppressed by raising the pH of the mobile phase. Unfortunately, this approach often results in a degradation in column performance [12]. Paired-ion chromatographic techniques [13] may overcome the problem of column degradation, but the reagent used for ion-pairing has a UV cut-off at about 240 nm and therefore prevents use of the more sensitive short-wavelength absorption. Since LAAM and its metabolites have very similar structures but different functional groups (Fig. 1) they are amenable to separation by normal-phase chromatography. The polar nature of the compounds of interest suggests that methanol, a solvent with a low UV cut-off and rather good polarity, might serve as the mobile phase. All of the compounds except MOL and NAM were separated but with a very long analysis time and peak tailing. This is probably the result of an acid—base interaction, where the mass transfer is slow between a weakly basic drug and the acidic sites of silica gel [14]. A small amount of ammonium hydroxide was added as a modifier to compete at the acidic sites of silica gel, leaving the compounds of interest free to adsorb at other sites where mass transfer is faster. Ammonium hydroxide will also increase the pH of the mobile phase, resulting in suppression of the ionization of the compounds of interest. The addition of this modifier substantially decreased analysis time, but MOL and NAM were still not resolved.

The addition of acetonitrile can improve the resolution of MOL and NAM without compromising the resolution of the other compounds of interest. Fig. 2 shows the effect of the addition of acetonitrile to the mobile phase on column capacity factor (k') and analysis time. When the concentration of acetonitrile exceeds 50%, the resolution between DNMOL and NMOL and between DNAM and NAM begins to decrease (not shown in Fig. 2). Since acetonitrile has a lower polarity than methanol, the analysis time increases as the concentration of acetonitrile in the mobile phase is increased (Fig. 2). Fig. 3 shows the effect of relatively small changes in the concentration of ammonium hydroxide on k', analysis time and peak height. As the concentration of ammonium hydroxide is increased from 0 to 0.02%, k' and analysis

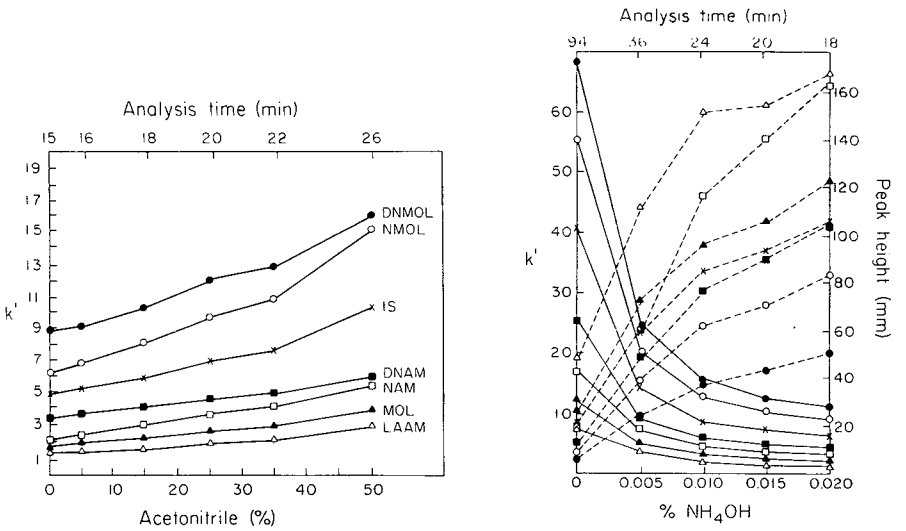

Fig. 2. Effect of increasing the percentage of acetonitrile in the mobile phase (methanol—acetonitrile) from 0% to 50% on the column capacity factor (k') of each compound of interest. The ammonium hydroxide modifier was kept constant at 0.015%. The chromatographic conditions are as described in Materials and methods. The abbreviations for LAAM and metabolites are as in Fig. 1 (IS = internal standard). The time required for a complete analysis is given on the upper abscissa.

Fig. 3. Effect of increasing the ammonium hydroxide modifier from 0 to 0.020% on the column capacity factor (k') of each compound of interest and on the peak height response. The mobile phase is methanol—acetonitrile (70:30, v/v). The chromatographic conditions are as described in Materials and methods. The abbreviations for LAAM and metabolites are as in Fig. 1. The time required for a complete analysis is given on the upper abscissa. (○) NMOL; (●) DNMOL; (□) NAM; (■) DNAM; (△) LAAM; (▲) MOL; (X) IS (internal standard).

time decrease while peak height is increased. The results shown in Figs. 2 and 3 demonstrate that by adjustment of the proportions of methanol, acetonitrile and ammonium hydroxide the system can be made optimal for quantitation by peak height measurement using the UV detector or for fractional collection of each compound of interest without cross-contamination.

A sample chromatogram demonstrating the resolution of the compounds of interest is given in Fig. 4. The use of elevated pH and methanol in a mobile phase can result in rapid deterioration of the column performance of a silica gel based packing material due to solubilization of the silica matrix. We have minimized this problem by placing a 20 × 4 mm guard column containing 40-μm silica (Vydac A, Varian Instrument Group) before the analytical column. The guard column serves to filter some contaminants in the applied samples and will saturate the mobile phase with silica to reduce the rate of dissolution of silica from the analytical column. These columns can be used for at least three months with only a slight loss in efficiency.

Column efficiency

The effects of flow-rate and temperature were investigated to determine optimum column efficiency. Since the mass diffusion term is much less im-

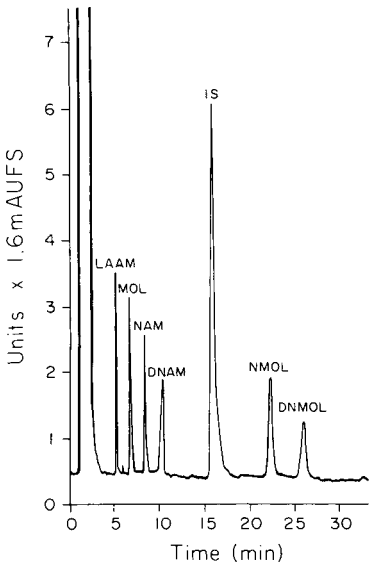

Fig. 4. Chromatogram of a calibration standard. To 1 ml of control human plasma were added LAAM and metabolites at 40 ng each and the internal standard (IS), propranolol, at 100 ng. The plasma extract was prepared as described in Materials and methods. The extract was reconstituted in 100 μl of methanol—acetonitrile (20:80, v/v) and 70 μl were injected. Chromatographic conditions are as described in Materials and methods. Abbreviations are as in Fig. 1.

portant in liquid chromatography than in gas chromatography, column efficiency is expected to be better at lower flow-rates due to good mass transfer. This is illustrated in Fig. 5 which shows the height equivalent to theoretical plates (HETP) obtained at different flow-rates. The HETP is quite low (< 0.09 mm) throughout the entire range and, except for MOL, the curves show a minimum at 50—70 ml/h. However, for a convenient analysis time we chose 80—90 ml/h. Increasing the column temperature from 20 to 50°C only slightly affects the column efficiency and analysis time. However, it is important to maintain the temperature of the column and detector constant when a wavelength in the far UV is used, since relatively small temperature fluctuations caused by heat generated from the pump or variations in room temperature will affect the precision of the retention time and the baseline. We chose to maintain the temperature of the column and detector cell at 30°C.

Reconstitution of the sample extract

The sample extract is dissolved in 100 μl of methanol—acetonitrile (20:80, v/v), a weaker solvent than the mobile phase, so that the sample will be concentrated at the column head as the reconstitution solution passes through the column.

Detection in the ultraviolet

The absorbance of LAAM and its metabolites increases significantly in the far UV. A wavelength of 218 nm was selected as a compromise between the

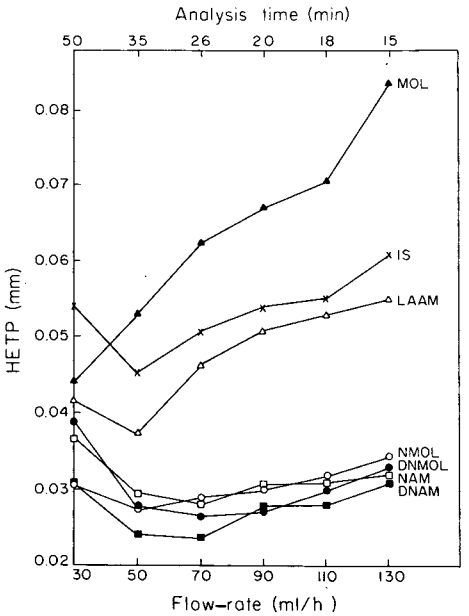

Fig. 5. Effect of the flow-rate of the mobile phase, methanol—acetonitrile (70:30, v/v) with 0.015% ammonium hydroxide on column efficiency as measured by height equivalent to theoretical plates (HETP). The chromatographic conditions are as described in Materials and methods. Abbreviations are as in Fig. 1 (IS = internal standard). The time required for a complete analysis is given on the upper abscissa.

maximum sensitivity and acceptable noise. At a cell temperature of 30°C (see above) the retention time of each compound is quite stable. The coefficient of variation for retention time was determined from eight consecutive injections of a mixture of the compounds of interest and was found to vary from 0.26% for LAAM to 0.73% for DNMOL. This reproducibility of retention time can permit the "blind" collection of fractions that correspond to the established retention times of the compounds of interest when the concentration of a sample falls below the lower limit of sensitivity of the UV detector. Samples collected in this manner can be analyzed using a sensitive radioimmunoassay procedure (studies in progress).

Extraction recovery and calibration curves

The optimal conditions for the simultaneous extraction of LAAM, metabolites and the internal standard, *l*-propranolol, require careful attention to pH and partition conditions. DNAM, a major metabolite is rapidly converted to the corresponding amide at pH 10.0 or greater [6]. The back-extraction of DNAM and DNMOL from *n*-butyl chloride into acid is incomplete and variable, therefore we found it necessary to evaporate the *n*-butyl chloride to dryness and reconstitute with a less polar solvent, *n*-hexane. The back-extraction from *n*-hexane into acid is nearly complete. The recovery of the compounds of interest is independent of concentration from 40 to 2000 ng/ml. After a correction for aliquot losses the mean recovery and coefficient of variation (C.V.) as

percentages are: LAAM = 95 (C.V. = 1.1), MOL = 88 (C.V. = 1.7), NAM = 89 (C.V. = 1.1), DNAM = 67 (C.V. = 3.7), NMOL = 85 (C.V. = 2.1), DNMOL = 77 (C.V. = 2.0).

The extraction procedure yields an extract that is free of peaks that interfere with the quantitation of the compounds of interest (Fig. 4).

Fig. 6 shows that, using the UV detector, standard calibration curves can be constructed that are linear from 10 to 100 ng. Although not shown in Fig. 6, the standard calibration curves are linear up to 2000 ng. The precision of triplicate determinations of 20 and 100 ng calibration standards is given in Table I. Using an initial volume of 1 ml, this system has a lower limit of sensitivity (signal-to-noise ratio of 5) of 10 ng/ml for DNAM, NMOL and DNAM and 6 ng/ml for LAAM, MOL and NAM.

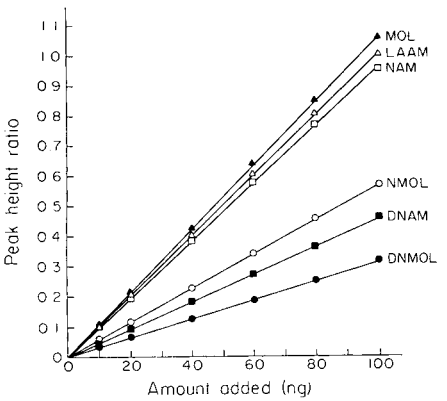

Fig. 6. Standard calibration curves for LAAM and metabolites recovered from control human plasma. Each point represents the mean of triplicate determinations. Abbreviations are as in Fig. 1.

TABLE I

PRECISION VALUES FOR THE CALIBRATION CURVE OF LAAM AND METABOLITES

Compound	Coefficient of variation (%)	
	20 ng	100 ng
LAAM	3.3	1.6
MOL	4.1	1.7
NAM	3.1	3.2
DNAM	5.2	4.8
NMOL	3.3	2.0
DNMOL	5.0	4.5

Potential interference

As shown in Table II, LAAM and metabolites are resolved from commonly used or abused narcotics and their metabolites. Thus, this method can be used to analyze samples from subjects taking or receiving the drugs given in Table II.

TABLE II

RESOLUTION OF LAAM AND METABOLITES FROM SELECTED NARCOTICS AND THEIR METABOLITES

Drug*	Column capacity factor (k')
Heroin	1.18
6-Monoacetylmorphine	1.37
Propoxyphene	1.52
Codeine	1.52
Morphine	1.59
Meperidine	1.71
LAAM	2.03
MOL	2.72
NAM	3.70
DNAM	4.70
Pentazocine	6.82
NMOL	10.57
Normeperidine	11.17
DNMOL	12.88
Methadone	13.14
Levorphanol	14.88
Norpropoxyphene	>15
Methadone metabolite 1	>15

*Abbreviations as in Fig. 1.

HPLC and liquid scintillation counting of radiolabeled samples

The availability of a tritiated form of LAAM with high specific activity led us to adapt the HPLC system we have described for the collection of fractions containing the radiolabeled compounds of interest so that they could be analyzed by liquid scintillation counting. The location and recovery of the compounds of interest are determined by adding unlabeled "carrier" in amounts such that any contribution to the UV detector response by the radioisotope is less than 5%. The limit of detection is a function of the specific activity of the radiolabeled drug and the average background. We can easily determine 1.0 ng/ml of [^{3}H]LAAM. The chromatographic conditions are adjusted to prevent cross-contamination of the peaks. We have found a satisfactory mobile phase to be methanol—acetonitrile (65:35, v/v) with 0.01% ammonium hydroxide (see also Figs. 2 and 3).

Human and animal studies

Examples of the application of the method using the UV detector for the quantitation of LAAM and metabolites are presented in Tables III and IV. LAAM and each of the five metabolites are found in the urine of maintenance patients (Table III). Fig. 7 shows the chromatogram of an extract of a sample of urine obtained from patient 4. At the low attenuation used to obtain Fig. 7 the peaks corresponding to the retention times of LAAM, NAM and DNAM are "off scale". However, peaks corresponding to MOL, NMOL and DNMOL are present and can be quantitated (Table III). These results confirm an earlier

TABLE III

LAAM AND METABOLITES IN MAINTENANCE-PATIENT BIOFLUIDS AT 24 HOURS AFTER A DOSE

Patient	Biofluid	Concentration (ng/ml)					
		LAAM	NAM	DNAM	MOL	NMOL	DNMOL
1	Plasma	41	140	80	nd*	nd	nd
1	Urine	1000	3050	4200	31	23	29
2	Plasma	12	28	26	nd	nd	nd
2	Urine	450	1500	2800	23	13	22
3	Plasma	24	68	57	nd	nd	nd
3	Urine	1450	2800	4000	46	26	40
4	Plasma	27	44	42	nd	nd	nd
4	Urine	600	1575	3700	14	22	46
5	Plasma	8	38	24	nd	nd	nd
5	Urine	50	575	1150	8	6	14

*nd = not detected.

TABLE IV

LAAM AND METABOLITES IN SHEEP PLASMA

The dose was an intravenous infusion of LAAM, 0.02 mg/kg per min for 6 h.

Time (h)	Plasma concentration (ng/ml)		
	LAAM	NAM	DNAM
0	0	0	0
0.25	97	13	0
0.5	116	19	0
1	228	54	6
2.5	253	97	23
4	340	159	50
6	423	211	78
24	36	72	168

qualitative study [6] which used mass spectrometry to identify LAAM, NAM, DNAM, MOL and NMOL in patients' urine. At the time of this report by Kaiko et al. [6] an analytical standard of DNMOL was not available. The discovery of DNMOL in patients' urine using HPLC will require confirmation using mass spectrometry (studies in progress). As we have reported earlier [6, 15], only LAAM, NAM and DNAM are present in detectable amounts in the plasma of LAAM maintenance patients (Table III). LAAM, NAM and DNAM are found in sheep plasma during and following the infusion of LAAM (Table IV). LAAM levels continued to rise during the 6-h infusion and were greater than those of NAM and DNAM. DNAM was not detected until 1 h after the start of the LAAM infusion. At 24 h (18 h after the cessation of the infusion) LAAM levels are lower than those of NAM and DNAM. These results suggest

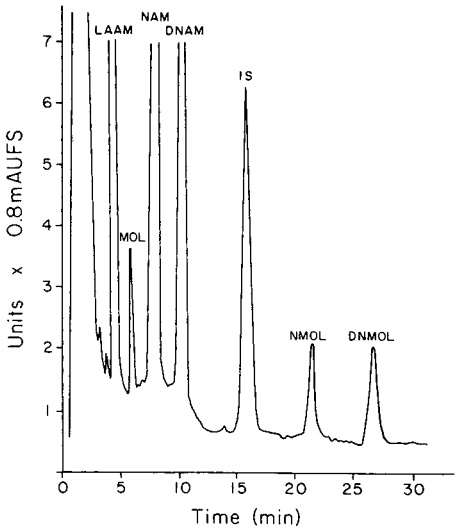

Fig. 7. Chromatogram of urine from a LAAM-maintenance patient. The extract was prepared from 2 ml of patient urine collected at 24 h after a dose, to which was added 50 ng of propranolol, the internal standard (IS). Extraction is as described in Materials and methods. Mobile phase is methanol—acetonitrile (75:25, v/v) with 0.008% ammonium hydroxide. Abbreviations are as in Fig. 1.

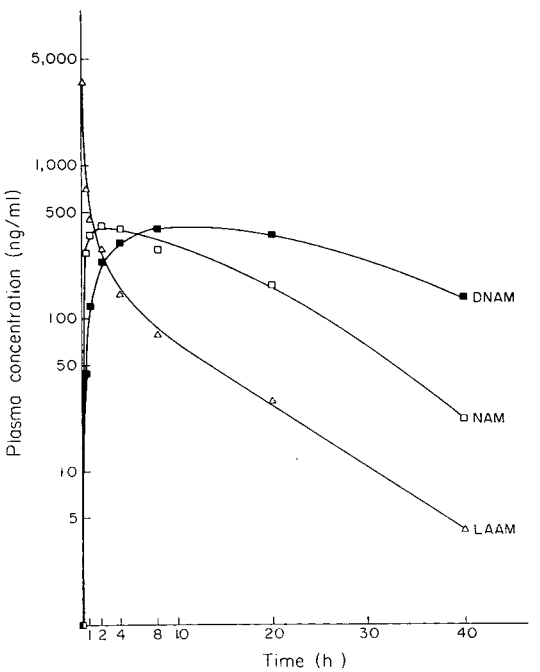

Fig. 8. Plasma levels of LAAM (△), NAM (□) and DNAM (■) following the intravenous injection of [^{3}H]LAAM (2.5 mg/kg, 130 μCi) to a rat.

that, in sheep, NAM and DNAM are persistent metabolites (see also ref. 15).

Fig. 8 demonstrates the results obtained when the HPLC system is combined with fraction collection and liquid scintillation counting to measure the disposition of [^{3}H] LAAM. Radiolabeled LAAM, NAM and DNAM were found in rat plasma following the intravenous administration of [^{3}H] LAAM. We could not demonstrate significant (> 1.0 ng/ml) radioactivity in the fractions that correspond to the UV peaks for MOL, NMOL or DNMOL. As would be expected from their respective product—precursor relationships, as LAAM levels fall, NAM and then DNAM increase. In the rat, at 40 h after dosing with [^{3}H] LAAM, DNAM levels are the highest and LAAM levels the lowest.

We have developed an HPLC method for the quantitative determination of LAAM and metabolites in biofluids. This system can be used to characterize the pharmacokinetics of LAAM in man and laboratory animals. In addition, the method can be used to determine the disposition of any one of the metabolites.

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DETERMINATION OF DIAZEPAM AND ITS MAJOR METABOLITES IN MAN AND IN THE CAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of diazepam, and its major metabolites, oxazepam, temazepam and nordiazepam in plasma, blood, and urine of humans and cats. The assay for the compounds involves extraction into benzene—methylene chloride (90:10) from plasma, blood or urine buffered to pH 9.0. In both species the overall recovery of diazepam and its major metabolites from plasma or blood ranged from 60 ± 3.2 to 89 ± 13% (S.D.) and for urine from 79 ± 7.9 to 93 ± 10.5% (S.D.).

The sensitivity limit of the assay using UV detection at 254 nm was 50 ng/ml of plasma and blood in both species except for human urine (post-Glusulase) which was 200 ng/ml. The HPLC assay was used to monitor the plasma concentration—time profile in humans following a 10-mg oral dose of diazepam and the blood concentration time profile of diazepam and nordiazepam in cats following a 10 mg/kg intravenous dose of either diazepam or nordiazepam. The HPLC assay data correlated well with data generated by an electron-capture—gas—liquid chromatographic assay.

INTRODUCTION

The pharmacology of diazepam is well defined in man and many animal species [1—3]. Metabolic studies in man and animals [4] have shown that the compound is biotransformed to form three major metabolites (Fig. 1). Several attempts have been made to determine diazepam and its major metabolites simultaneously by electron-capture—gas—liquid chromatography (EC—GLC) [5—7], thin-layer chromatography (TLC) [8] and high-performance liquid chromatography (HPLC) [9, 10]. In general, the EC—GLC and TLC assays either could not separate diazepam and its three major metabolites simultaneously, were time consuming, needed a large sample (1.0 ml or greater),

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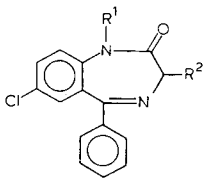

Compound	R^1	R^2
Diazepam	CH_3	H_2
Nordiazepam	H	H_2
Temazepam	CH_3	OH
Oxazepam	H	OH

Fig. 1. Chemical structures of diazepam and its metabolites.

and/or had a long analysis time making the assay impractical for routine use in pharmacokinetic studies where rapid sample throughput is very desirable.

The purpose of the study was to develop an analytical method suitable for pharmacokinetic studies capable of determining diazepam and its three major metabolites simultaneously using small samples of biological fluid. Previous studies in the cat [11] using ^{14}C-labelled diazepam indicated that hydroxylation was a major biotransformation pathway in this species and prompted further study.

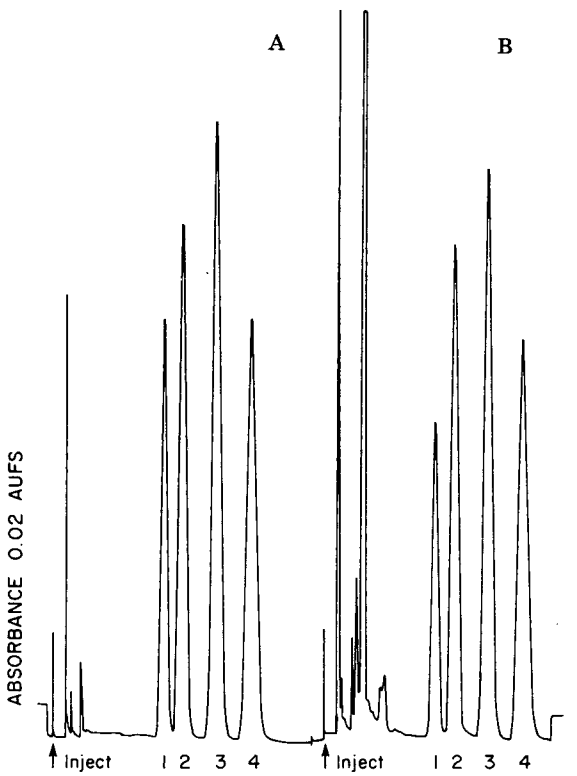

Fig. 2. Chromatograms of (A) standard mixture of 200 ng of each compound, and (B) human plasma containing 400 ng of each compound per ml taken through the extraction procedure. Peaks: 1 = oxazepam (k' = 6.0), 2 = temazepam (k' = 7.2, α = 1.2), 3 = nordiazepam (k' = 9.2, α = 1.3), 4 = diazepam (k' = 10.8, α = 1.2).

EXPERIMENTAL

HPLC parameters

Column parameters. The column used was a prepacked 30 cm × 3.95 mm I.D. stainless-steel column containing Bondapak (10 μm) C_{18} reversed-phase packing (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters. A Waters Assoc. high-performance liquid chromatograph Model ALC/GPC-204/6000A with a Model U6K injection system and a Model 440 UV detector with a 254 nm wavelength kit was used. All chromatograms were recorded on a 10-mV Hewlett-Packard recorder at a chart speed of 0.25 in./min.

The mobile phase used was methanol 550 ml diluted to 1000 ml with distilled deionized water. The solvent flow-rate was 2.0 ml/min.

Representative chromatograms of human plasma, human urine (pre- and post-Glusulase) and cat blood extracts, the retention times (t_R), capacity factors (k'), and the separation factor (α) of the compounds analyzed under the above conditions are given in Figs. 2, 3 and 4.

Analytical standards

All analytical standards were of pharmaceutical grade purity (> 99%). These included diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodia-

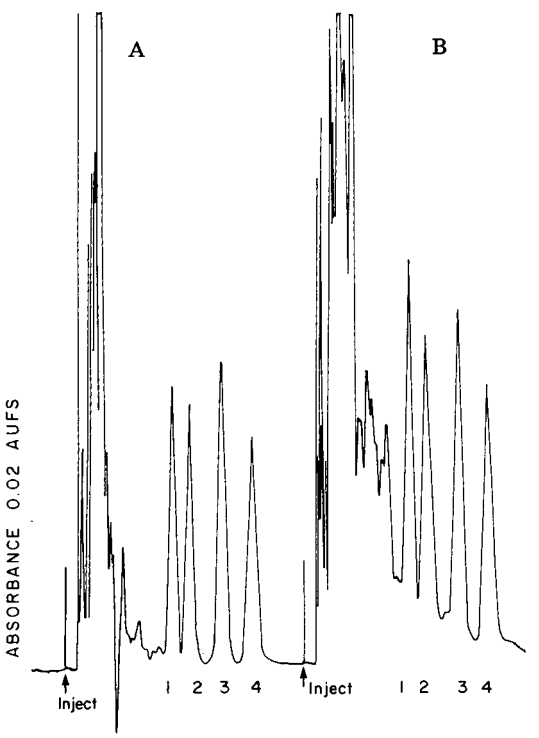

Fig. 3. Chromatograms of human urine containing 400 ng of each compound per ml, extracted (A) pre-Glusulase, and (B) post-Glusulase incubation. Peaks: 1 = oxazepam (k' = 8.0), 2 = temazepam (k' = 9.5, α = 1.2), 3 = nordiazepam (k' = 12.5, α = 1.3), 4 = diazepam (k' = 15.0, α = 1.2).

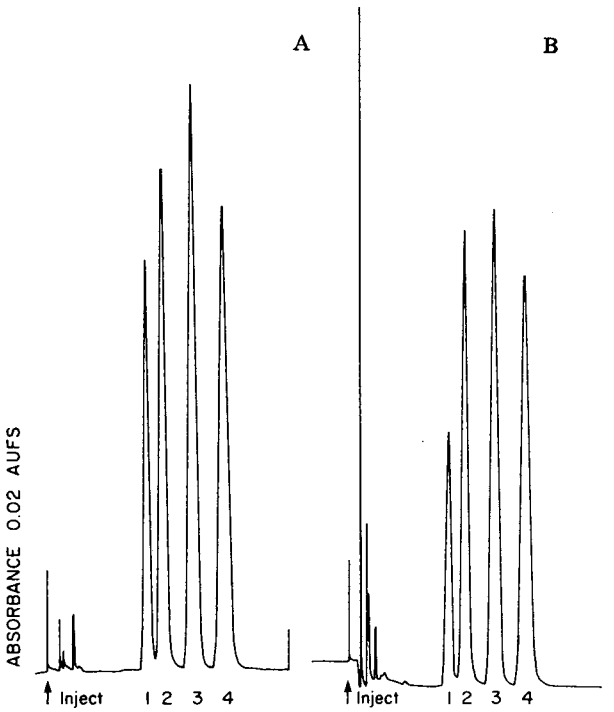

Fig. 4. Chromatograms of (A) standard mixture containing 200 ng of each compound, and (B) cat blood containing 400 ng of each compound per ml taken through the extraction procedure. Peaks: 1 = oxazepam (k' = 7.2), 2 = temazepam (k' = 8.3, α = 1.1), 3 = nordiazepam (k' = 11.0, α = 1.2), 4 = diazepam (k' = 13.8, α = 1.2).

zepin-2-one, $C_{16}H_{13}N_2OCl$, MW 284.74), nordiazepam (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{15}H_{10}N_2OCl$, MW 270.72), temazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{16}H_{13}N_2O_2Cl$, MW 300.74), and oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{15}H_{10}N_2O_2Cl$, MW 286.72).

Preparation of standard solutions

Weigh 10 mg of each of the compounds into a 100-ml volumetric flask and dissolve in 1 ml of ethanol. Dilute to 100 ml with benzene to yield a stock solution containing 100 μg/ml of each component. Dilute 1 ml of the stock solution to 100 ml with benzene to yield a working solution containing 1.0 μg/ml.

Aliquots of the respective working solutions (50—1000 μl) are transferred into separate 15-ml centrifuge tubes, evaporated to dryness under nitrogen at 60°C, and the residues are dissolved in 50 μl methanol to yield mixed standard solutions containing each of the four compounds in the amounts indicated

below:

Mixed standard solutions	Compound (ng/50 μl)
A	50
B	200
C	400
D	800
E	1000

Twenty-five microliters of the mixed standard solutions (A, B, C, D or E), are injected as external standards for establishing the HPLC parameters. Separate aliquots (50—1000 μl) of the working solution (1 μg/ml) are transferred into 15-ml centrifuge tubes, evaporated to dryness under nitrogen at 60°C, the residues are dissolved in an appropriate volume (0.1—1.0 ml) of plasma, blood or urine of either human or cat and are used as the processed standards to establish a calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery.

Reagents

All reagents used were of analytical reagent grade (> 99% purity). The inorganic reagents used are 1.0 *M* borate—Na_2CO_3—KCl buffer (pH 9.0) and 1.0 *M* phosphate buffer (pH 5.3) prepared as described [5].

The organic solvents used (nanograde purity) are methylene chloride and benzene (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

Calibration of diazepam and its major metabolites by HPLC

A calibration (external standard) curve of peak height of each component versus concentration in the range 25—500 ng per 25 μl injected is constructed. Calibration curves of both the external and the recovered standards were prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Analysis of human plasma or cat blood

Into a 15-ml centrifuge tube (PTFE No. 13 stoppered) add 0.5—1.0 ml of human plasma or 0.1—1.0 ml of cat blood, 0.2—1 ml of 1.0 *M* H_3BO_3—Na_2CO_3—KCl buffer (pH 9.0), and mix well on a Vortex action mixer. Extract the mixture with 5 ml of benzene—methylene chloride (90:10) by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes per min. Along with the samples, run six 1-ml specimens of control human plasma, urine, cat blood and urine. One used as a control blank and five containing 50 μl of standard solutions, A—E evaporated to dryness before adding the various biological fluids. Centrifuge the samples at 2200 rpm for 5 min (Damon/IEC Centrifuge Model PR-J, rotor No. 250 at 1300 *g*) and transfer a 4.8-ml aliquot of the upper organic layer into a 5-ml conical centrifuge tube. Evaporate the organic layer to dryness at 60°C in an N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean dry nitrogen. Dissolve the residue in a 50-μl aliquot of the mobile phase used in the

respective assay and inject a 25-μl aliquot for HPLC analysis.

Analysis of human or cat urine

Unconjugated fraction. One ml of human or cat urine is processed exactly as described for the analysis of human or cat blood. This fraction represents intact drug and unconjugated metabolites.

Conjugated fraction. The urine specimen remaining after the extraction of the unconjugated fraction is titrated with 1.0 *N* HCl to pH 5.3 and buffered with 1.0 *M* phosphate buffer. Add 2% Glusulase® enzyme preparation containing 150,000 units of glucuronidase and 100,000 units of sulfatase per ml (Endo Labs., Garden City, NY, U.S.A.) by volume of sample taken for analysis and incubate in a Dubnoff metabolic shaker at 37°C for 12—18 h (overnight). After cooling, the pH of sample is adjusted to 9.0 with 2 *N* NaOH and the sample extracted as described for plasma or blood.

Calculations

The concentration of each compound in the unknowns is determined by interpolation from the respective calibration curve of the recovered standards processed along with the unknowns, using the direct calibration (peak height versus concentration) technique. The percent recovery of each compound is determined by comparing the slope value (peak height per ng of compound) of the recovered standard curve to that of the external standard curve.

RESULTS AND DISCUSSION

The utility of EC—GLC in the analysis of diazepam and its major metabolites is well documented [5—7]. Although it is inherently more sensitive than HPLC analysis (with UV detection) for the determination of diazepam and nordiazepam per se, the analysis of temazepam and oxazepam requires silylation to the trimethylsilyl ether derivative, not only for enhanced sensitivity and chromatographic behavior, but also to ensure thermal stability, especially of oxazepam. The procedural details required make the simultaneous analysis of all four compounds by EC—GLC [5] a difficult procedure, at best.

The intrinsic UV absorbance of the benzodiazepines and their amenability to HPLC analysis, especially of thermally unstable compounds such as oxazepam or those that require derivatization prior to EC—GLC analysis (temazepam) have been used to advantage in pharmacokinetic studies [9, 12, 13].

Reversed-phase HPLC is preferred for the analysis of benzodiazepines since it provides better separation than does adsorption chromatography [12]. The pH of extraction and solvents used were selective for the quantitative extraction of the respective compounds of interest, evidenced by the absence of endogenous interfering peaks in the retention areas of diazepam and its major metabolites. All the compounds have sufficient UV absorbance at 254 nm for their accurate quantitation in the nanogram range.

The sensitivity of the assay for all four compounds in human plasma, cat blood, human urine (pre-Glusulase) and cat urine (both pre- and post-Glusulase

incubation) was 50 ng/ml. The sensitivity for all four compounds in human urine post-Glusulase, however, was 200 ng/ml.

Statistical validation

The overall mean recovery of diazepam and its major metabolites over the concentration range of 50—1500 ng/ml from various biological fluids is presented in Table I. The recovery of temazepam and nordiazepam was more consistent ranging from 81—89%, whereas that for diazepam (73—93%) and oxazepam (60—87%) showed a greater variation in the four biological fluids studied.

The intra-assay variability of diazepam and its metabolites recovered from human plasma, urine, cat blood and urine is presented in Table II. The mean intra-assay variability for all four compounds ranged from 4.2—6.9% over the concentration range of 50—1500 ng/ml.

TABLE I

OVERALL PERCENT RECOVERY (± S.D.) OF DIAZEPAM AND ITS MAJOR METABOLITES FROM VARIOUS BIOLOGICAL FLUIDS

	Concentration range (ng/ml)	Human plasma	Cat blood	Human* urine	Cat urine
Diazepam	50— 800	73 ± 5.0	74 ± 5.6	93 ± 10.5	79 ± 7.9
Nordiazepam	50— 800	84 ± 4.8	85 ± 8.0	85 ± 6.2	84 ± 12.1
Temazepam	50—1500	89 ± 13.0	81 ± 9.0	87 ± 11.3	88 ± 4.1
Oxazepam	50—1500	60 ± 3.2	63 ± 7.0	81 ± 10.4	87 ± 6.3

*The limit of quantitation for all four compounds in human urine (post-Glusulase) is 200 ng/ml.

TABLE II

INTRA-ASSAY VARIABILITY OF DIAZEPAM AND ITS MAJOR METABOLITES IN HUMAN PLASMA, URINE (PRE- AND POST-GLUSULASE), CAT BLOOD AND URINE (PRE- AND POST-GLUSULASE)

In all cases $n = 5$.

	Amount added (ng/ml)	Amount recovered (ng/ml)	Standard deviation (%)
Diazepam	50*	54	7.7
	50**	50	8.0
	50***	50	8.0
	50§	50	4.0
	50§,§§	50	4.0
	100*	97	4.9
	200**,§§	200	6.5
	300*	300	7.4
	300***	300	5.3
	300§	300	13.3
	300§,§§	300	7.3
	1500**	1500	8.5
	1500**,§§	1500	4.2
		Average	6.9

(Continued on p. 102)

TABLE II (continued)

	Amount added (ng/ml)	Amount recovered (ng/ml)	Standard deviation (%)
Temazepam	50*	55	2.0
	50**	50	6.0
	50***	50	8.0
	50§	50	0.0
	50§,§§	50	0.0
	100*	96	5.0
	200**,§§	195	4.1
	300*	301	6.7
	300***	300	4.7
	300§	300	10.0
	1500**	1500	5.5
	1500**,§§	1500	3.6
		Average	4.6
Nordiazepam	50*	54	0.0
	50**	50	2.0
	50***	50	8.0
	50§	50	0.0
	50§,§§	50	0.0
	100*	96	5.0
	200**,§§	200	4.2
	300*	300	7.4
	300***	300	4.7
	300§	300	9.3
	300§,§§	300	4.0
	1500**	1500	6.3
	1500**,§§	1500	3.5
		Average	4.2
Oxazepam	50*	51	4.3
	50**	50	9.0
	50***	50	4.3
	50§	50	8.9
	50§,§§	50	0.0
	100*	98	3.6
	200**,§§	200	4.3
	300*	300	6.9
	300***	300	2.6
	300§	300	13.3
	1500**	1500	7.9
	1500**,§§	1500	2.4
		Average	5.4

* In human plasma.
** In human urine.
***In cat blood.
§ In cat urine.
§§ Pre- and post-Glusulase.

The inter-assay variability of diazepam and its major metabolites recovered from human plasma and cat blood is presented in Table III. The mean inter-assay variability for all four compounds ranged from 6.2—8.4% over the concentration range of 50—800 ng/ml.

TABLE III

INTER-ASSAY VARIABILITY OF DIAZEPAM AND ITS MAJOR METABOLITES IN HUMAN PLASMA AND CAT BLOOD

	Amount added (ng/ml)	Amount recovered (ng/ml)	n	Standard deviation (%)
Diazepam	50	54	5	11.0
	100	104	5	6.7
	100*	109	4	12.0
	150	154	5	14.9
	200	197	4	8.5
	200*	187	4	9.0
	300	303	4	2.9
	400*	394	3	5.3
	800*	789	3	4.9
				Average 8.4
Nordiazepam	50	51	6	15.0
	100	106	6	6.0
	100*	111	4	12.0
	200	197	6	4.1
	200*	185	4	3.7
	300	303	4	2.0
	800*	853	3	5.7
				Average 6.9
Temazepam	50	53	6	12.6
	100	102	6	5.4
	100*	103	4	1.3
	150	141	4	9.1
	200	196	6	7.1
	200*	191	4	13.0
	300	304	4	2.9
	400*	402	4	4.1
	800*	805	4	0.3
				Average 6.2
Oxazepam	50	51	6	12.8
	100	100	6	5.7
	100*	99	4	5.9
	150	147	4	6.3
	150*	150	3	16.0
	200	199	6	5.1
	200	195	4	8.4
	300	298	4	2.0
	800	817	2	2.3
				Average 7.2

*Data from cat blood.

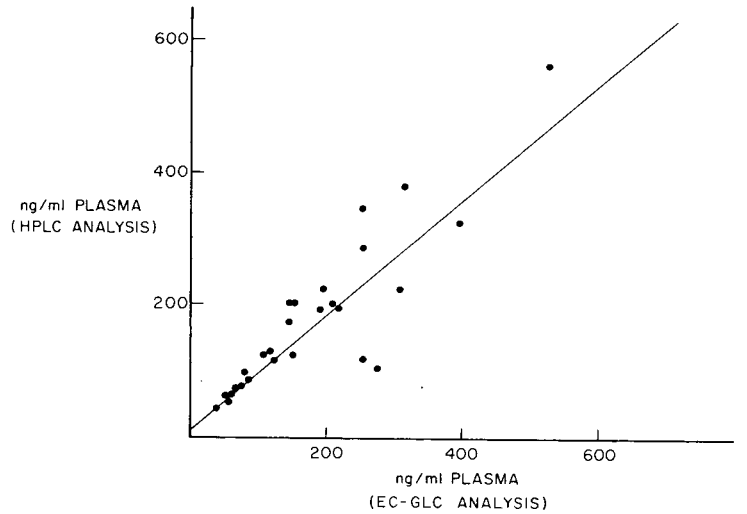

Fig. 5. Plasma concentrations (ng/ml) of diazepam in man following a single 10-mg oral dose, determined by HPLC vs. EC—GLC. $y = 0.938\,x + 15.9$; $r^2 = 0.831$, $r = 0.912$.

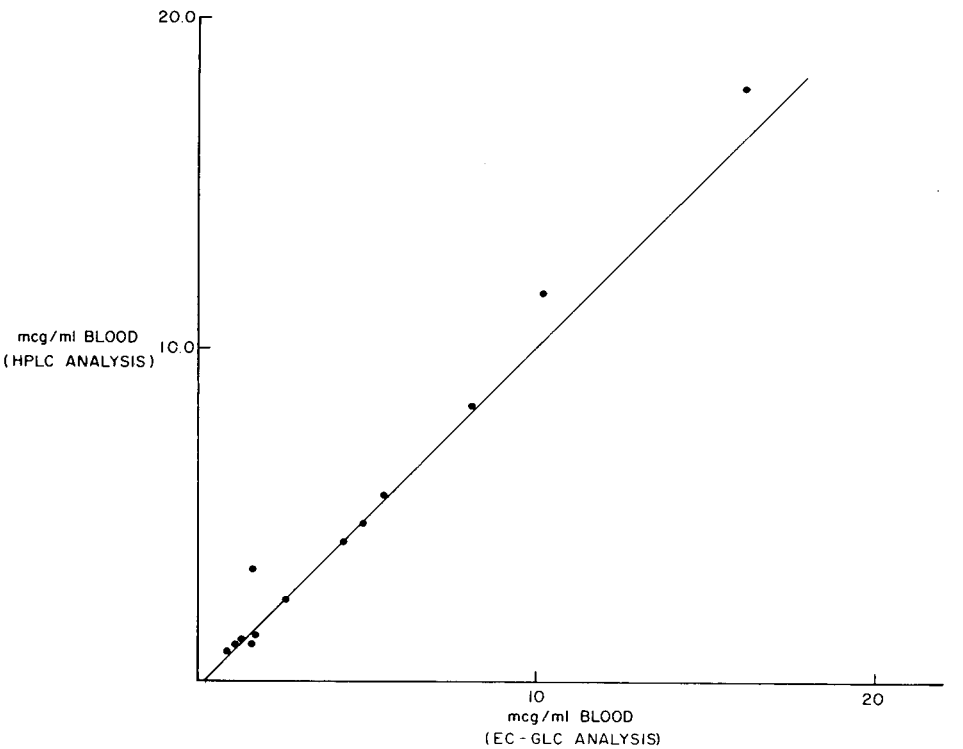

Fig. 6. Blood concentrations (μg/ml) of diazepam in a cat following a single intravenous dose of 10 mg/kg determined by HPLC vs. EC—GLC. $y = 1.10\,x - 0.17$; $r^2 = 0.985$, $r = 0.992$.

Application of the method in biological specimens

Human plasma and cat blood samples following diazepam administration, and cat blood samples following nordiazepam administration were analyzed by the HPLC assay method reported, and validated against the EC—GLC method previously reported [7].

A comparison of the data (HPLC vs. EC—GLC) from three human subjects, Fig. 5 (diazepam) and one cat, Fig. 6 (diazepam) and Fig. 7 (nordiazepam) determined by regression analysis showed a high degree of correlation (r = 0.912, 0.992, and 0.989, respectively) between the two methods, thus validating the clinical utility of the HPLC method for the analysis of biological samples.

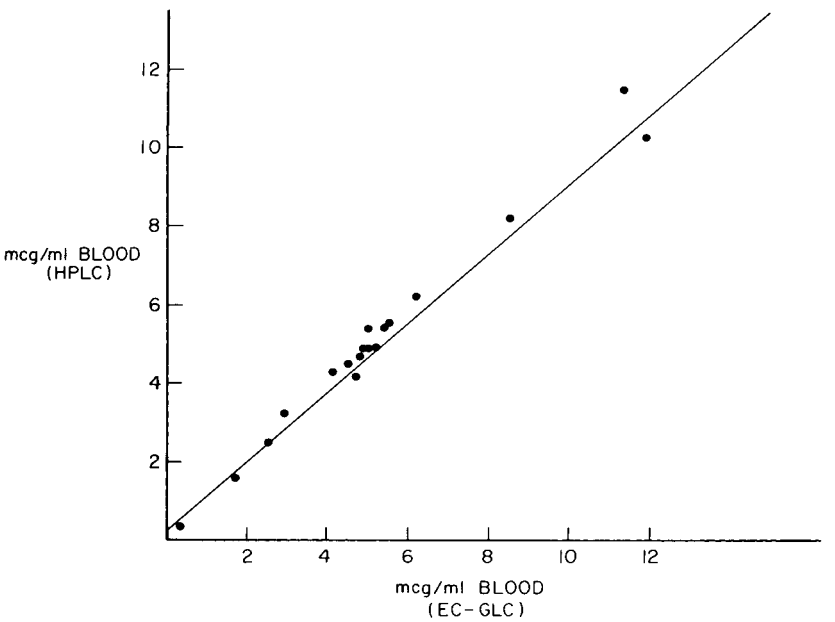

Fig. 7. Blood concentrations (μg/ml) of nordiazepam in a cat following a single intravenous dose of 10 mg/kg determined by HPLC vs. EC—GLC. $y = 0.924\ x + 0.300$; $r^2 = 0.978$, $r = 0.989$.

CONCLUSIONS

A sensitive and specific reversed-phase HPLC assay was developed for the determination of diazepam and its major metabolites in a variety of biological fluids: blood, plasma and urine. The method was applied to pharmacokinetic studies of diazepam in man and of diazepam and nordiazepam in the cat, and correlated well against the established EC—GLC assay, validating its clinical utility.

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Note

Determination of rat brain tissue catecholamines using liquid chromatography with electrochemical detection

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The determination of catecholamine levels in the brain as well as in other biological tissues has been the focus of many investigators for a number of years. Liquid chromatography coupled with electrochemical detection (LC—EC) potentially has several advantages over fluorometric or radioenzymatic assays. LC—EC not only is more rapid and specific, but has the capacity to examine the metabolites as well as the parent catecholamines. Although previously published LC—EC procedures which use Waters μBondapak C_{18} columns [1—3] show that norepinephrine (NE) is separated from the solvent front, this separation is not consistent from column to column and deteriorates rapidly with column usage. Without an integrator, quantitation of NE is inconsistent because it is often not adequately resolved from the solvent front. When a peak is not adequately resolved, drawing a baseline is subjective and therefore not consistent from sample to sample. An electronic integrator determines a consistent baseline, even for unresolved peaks and therefore consistent values are obtained. This paper describes an improved LC—EC assay using a column with a straight-chain octadecyl (C_{18}) hydrocarbon bonded chemically to silica specifically designed to use with ion-pair agents.

MATERIALS AND METHODS

All the catecholamines and the metabolites were purchased from Sigma (St. Louis, MO, U.S.A.). Regis (Morton Grove, IL, U.S.A.) was the source of our sodium octyl sulfonate. Acid washed alumina was purchased from Bioanalytical

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Systems (West Lafayette, IN, U.S.A.) or alumina obtained from Calbiochem (Los Angeles, CA, U.S.A.) was washed by the procedure of Anton and Sayre [4]. Methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

The chromatography was performed with a Model 110A pump (Altex), Model 210 valve with a 20-μl loop (Altex), and a reversed-phase Altex Ultrasphere Ion Pair C_{18} column (25 × 0.46 cm I.D.). The column eluent was monitored with an electrochemical detector Model LC-4 and a glassy carbon electrode from Bioanalytical Systems. The detector potential was +0.72 V vs. an Ag/AgCl reference electrode. The flow-rate was maintained at 1.5 ml/min. The mobile phase was 1 volume of methanol and 9 volumes of 0.1 *M* potassium phosphate (pH 3.0), 0.2 m*M* sodium octyl sulfonate, and 0.1 m*M* EDTA. Water was deionized and the buffer was filtered through Millipore HAWP 0.45-μm filters. Millipore EHWP 0.5-μm filters were used to filter the methanol. The column was used at room temperature, but the buffer was maintained at 40—50° C to prevent bubble formation in the detector cell.

Male Sprague-Dawley rats (Holtzman) were killed by decapitation. Brains were rapidly removed, dissected on ice, frozen on dry ice, and stored at −80°C until assayed. The dissection was completed as follows: the cerebellum was peeled off the brain stem before the brain was removed from the skull. A cut was made through the corpus callosum and the cortex was peeled back, exposing the hippocampus and caudate which were carefully removed. Remaining white matter was removed from the cortex. To obtain the hypothalamus and thalamus, cuts were made anterior and posterior to the median eminence followed by sagital cuts on the two sides of the median eminence and a horizontal cut through the mamillothalamic tract. The dorsal tissue piece was used for thalamus and the ventral portion as the hypothalamus. Tissue was kept frozen at −80°C until assayed. No differences in NE or dopamine (DA) levels are observed upon storage for up to one month. However, tissues that were stored frozen for nine months at −80°C had catecholamine levels 30 to 40% of those assayed within one month of sacrifice.

Frozen tissues were weighed and homogenized on ice in 3 ml of 0.05 *M* perchloric acid containing varying amounts of dihydroxybenzylamine (DBA) with a Polytron distributed by Brinkmann. Whole brains were homogenized in 10 ml of 0.05 *M* perchloric acid containing 100 ng DBA per ml for NE and DA determinations. For determining NE and DA in cerebral cortex only one side was homogenized with 90 ng DBA. One of the caudate nuclei was homogenized with 450 ng of DBA to determine DA and the other with 45 ng of DBA to determine NE. Both hippocampi were homogenized with 30 ng DBA. The hypothalamus was homogenized with 60 ng DBA, the thalamus with 45 ng DBA, the cerebellum with 90 ng DBA, and the midbrain with 90 ng DBA. From each homogenate two 1-ml aliquots were placed in 12 × 75 mm polypropylene tubes (Sarstedt 526). One of the two aliquots was supplemented with 1 μg/ml NE and/or DA in 0.05 *M* perchloric acid to be used as working standards. The following provided reasonable supplements: whole brain, 50 ng NE and 25 ng DA; cortex, 10 ng NE and 10 ng DA; caudate for DA, 50 ng DA; caudate for NE, 5 ng NE; cerebellum, 10 ng NE; hippocampus, 5 ng NE and 5 ng DA; hypothalamus, 10 ng NE and 5 ng DA; thalamus 5 ng NE and 2.5 ng DA; and midbrain, 10 ng NE and 5 ng DA.

All the samples were centrifuged at 9000 *g* for 15 min. Supernatants from the samples were transferred to 12 × 75 mm conical polystyrene tubes (Sarstedt 477) containing 10 mg acid washed alumina and 1 ml of 0.5 *M* Tris—HCl, pH 8.6. The tubes were capped and shaken for 15—30 min at top speed on a New Brunswick Scientific Shaker. After allowing the alumina to settle the supernatants were aspirated. The alumina was washed three times with 1 ml 5 m*M* Tris—HCl, pH 8.6. Elution of the catecholamines was with 100 μl of 0.2 *M* acetic acid. The 100-μl aliquots of 0.2 *M* acetic acid containing the eluted catecholamines were filtered with a Flath-Lundin filter syringe purchased from Hamilton (Reno, NV, U.S.A.). Millipore HAWP 0.45-μm filters were used. A 20-μl aliquot of the filtered samples was injected. Catecholamine levels were calculated as described by Felice et al. [1].

RESULTS AND DISCUSSION

Fig. 1A shows an isocratic separation of pure standards of NE and DA as well as a number of their major metabolites using the methanol—phosphate mobile phase described in detail in the Methods section. From this chromatogram it is clear that NE is markedly separated from the solvent front. Further-

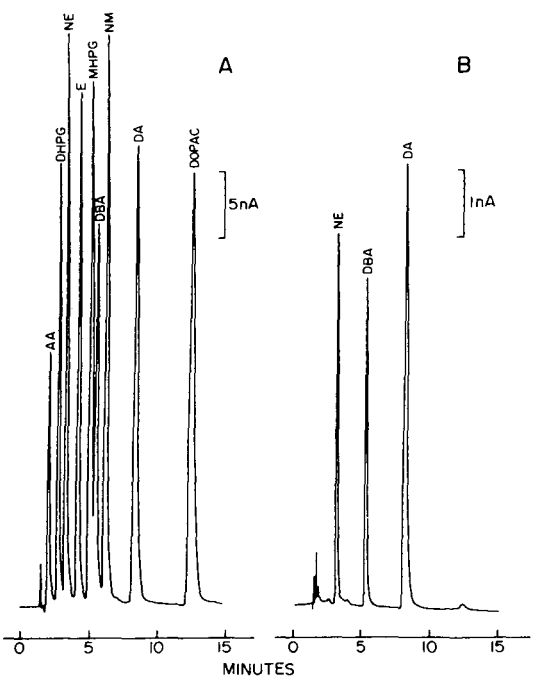

Fig. 1. Chromatograms of standards (A) and of catecholamines from adult rat whole brain with DBA added as an internal standard (B). Details for the chromatographic conditions and the sample preparations are in the Methods section. For (A) abbreviations and amounts injected in nanograms: AA = ascorbic acid, 15; DHPG = dihydroxyphenylglycol, 8; NE = norepinephrine, 15; E = epinephrine, 30; MHPG = hydroxymethoxyphenylglycol, 25; DBA = dihydroxybenzylamine, 10; NM = normetanephrine, 400; DA = dopamine, 15; and DOPAC = dihydroxyphenylacetic acid, 25.

more, NE is not only resolved from ascorbic acid but also from dihydroxyphenylglycol (DHPG), a major NE metabolite, which is not the case with another widely used LC column [1]. Under these conditions DA elutes from the column within 10 min. That is a remarkably rapid elution with baseline separations for NE, DBA and DA.

The mobile phase we used with the Altex Ultrasphere C_{18} Ion Pair column contained a relatively large percentage of methanol and a relatively low concentration of ion-pairing agent. Therefore, there are a number of things one can do to the mobile phase to further separate these and other catecholamines and their metabolites. Decreasing the methanol concentration and/or increasing the ion-pair concentration will increase retention times. With use, LC columns generally lose some of their resolving power(s); therefore the ability to dramatically increase the polarity of the solvent should lengthen the expected useful lifetime of the column. On the other hand, even when we used the more polar mobile phases containing no methanol, as described by Felice et al. [1] and Asmus and Freed [5], NE did not completely separate from the solvent front on a Waters μBondapak C_{18} column. The addition of more ion-pairing agent markedly increased the retention time of DA but had little or no effect on the retention time for NE. EDTA is an essential component of the mobile phase, since it dramatically decreases the spread of the solvent front. Without EDTA; ascorbic acid, DHPG and NE are not resolved from the solvent front. A possible explanation for this effect is that the samples are in acid which may cause metal ions to be injected from the syringe, the injector, or the pumping system which are oxidized and consequently observed with the electrochemical detector.

Fig. 1B is a chromatogram of an alumina extraction from a male Sprague-Dawley rat whole brain that included DBA as an internal standard. The amount of NE, 345±10 ng/g wet weight and DA, 818±21 ng/g wet weight in whole brain that we calculated using this LC—EC method is comparable to that obtained by other investigators [1, 6—8]. Calculations were performed using DBA as an internal standard as described in detail by Felice et al. [1]. The amounts of NE and DA found in the various regions of male Sprague-Dawley rat brains are presented in Table I. These values compare favorably with those reported by other investigators [1, 6, 7].

TABLE I

NOREPINEPHRINE AND DOPAMINE CONTENT OF SEVERAL RAT BRAIN REGIONS

Each region was assayed in duplicate from 3 to 14 animals. Results are expressed as a mean ± S.E.M.

Region	Norepinephrine (ng/g of tissue)	Dopamine (ng/g of tissue)
Whole brain	345 ± 10	818 ± 21
Cerebral cortex	205 ± 10	51 ± 4
Cerebellum	124 ± 15	13 ± 1
Caudate nucleus	277 ± 37	9253 ± 213
Hippocampus	201 ± 21	11 ± 0.5
Hypothalamus	1446 ± 87	298 ± 33
Thalamus	554 ± 41	191 ± 31
Midbrain	392 ± 7	129 ± 17

This method gave linear responses both with the detector and with the tissue sample in the tested range of 1—100 ng NE, DA, and DBA. The extraction method has not been optimized for maximum recovery. Using 0.2 *M* acetic acid to extract catecholamines from alumina gave recoveries of approximately 40%. Acetic acid was chosen because it probably is least detrimental to the column packing material and the sample injection valve. In addition, it is volatile which means concentrating samples does not increase the acid concentration.

Using this method we could assay five samples per hour. The system stabilizes very quickly (15—20 min) if one leaves the power on overnight for the electrochemical detector and keeps the column equilibrated with the methanol—phosphate mobile phase by pumping at 0.1 ml/min. We found it takes 3—4 h to obtain a stable baseline if the power is turned off overnight. A similar amount of time is needed to re-equilibrate the column if one stores the column in 70 to 100% methanol.

With a buffered mobile phase the column must have a continual flow-rate to avoid salts crystallizing on the column. We have found our baseline to be stable enough that we anticipate one could successfully analyze catecholamines with a fully automated system, i.e. an autosampler and an integrator.

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Note

Amperometric determination of 3-methoxy-4-hydroxyphenylethyleneglycol in human cerebrospinal fluid

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3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) is the major central nervous system metabolite of norepinephrine [1—3] and measurement of MHPG levels in human cerebrospinal fluid (CSF) are an indication of central norepinephrine turnover. Methods which have been employed for the determination of MHPG in CSF include gas chromatography with electron-capture [4—7] or mass spectroscopic [8—12] (GC—MS) detection, fluorimetry [13, 14] and liquid chromatography (LC) [15, 16]. As methods of analysis have improved, reported mean values for free MHPG in normal or control human CSF have decreased from ca. 30 ng/ml [13, 14] and above [2, 7] to around 10 ng/ml [8, 10, 12, 17, 18]. Also, more recent reports state that the preponderance of the MHPG in human CSF is in the unconjugated (free) form [8, 10—12, 18]. In general, the procedures used have been lengthy and complicated. We present here a simple and rapid LC—amperometric method.

MATERIALS AND METHODS

MHPG standard was purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution of 100 ng/ml in 0.1% (w/v) ascorbate was stable for up to three months.

The LC system was composed of an Altex 110A pump, a Rheodyne 71-20 injection valve with 100-μl sample loop (Rainen Instrument, Brighton, MA, U.S.A.), and a μBondapak C_{18} reversed-phase column (300 mm × 3.9 mm I.D., average particle size 10 μm, Waters Assoc., Milford, MA, U.S.A.). The ampero-

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metric detector consisted of a Bioanalytical Systems (West Lafayette, IN, U.S.A.) electrochemical controller (LC-2A), a glassy carbon working electrode, an Ag/AgCl reference electrode, and a Kel-F thin-layer detector cell and reference electrode compartment. A ca. 50-μm spacer gasket was used, and the working electrode was set at +0.75 V versus the reference electrode. The citrate—acetate buffer [19] solvent system was adjusted to pH 5.15 ± 0.01 and delivered at a flow-rate of 2.0 ml/min. The buffer was degassed by heating at ca. 45°C on a stirrer hot plate: the column apparently remained at room temperature as no change in retention time of MHPG standard was observed when the buffer was not warmed.

MHPG was determined in centrifuged (ca. 10,000 *g* for 2 min) lumbar CSF by injecting 20—50 μl of the otherwise unprocessed CSF. A 1-ng MHPG standard was injected after every two samples. A single-point standard (quantified by peak height) was used as the response was linear over the working range (0.1—1.0 ng). The full-scale sensitivity was usually set at 2 nA. Absolute background levels varied from ca. 0.5 to 3 nA, depending upon the particular electrode. The glassy carbon electrode was cleaned daily by wiping gently with a damp tissue.

RESULTS AND DISCUSSION

Chromatograms obtained from two different CSF samples are shown in Fig. 1; in each, 40 μl of centrifuged human lumbar CSF were injected directly. The difference in retention time observed for the MHPG peaks is due to the use of two different C_{18} μBondapak columns. The column employed for sample No. 1 had shown, even initially, lower retention times. The more extensive use of that column lowered retention times further and also lowered efficiencies to those observed. The MHPG peak was well formed and adequately separated from neighboring peaks using the pH 5.15 solvent system. In order to further establish the identity of the peak, CSF was also chromatographed using solvent systems of pH 3.75 and 6.00. Under these conditions, the peak still co-eluted with MHPG standard, having a lower retention time at lower pH and a greater retention at higher pH (retention time ratios at pH 3.75, 5.15, and 6.0, were 0.5, 1.0 and 1.1, respectively). At pH values other than ca. 5.15, the MHPG peak was not as well separated from nearby peaks. A similar study with 0.5% and 5% methanol added to the pH 5.15 buffer also confirmed the identity of the peak while giving inadequate separation. A very recent high-performance liquid chromatography—amperometric method [16] for determining MHPG in CSF apparently suffers from some degree of interference as a broadened peak was observed in samples.

A standard addition study with free MHPG (10, 20, and 40 ng/ml added) gave a linear recovery of 95.1 ± 5.9%. The compound was determined in a single CSF sample with a coefficient of variation of 7.3% (10.3 ± 0.75 ng/ml, mean ± S.D., n = 5). CSF samples examined to this point have had concentrations of 6.7—14.8 ng/ml, in agreement with GC—MS reports [8, 10, 12, 17, 18]. In order to speed up the determination, the late-eluting peaks can be rapidly removed by injecting about 1.0 ml of methanol. The detector cell was bypassed for 3—4 min while the methanol eluted. A steady baseline was obtained within 1 min of switching the detector back on-stream.

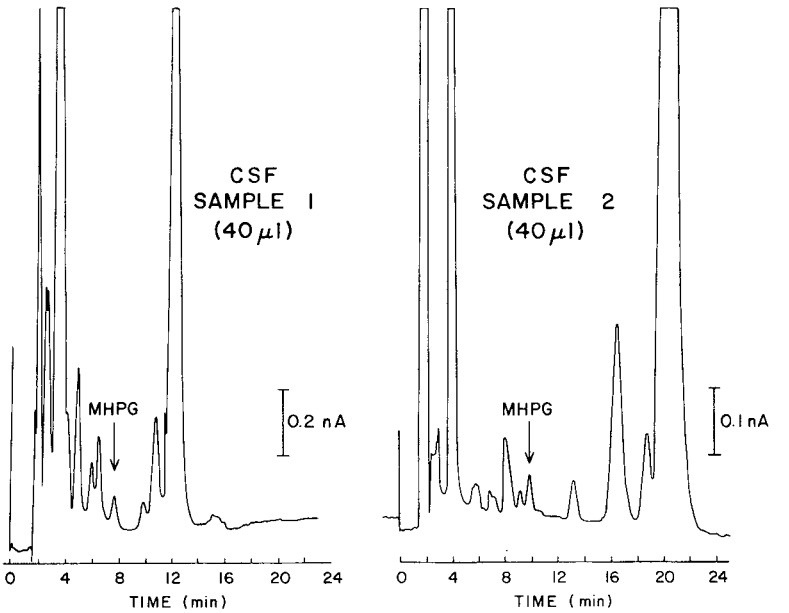

Fig. 1. The determination of MHPG in two different CSF samples using different C_{18} columns. The MHPG levels for the samples shown are 6.7 ng/μl (sample 1) and 10.5 ng/ml (sample 2). Electrode sensitivities established at the time each sample was run were 0.336 and 0.125 nA per ng MHPG injected for samples 1 and 2 respectively.

An attempt was made to extend the method to the determination of free MHPG in human urine and plasma. In urine, a peak co-eluted with MHPG standard using the pH 5.15 solvent system. However, when the peak was collected and re-chromatographed using a solvent system containing 5% methanol, the retention time was 4.5 min versus 4.3 min for MHPG standard (α = 1.07). In plasma, a poorly shaped, presumably merged, peak was observed, which when quantitated gave a much higher than expected concentration (ca. 50 ng/ml versus reported values of ca. 5—10 ng/ml).

We believe the method to be much simpler, less expensive, and more rapid than existing methods. Amperometric detection of LC eluents is not without its practical problems due to bubble formation, electrode passivation, and system shielding, etc. However, the advantages of the method are more than ample compensation for the occasional problem.

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Note

Carrier-ampholyte displacement chromatography (chromatofocusing) on ion-exchange papers

Application to the separation of haemoglobin variants

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Ion-exchange chromatography is the most frequent method used to perform separations of proteins. Proteins fixed on a chromatographic support are eluted either by changing the pH and/or ionic strength of eluent, or by using an elution reagent able to displace proteins selectively from the support. The use of carrier ampholytes in displacement chromatography on an ion-exchange column for liquid chromatography has been described [1—6]. This paper describes carrier ampholytes as developers in ion-exchange paper chromatography applied to the study of human haemoglobins. The behaviour of some human haemoglobins in the presence of carrier ampholytes in an ion-exchange column for liquid chromatography has been previously described [7].

EXPERIMENTAL

All haemoglobin samples were prepared from red cell lysates as described

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elsewhere [7]. However, pure foetal haemoglobin components F_0 and F_1, used for controls, were obtained by preparative isoelectric focusing of cord blood hemolysates [8]. Haemoglobin concentrations were adjusted to 4% (w/v) with distilled water.

The chromatographic support was an anion-exchange paper with diethylaminoethyl (DEAE) groups (Whatman DE-81). Paper strips were cut to the specific size of 200 mm × 45 mm. The equilibration buffer was tris(hydroxymethyl)aminomethane (Tris), 0.05 *M* HCl (pH 8.9), KCN 0.01%. Aqueous solutions of carrier ampholytes used as developers were prepared from commercial Ampholines^R (LKB, Bromma, Sweden) or Pharmalytes^R (Pharmacia, Uppsala, Sweden); the pH values of the solutions were not adjusted.

Chromatography was performed as follows. Haemoglobin samples were diluted down to 1/50 with the equilibration buffer; 2 µl of each diluted sample were deposited on the chromatographic paper in saturating conditions [9] at point 0, 20 mm from the lower edge of the paper. Ascending development was made at 4°C for 12—14 h in a chromatographic chamber in saturating conditions.

RESULTS AND DISCUSSION

Aqueous solutions of Ampholine in the pH range 6—8 (1.2%, w/v) and Pharmalyte in the pH range 6.5—9 (3%, w/v) have been compared as developers. A typical separation obtained between some haemoglobin variants with the above Ampholine solution is shown in Fig. 1. The corresponding R_F values are given in Table I.

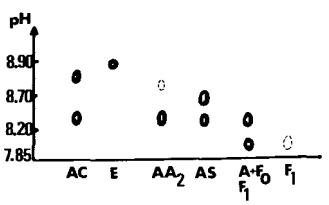

Fig. 1. Paper chromatogram of some haemoglobin variants. Solvent: Ampholine pH range 6—8 solution (1.2%, w/v). Equilibration buffer: 0.05 *M* Tris · HCl (pH 8.9), 0.01% KCN. Spots with broken line: haemoglobin variants. R_F values increase in the order: HbF_1, HbA (HbF_0), HbS, HbA_2, HbC, HbE.

TABLE I

R_F VALUES OBTAINED WITH AMPHOLINE SOLUTIONS IN THE pH RANGE 6—8

Ampholine concentration (pH 6—8)	HbA	HbA_2	HbF_0	HbS	HbC
1.2% (w/v)	0.13	0.24	0.13	0.19	0.26
4.0% (w/v)	0.45	0.60	0.42	0.54	0.61

No separation was obtained with the Pharmalyte solution tried; i.e. the pH varying from 8.16 (point 0: initial application) to 8.90 (solvent from at 60 mm). However, a clear-cut separation of haemoglobins, similar to that obtained with Ampholine in the pH range 6—8, was obtained with another Pharmalyte solution: 6% (w/v), pH range 6.5—9. The pH values detected varied from 8.13 to 9.00 for a maximal migration distance of 180 mm.

The influence of the pH range of the carrier ampholytes was studied with the haemoglobin samples previously shown in Fig. 1. Optimal conditions of separation were obtained with Ampholine of pH range 6—8. The effect of the concentration of the carrier ampholytes on R_F values was also determined, and the results are shown in Table I.

As an additional assay, a pre-run was done before the application of the haemoglobin samples and the pH determined on the chromatographic support. But there was no definite improvement in the resulting power of resolution, although the solutions for pre-run and elution were the same.

The influence of the position of the sample application on the chromatographic paper was tested using a mixture of HbC and HbS. With an Ampholine solution of pH range 6—8 (1.2%, w/v) the separation between the haemoglobins was clear-cut at the initial point 0 (pH determined was 7.50—7.60), but the separation was partial when application was made at 30 mm (pH 8.30—8.40), and no separation occurred with initial applications at 60 mm, 90 mm, 120 mm, where the respective pH values measured were 8.60, 8.80—8.90 and 8.90—9.00.

In ampholyte-displacement chromatography (ADC) proteins fixed on the chromatographic support are selectively displaced by carrier ampholytes. The interactions between carrier ampholytes and ion exchanger induce the formation of an "internal" pH gradient and a displacement of counter-ions (i.e. chloride ions) by ampholytes. The migration order obtained by carrier ampholytes on ion-exchange paper is identical to that obtained for the same haemoglobins on an ion-exchange column with either DEAE-cellulose or DEAE-Sephadex supports and an "external" pH gradient maker [10].

A clear-cut separation between HbA (or HbF_0) and HbF_1 is shown in Fig. 1, but no separation is obtained between HbA and HbF_0 when ion-exchange papers are equilibrated with Tris · HCl, KCN (pH 8.9) buffer and the Ampholine solution of pH range 6—8 (1.2%, w/v) is the developer. The equilibration of ion-exchange paper with 0.2 *M* glycine, 0.01% KCN buffer [11] increases the power of resolution of Ampholine 6—8 solvent between HbA and HbF_0.

Isoelectric points, p*I*, of the two components of foetal haemoglobin differ by 0.3 pH units. The p*I* of HbA and HbF_0 differ by 0.2 pH units [12, 13]. An approach to the influence of p*I* on the migration order of haemoglobins in paper ADC may be obtained by introducing relative mobilities, R_M, of the haemoglobins. A linear relationship exists between R_M and p*I* for two different Ampholine pH range 6—8 concentrations: 1.2% (w/v) and 4% (w/v) (Fig. 2). The p*I* values of haemoglobin variants are reported elsewhere [12, 13].

Carrier ampholytes as developers in ion-exchange paper chromatography strongly diminish tailings [14]. The carrier ampholyte solutions are usable for several runs. The ADC method avoids an exposure of proteins to large pH

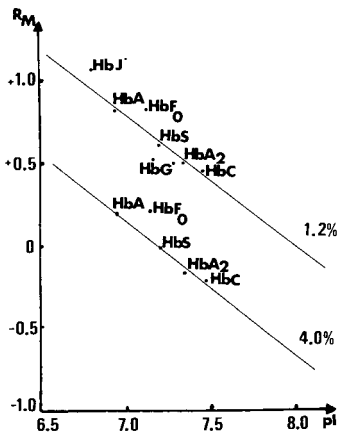

Fig. 2. Relative mobilities, R_M, as related to isoelectric points, pI, of some haemoglobin variants, for two different concentrations of Ampholine solutions, 1.2% (w/v) and 4% (w/v), in the same pH range 6—8. Equilibration buffer: 0.05 M Tris · HCl (pH 8.9), 0.01% KCN. HbJ* = haemoglobin variant with HbJ Oxford mobility in isolelectric focusing. HbG° = haemoglobin G Philadelphia.

variations during the chromatographic process and, as compared with conventional chromatographic techniques, the effects of temperature changes are also reduced.

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Note

Rapid gas chromatographic method for the determination of carbamazepine and unrearranged carbamazepine-10,11-epoxide in human plasma

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Several gas—liquid chromatographic (GLC) methods for the determination of carbamazepine in plasma have been proposed [1—4]. Prolonged extraction procedures or carbamazepine derivative formation, in consequence of its thermal instability, are needed with these methods. Moreover, carbamazepine-10,11-epoxide (CBZ-E) is always degraded to 9-acridine carboxaldehyde [5]. We describe here a rapid GLC method for the routine assay of carbamazepine and CBZ-E, without the necessity of derivatization.

EXPERIMENTAL

Materials

Solvents used were: methylene chloride, acetone, petroleum ether (b.p. 40—70°C), diethyl ether (without further purification). N,O-Bis(trimethylsilyl)acetamide (BSA) or hexamethyldisilazane (HMDS) (both from Carlo Erba, Milan, Italy) were the silylating agents for the sorbent.

Standards

Internal standard. Dissolve 20 mg of 10,11-dihydrocarbamazepine (99%; Aldrich, Milwaukee, WI, U.S.A.) in 100 ml of acetone and keep the solution frozen at −20°C in screw-capped tubes.

Drug standards. Carbamazepine (Tegretol) was from Ciba-Geigy (Basel, Switzerland). Carbamazepine-10,11-epoxide was synthesized according to the method of Baker et al. [5]. The stock drug standards were prepared in the following concentrations: carbamazepine 10 mg and CBZ-E 5 mg per 100 ml of acetone. These standards were stored in the freezer at −20°C.

Standard curves. These were prepared for each drug. Each serum plus standard was extracted and chromatographed in duplicate as if it were a patient's sample. The mean value of the relative peak area ratios of each standard was plotted against the concentration of the respective standard (Fig. 1).

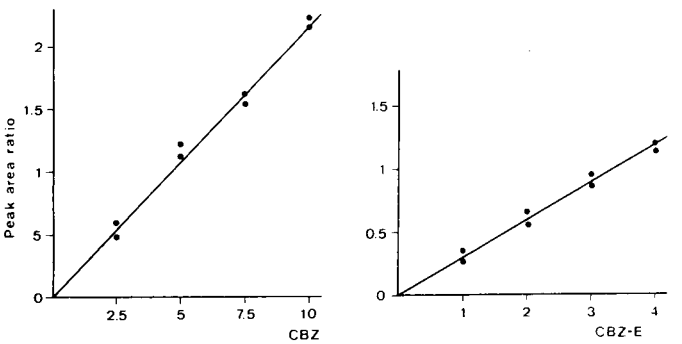

Fig. 1. Calibration curves relating peak area ratios (carbamazepine/internal standard and CBZ-E/internal standard) to drug concentrations in the extracted plasma.

Instrumentation and analysis conditions

Chromatography was performed on a Fractovap 2400 T instrument (Carlo Erba) equipped with a flame ionization detector. Mass spectra were performed on an A.E.I./MS 902 spectrometer (Varian Instruments, Turin, Italy).

The column was 1.80 m × 4 mm I.D. borosilicate glass tubing. It was packed with 3% OV-17 on 80—100 mesh (or 100—120 mesh) silanized Varaport 30 (Varian). The column was conditioned at 270°C for 12 h, without nitrogen flow. The oven temperature was maintained at 270°C. Detectors and injection ports were heated, respectively, to 280°C and 290°C. Gas flow-rates were adjusted at 70 ml/min (nitrogen), 35 ml/min (hydrogen) and 300 ml/min (air). The electrometer was operated in the range of 10^{-10} A/mV and the amplifier output was attenuated at 2. The peaks were recorded at a chart speed of 10 mm/min.

The mass spectrometer was used under the following conditions: ionization energy 70 eV, injector temperature 270°C, oven temperature 265°C, helium flow-rate 40 ml/min. The sample introduction was either direct or by GLC procedure (column and packing as described above).

Silylation was performed on coated support by five injections of 10 µl of silylating agent repeated every 5 min, with slow carrier flow-rate. The oven temperature was maintained at 80—100°C for HMDS, or at 140—160°C for BSA. During this procedure the end of the column was disconnected from the detector, to avoid silica deposits.

Extraction

Forty microlitres of internal standard solution were placed in a glass-stoppered centrifuge tube. After evaporation of the solvent under nitrogen stream, 1 ml of plasma was added and alkalinized by 0.2 ml of 1.5 *N* NaOH (or 3 *N* NaOH when primidone was present in plasma). To the sample was added 1 ml of methylene chloride and, after shaking, 2 ml of petroleum ether. Three layers were formed. Then the sample was gently swirled for a few minutes without mixing the layers. After discarding the petroleum ether by aspiration, 6 ml of methylene chloride were introduced into the glass tube, which was subsequently shaken on a horizontally rotating device (18 rotations/min) for 15 min. The sample was then centrifuged at 314 *g* for 15 min, at −4°C. Occasionally an emulsion formed, but it was easily dispersed by stirring with a glass rod and recentrifugation. The upper layer was removed by aspiration, whereas the rest of the solution was transferred to a conical tube and evaporated in a water bath (35°C) by a gentle nitrogen stream.

Chromatography and mass spectrometry

The dried residue was reconstituted with 20 μl of acetone. This solution (1—1.5 μl) was injected (for chromatography or mass spectrometry) following the analysis conditions described above. Plasma peaks were identified by comparing their retention times with those obtained by injection of authentic samples of carbamazepine or CBZ-E. Drug concentrations were calculated from the standard curve.

RESULTS

Results are summarized in Figs. 1—3 and in Table I. The peak area ratio of carbamazepine and CBZ-E to internal standard (cyheptamide) was plotted against the peak areas obtained from the same anticonvulsants, as added to a

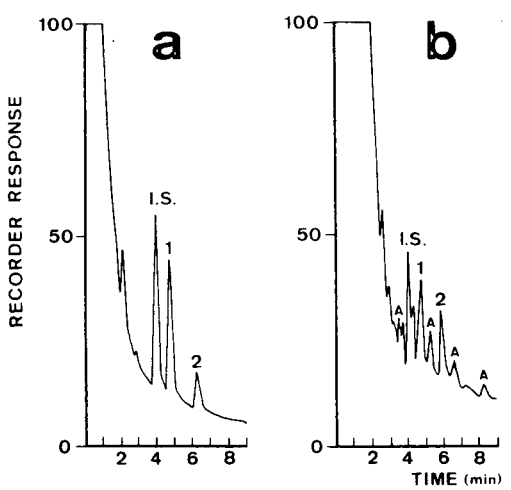

Fig. 2. Typical chromatograms from plasma, (a) obtained with this method; (b) obtained with a different extraction procedure. 1 = Carbamazepine; 2 = CBZ-E; I.S. = cyheptamide; A = artifacts due to lipidic contamination.

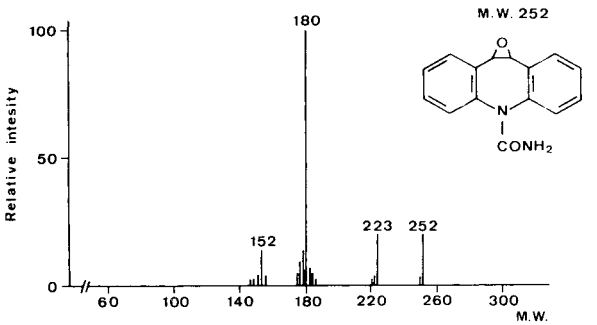

Fig. 3. GLC—mass spectrum of CBZ-E obtained from plasma. This spectrum is identical to that obtained from an authentic sample of CBZ-E and to the mass spectrum of CBZ-E obtained using the direct inlet system.

TABLE I

ANALYTICAL RECOVERIES

$n = 20$.

Drug added to plasma	conc. (μg/ml)	Mean conc. found (μg/ml)	S.D. (μg/ml)	Recovery (%)
Carbamazepine	3	2.9	0.3	97
	6	5.8	0.2	96
	9	8.7	0.1	96
	12	11.8	0.4	98
	15	15.1	0.3	101
Carbamazepine epoxide	1	0.95	0.1	95
	1.5	1.4	0.2	93
	2.5	2.3	0.2	92

plasma pool in a known amount. The resulting regression slope was linear, both for direct drug injection and after drug extraction from plasma or water. The recovery of the two compounds varied between 96 and 101% for carbamazepine and from 92 to 95% for CBZ-E. The standard deviation among recovered amounts of the two drugs in different determinations varied from 0.1 to 0.4 μg/ml. The GLC—mass spectrum of compound 2 (Fig. 2) extracted from plasma was identical to both the GLC—mass spectrum of an authentic CBZ-E sample and the mass spectrum of compound 2 obtained by the direct inlet system (Fig. 3). Plasma free from carbamazepine or CBZ-E was carried through the entire analytical procedure and no normally occurring interferences were seen. On the other hand, primidone, when present in a patient's plasma, produced interference only when the plasma was not sufficiently alkaline.

DISCUSSION

There are some points deserving discussion because of their importance in obtaining good reliability for this method.

Packing of the column must be very carefully performed. Bad packing causes the conversion of carbamazepine to an unknown compound quantitatively cor-

related and showing a retention time longer than 17 min. This partial conversion may depend on chemical interaction between carbamazepine and support occurring in poorly filled spaces of a column that is imperfectly packed. Naturally occurring impairment of the packing can be avoided by conditioning the column without carrier flow.

The residual active sites on the coated support are blocked by "in place silanizing" [6, 7]. We avoided degradation of the studied compounds, seen with chlorinated silanizing agents (trimethylchlorosilane, dimethyldichlorosilane), by using HMDS or BSA. Chlorinated agents would produce the primary chlorosilyl ether with residual active silanol groups, while HMDS and BSA would produce, respectively, NH_3 and acetamide, undergoing complete elimination by the carrier gas. Therefore, the good results obtained using HMDS or BSA should depend on non-acidic columns: it is well-known, in fact, that carbamazepine or CBZ-E easily react in acidic conditions.

Lipidic contamination of the dry residue, responsible for artifacts (see Fig. 2), is completely avoided without making the routine procedure impracticable, by our three-phase extraction.

One of the most frequent problems is degradation of carbamazepine and CBZ-E molecules. This may occur because of contact with metallic parts of the injector, or of combination with residual molecules remaining in the column after the previous determination of other compounds, or from use of chloride solvents that may contain trace amounts of acids, or from choosing the wrong support. Therefore, we recommend the use of glass columns long enough to permit a direct "in column" injection. Columns should be reserved exclusively for the determination of carbamazepine and CBZ-E and supports should be analogous to Varaport 30. Finally, it is advisable to use cyheptamide as internal standard, because of its structural analogy with the studied compounds. We suggest the use of analytical grade cyheptamide, to avoid interfering peaks due to impurities.

In conclusion, this method is reliable, rapid and suitable for the routine determination of carbamazepine and its major metabolite, without derivatization, which could be important for pharmacokinetic studies [8].

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Note

Plasma concentrations of temazepam, a 3-hydroxy benzodiazepine, determined by electron-capture gas—liquid chromatography

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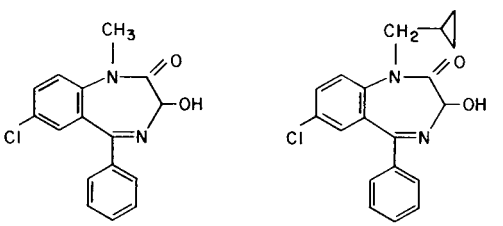
Temazepam (Fig. 1) is a 1,4-benzodiazepine used clinically for the treatment of insomnia [1—3]. Temazepam is biotransformed in humans by glucuronide conjugation of the 3-hydroxy substituent, yielding a water-soluble glucuronide metabolite excreted in urine [4]. This paper describes a gas—liquid chromatographic method for the analysis of temazepam in human plasma following therapeutic doses.



TEMAZEPAM *3-HYDROXY-PRAZEPAM*

Fig. 1. Structures of temazepam and its internal standard, 3-hydroxy-prazepam.

EXPERIMENTAL AND RESULTS

Apparatus and chromatographic conditions

The instrument used is a Hewlett-Packard Model 5750 gas chromatograph equipped with a 2-mCi ^{63}Ni electron-capture detector operated in the pulsed

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mode with a pulse interval of 150 μsec. The column is coiled glass, 183 cm × 2 mm I.D., packed with 3% SP-2250 on Supelcoport (80—100 mesh). The carrier gas is ultrapure helium at a flow-rate of 50 ml/min. The purge gas is argon—methane (95:5), at a flow-rate of 80 ml/min. Operating temperatures are: injection port, 310—320°C; column, 280°C; detector, 320°C. The column is primed daily by an injection of 2—3 μl of a solution of azolectin dissolved in benzene (1 mg/ml).

Stock solutions

Ten milligrams of temazepam (TMZ) kindly supplied by Sandoz (East Hanover, NJ, U.S.A.) and of its structural analog, 3-hydroxy-prazepam (3-OH-PRZ) kindly supplied by Warner-Lambert (Morris Plains, NJ, U.S.A.) (Fig. 1) are each dissolved in 2—3 ml of absolute ethanol, then diluted to 100 ml with benzene. The stock solutions, containing 100 μg/ml, are stored in amber-colored bottles at 40°C. Working standards containing 1 μg/ml of TMZ and 5 μg/ml of 3-OH-PRZ are prepared as needed by appropriate dilution with benzene.

Preparation of samples

3-OH-PRZ serves as the internal standard. A constant amount (250 ng) is added to a series of 13-ml round bottom glass culture tubes equipped with PTFE-lined screw-top caps. Calibration standards are prepared by adding TMZ from the working standard solution in the following amounts: 25, 50, 75, 100, 200, and 300 (or 400) ng. The contents of both the calibration and sample tubes are evaporated to dryness at 40—50°C under mildly reduced pressure.

One ml of drug-free control plasma is added to each of the calibration tubes, and 1 ml of unknown plasma is added to each of the sample tubes which contain only internal standard. Calibration standards are extracted and analyzed daily together with each set of unknowns. No buffering or other sample preparation is required. When plasma TMZ concentrations exceeding 300—400 ng/ml are anticipated, plasma aliquots of less than 1 ml can be analyzed. The final volume is adjusted to 1.0 ml by addition of distilled water.

Extraction

After addition of 3—5 ml of benzene (containing 1.5% isoamyl alcohol), the tubes are agitated gently in the upright position on a vortex-type mixer for 60 sec, then centrifuged at room temperature for 10 min at 400 *g*. An aliquot of the organic layer is transferred to another tube, and the procedure is repeated. The combined organic extracts are evaporated to dryness at 40°C under mildly reduced pressure. The residue is redissolved in 50—100 μl of benzene (containing 15% isoamyl alcohol), of which 1—3 μl is injected into the chromatograph.

Evaluation of the method

Under the described chromatographic conditions, approximate retention times for TMZ and 3-OH-PRZ are 4 min and 6 min, respectively (Fig. 2).

A linear relationship exists between concentrations of TMZ in the calibration standards and the peak height ratio of TMZ to its internal standard. The day-to-

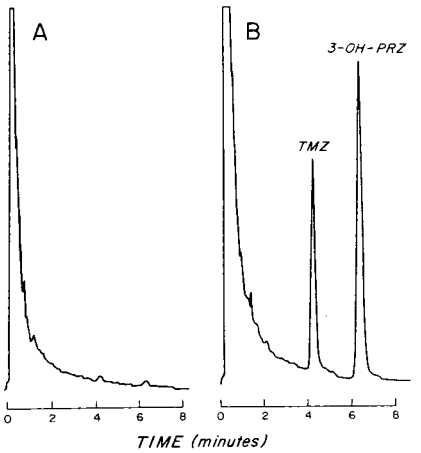

Fig. 2. (A) Chromatogram of a drug-free control plasma extract; (B) the same sample to which was added temazepam (TMZ), 100 ng/ml, and 3-hydroxy-prazepam (3-OH-PRZ), 250 ng/ml.

day coefficient of variation in the slope of the calibration curves was 6.9% (n = 18).

The sensitivity limits are approximately 5 ng of TMZ per ml of original sample. Coefficients of variation for identical samples (n = 8 at each concentration) were 5.4% at 25 ng/ml, 5.5% at 50 ng/ml, and 8.7% at 200 ng/ml. The mean deviation between 42 randomly selected duplicate samples was 6.2%. As in the case of other benzodiazepines [5, 6], recovery of TMZ and 3-OH-PRZ is more than 95%.

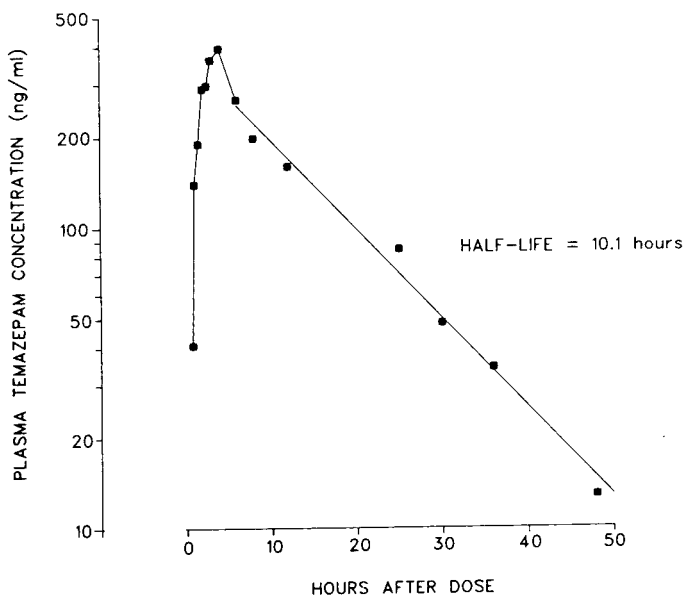

Fig. 3. Plasma temazepam concentrations for 48 h after a single 30-mg dose administered to a healthy volunteer.

Clinical pharmacokinetic study

A healthy 29-year-old female participated after giving informed consent. A single 30-mg dose of TMZ (Restoril^{R}, Sandoz) was administered with 100 ml of water after an overnight fast. Venous blood samples were drawn into heparinized tubes at multiple points in time over 48 h. Plasma was separated and frozen until assay. Plasma concentrations of TMZ were determined as described above.

A peak TMZ concentration of 392 ng/ml was measured in the sample drawn 3.0 h after the dose. Elimination proceeded with a half-life of 10.1 h (Fig. 3).

CONCLUSION

This paper describes a rapid and sensitive method for the quantitation of TMZ in human plasma. Both TMZ and 3-OH-PRZ are extracted into the organic solvent at physiologic pH using a double extraction technique. The organic solvent is then evaporated, and the redissolved residue is injected directly into the chromatograph. Plasma samples are consistently free of contaminants in the areas corresponding to retention times of TMZ and 3-OH-PRZ, making extensive cleanup unnecessary. The underivatized compounds produce symmetric, gaussian peaks under the described conditions largely due to the excellent performance of the 50:50 phenyl methyl silicone liquid phase (SP-2250) that was utilized.

Application of the method to pharmacokinetic studies of TMZ in humans is illustrated. Consistent with previous reports [7], the elimination half-life of TMZ appears to fall in the short to intermediate range and is similar to half-life values reported for oxazepam and lorazepam, two other 3-hydroxybenzodiazepines in wide clinical use [8, 9].

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CHROMBIO. 710

Note

Assay of prolintane in plasma by capillary gas chromatography with nitrogen-selective detection

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Prolintane (Fig. 1) has been used as a stimulating antidepressant drug since 1962. In this communication a method for the assay of prolintane in plasma is described, which is much more sensitive than the previously published methods using thin-layer chromatography (TLC) [1, 2] and gas chromatography (GC) with packed columns and flame ionization detection (FID) [3—5]. These procedures were insufficient for measuring plasma levels after therapeutic doses, the basis of pharmacokinetic studies in man. Recently the use of a capillary column together with a nitrogen-selective detector has proved to be a very sensitive and thus suitable instrument for such an analysis [6, 13].

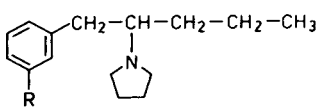

Fig. 1. Structural formulae of prolintane (R = H) and internal standard (R = CH_3).

EXPERIMENTAL

Reagents and chemicals

Prolintane [(*R,S*)-1-phenyl-2-(N-pyrolidyl)-pentane] and the internal standard [(*R,S*)-1-(3-tolyl)-2-(N-pyrolidyl)-pentane] were of analytical grade. ^{14}C-Labelled prolintane (specific activity 12.6 μCi/mg $\equiv 4.62 \times 10^5$ dps mg^{-1}) was synthesized in the isotope laboratory of the Biochemical Department of Dr. Karl Thomae GmbH. It was labelled in the carbon-2 position of the pentane chain.

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n-Hexane and ethyl acetate were from Mallinckrodt (Wesel, G.F.R.) and were Nanograde quality, Nos. 4159 and 3427, respectively. Hydrochloric acid and sodium hydroxide were purchased from Merck (Darmstadt, G.F.R.); they were p.a. quality, Nos. 319 and 6498, respectively. Thin-layer chromatography was performed with silica-gel plates (Merck, No. 5715) in the solvents cyclohexane—diethylamine—benzene (75:20:15, v/v) and benzene—dioxane—aqueous ammonia (60:35:1, upper phase).

Apparatus

The gas chromatograph used was a Hewlett-Packard 5840 equipped with an N—P detector Model 18848A and an autosampler No. 7672. The column was a WCOT, 25 m × 0.3 mm I.D., Duranglass, the stationary phase Carbowax 20M, purchased from Perkin-Elmer (Überlingen, G.F.R.) and Bebjack (Kissing, G.F.R.). Operating conditions were: injector temperature 230°C, detector temperature 230°C. After injection in the splitless mode, the split was opened 1 min later and the temperature program was started. The column temperature was programmed from 70°C to 170°C at 10°C/min and then 15 min isothermal.

Gas flow-rates were: carrier (helium) 2 ml/min, hydrogen 3 ml/min, air 50 ml/min, auxiliary gas (helium) 35 ml/min. The helium was purified by an Oxisorb kit (Messer-Griesheim, Frankfurt, G.F.R.) to improve the service life of the column. The evaporator used was a Vortex-Evaporator (Searle-Buchler, Fort Lee, NJ, U.S.A.).

Analytical procedures

Extraction. Blood was sampled via heparinized syringes and centrifuged. The plasma obtained was stored frozen at —30°C in glass tubes. It was thawed at room temperature and 2-ml portions were pipetted into 25-ml centrifuge tubes. Then 200 μl of water containing 183.4 ng of the internal standard (as the citrate ≡ 100 ng of base) were added, followed by 2 ml of 0.2 *N* NaOH and 10 ml of *n*-hexane. The samples were mixed for 15 min on a shaking machine.

After centrifuging, the plasma phase was frozen at —20°C. The organic phase was transferred to another tube and extracted with 1 ml of 0.2 *N* HCl. The water phase was frozen, and the organic layer discarded. After thawing the water phase was made alkaline with 1.5 ml of 0.2 *N* NaOH and extracted with *n*-hexane. The hexane phase was concentrated in silanized tubes in the evaporator and reconstituted with 50 μl of ethyl acetate and transferred to the autosampler; 2 μl were injected into the chromatograph.

Preparation of the calibration curve. The calibration curve was constructed by preparing a stock solution containing 1.168 μg/ml prolintane hydrochloride in plasma. After incubation (2 h, 37°C) this plasma was diluted with non-spiked plasma to obtain a concentration range of 10—100 ng/ml. By this procedure we hoped to attain an equilibration of prolintane to plasma proteins. These standard samples were analysed by the same procedure as described above.

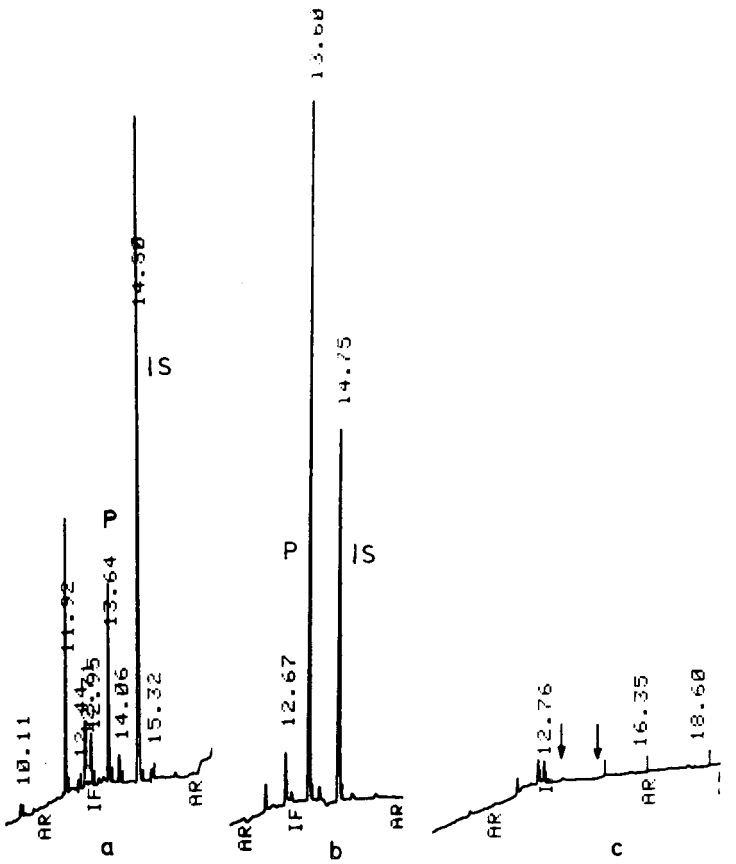

Fig. 2. Chromatograms showing human plasma levels of prolintane at concentrations of (a) 7.7 ng/ml, (b) 52.8 ng/ml, and (c) blank. P = Prolintane, IS = internal standard.

RESULTS AND DISCUSSION

The use of an N-FID instead of an FID increases the selectivity. In Fig. 2 the gas chromatograms of extracts from plasma compared with the plasma blank are shown. The blanks are very low (< 1 ng/ml) and nearly constant for all volunteers. The detection limit of the assay is about 1 ng/ml, the detection limit of the pure substance lower than 50 pg. The detection limit is determined by the variation of the blanks [7] and not by the detection limit of the system, as we inject only 1/25th of the extract.

The back-extraction is necessary for two reasons. First, we attain selectivity, as interfering non-basic compounds are removed. Without back-extraction we have a detection limit of about 5 ng/ml. Secondly, this procedure is necessary to improve the life of the Carbowax column, as with each injection the column would be coated with about 100 μg of triglycerides and 100 μg of cholesterol.

The calibration curve is linear between 10 and 100 ng of prolintane per ml (the range of therapeutic doses). It is described by the equation

$y = bx + a$, where $a = -0.021$, $b = 3.09 \times 10^2$, and $r = 0.9989$.

TABLE I

REPRODUCIBILITY OF PROLINTANE DETERMINATION ON THE SAME DAY (A) AND ON DIFFERENT DAYS (B)

	Plasma level (ng/ml)	Mean peak ratio	No. of determinations	S.D. (%)
A	10	0.268	5	4.5
	25	0.719	5	2.1
	100	3.07	5	2.7
B	20	0.58	4	8.5
	100	3.07	4	8.7

The reproducibility of the method was studied by preparing plasma samples containing different amounts of prolintane and analysing them on different days. The results are shown in Table I.

By liquid scintillation counting, the recovery of ^{14}C-labelled prolintane was found to be 69.7% ± 2.2% (n = 5) at a concentration of 50 ng/ml and 72.6% ± 2.8% (n = 5) at a concentration of 200 ng/ml. During this experiment 100 ng of internal standard were present to reproduce the same conditions as for the construction of the calibration curve. This relatively low recovery for the whole procedure is due to the fact that we used only single extraction steps. But as we used a very similar internal standard the variation of the recovery does not influence the precision of the assay. The use of silanized glass is strongly recommended to minimize losses caused by adsorption. This is of special importance as we used hexane without an alcohol. The use of hexane gives the lowest blank (cf. selectivity) and has an appropriate extraction efficacy for prolintane (Table II).

TABLE II

PARTITION RATIOS FOR PROLINTANE BETWEEN BUFFERS AND DIFFERENT SOLVENTS

	Partition ratio (C_{org}/C_{aq})		
	Hexane	Ethyl acetate	Toluene
NaOH 0.1 *N*	>100	26	>100
Phosphate buffer (pH 6.9)	3.5	3.0	8.0
HCl 0.1 *N*	0.02	0.06	0.05

The selectivity of the method was tested by two experiments. First, we applied 10 mg/kg [^{14}C]prolintane to six rats (200 g) and killed them 1 min, 3 min, 10 min after application. The plasma was extracted according to our procedure and the extracts analysed by TLC in different solvent systems. The hexane extract contained only the parent compound, quite different to extract obtained with other extraction solvents (ethyl acetate, chloroform). This means that polar metabolites are not extracted by our procedure. For legal reasons this

experiment with the labelled compound could only be done in animals. Secondly, identification of the compounds eluting from the gas chromatograph was further carried out by GC—mass spectrometry (MS) (Finnigan 3300 with computer system 6015). The mass spectrum of prolintane shows no molecular peak. The base peak is at *m/z* 126. The spectrum at the retention time of prolintane showed no interference with other substances.

For routine pharmacokinetic analysis the combination of glass capillary GC with N-FID is superior to glass capillary GC—MS as it is more robust against contamination from endogenous substances. In this special case the usable selected ion monitoring trace is at *m/z* 126. This means that it is easily disturbed by fragmentation ions arising from endogenous material. This fact was emphasized recently in the literature [8].

The method is useful for serial analyses. As the extraction steps are performed in centrifuge tubes and the phase separation is done by freezing out, 40 samples can be analysed a day by one person. The advantages of phase separation via centrifugation and freezing out (the latter step was first described by Diekmann [9] are as follows: (1) All samples can be handled simultaneously (depending on equipment). (2) There are never problems with the formation of emulsions. The small gelatinous phase between the organic and water phase (containing lipids) is solid too. (3) The water content of the organic phase is much smaller at −20°C than at 20°C, thus no drying is necessary. (4) No additional glassware such as separation funnels and pipettes (adsorption, contamination) are used to separate the water phase from the organic phase. Using an autosampler, the long time needed for an analysis run (30 min) is of minor importance as day and night operation is possible.

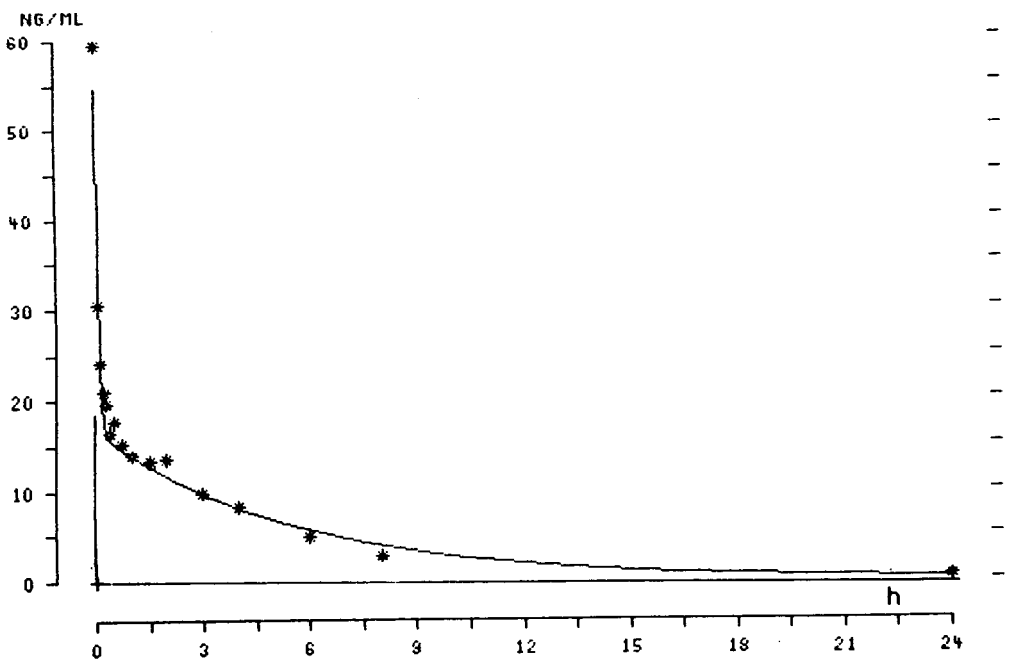

Fig. 3. Plasma level of prolintane in human subjects (n = 5) following intravenous administration of 0.15 mg/kg by a 45-sec infusion. *, Experimental values; —, computer-fitted curve.

APPLICATION

Fig. 3 shows the mean plasma level of five human volunteers after a dose of 0.15 mg/kg prolintane by a short (45-sec) infusion. The values measured are fitted with a two-compartment model with one side-compartment "Thomae Topfit" [10]. The terminal elimination half-life is about 4.5 h under controlled urinary pH and is determined by the back-diffusion of prolintane from the tissues. Compared to earlier studies [5], in which only total radioactivity was measured, we find that prolintane is rapidly metabolised. The kinetics of prolintane are characterised by a large first-pass effect [11]. This can be demonstrated easily by the intravenous experiment, from the total clearance Cl_{tot} [12], which in this case is 1.6 l/min. This clearance is of the magnitude of the hepatic blood-flow.

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Note

Determination of cyclizine and norcyclizine in plasma and urine using gas—liquid chromatography with nitrogen selective detection

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(First received June 20th, 1980; revised manuscript received August 11th, 1980)

Cyclizine is a clinically useful drug of the benzhydrylpiperazine series [1] and norcyclizine is its demethylated metabolite (Fig. 1). Previous methods for the estimation of these substances in biological material were complexing with methyl orange [2] and derivatisation with tritiated acetic anhydride [3]. The accuracy of these methods is limited at low concentrations owing to non-selective reactions with naturally occurring substances.

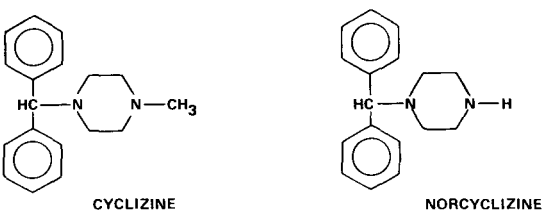

Fig. 1. Molecular structure of cyclizine and its demethylated metabolite, norcyclizine.

The method described below, which employs gas—liquid chromatography (GLC) with a nitrogen selective alkaline flame ionisation detector (AFID) can measure cyclizine in plasma and urine with adequate sensitivity for pharmacokinetic studies. The method can also measure norcyclizine in urine.

MATERIALS AND METHODS

Chemicals

Cyclizine, norcyclizine and chlorcyclizine were obtained from the Wellcome Foundation (Dartford, Great Britain) and recrystallised as hydrochlorides

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before use. Dowtherm A, an eutectic mixture of biphenyl and dibenzofuran, was obtained from Fluka (Fluorochem, Glossop, Great Britain); cyclohexane AR, which was glass distilled before use, and isopropanol (HPLC grade) were obtained from Fisons (Loughborough, Great Britain). All aqueous solutions were made up using glass distilled deionised water.

Glassware

Screw-capped tubes (20 ml Soveril, type 611-03; V.A. Howe, London, Great Britain) were used for the extractions. Microtubes of 3.5 ml maximum capacity [Du-Pont (U.K.) part No. 834078; Hitchin, Great Britain] were used for the solvent evaporation stages. The microtubes end in a fine point which was useful for manipulation of the concentrate. All glassware was washed with hydrochloric acid (2 *M*) and rinsed with deionised water before use. The microtubes were dried at 210°C in a vacuum oven before use.

Determination of plasma cyclizine

Standard solutions of cyclizine ranging from 0—500 ng/ml were prepared by dilution of the aqueous stock solution (1 mg/ml) with heparinised human plasma. An aliquot of the standard or sample (1 ml) was placed in an extraction tube followed by chlorcyclizine (internal standard, 50 μl; 10 μg/ml) sodium hydroxide (2 ml; 2 *M*) and cyclohexane (10 ml). Cyclizine and chlorcyclizine were then extracted into cyclohexane by rocking the tube for 20 min along its long axis at 25 oscillations per min (flat bed shaker) after which the liquid phases were separated by centrifugation (1200 *g* for 10 min). As much of the cyclohexane (top layer) as possible was removed and transferred to another extraction tube containing hydrochloric acid (2 ml; 2 *M*). The cyclizine and chlorcyclizine were back extracted into the acid layer and the organic layer was removed and discarded. The cyclizine and chlorcyclizine were re-extracted into cyclohexane by adding sodium hydroxide (2 ml; 4 *M*) and cyclohexane (4 ml). The cyclohexane (3 ml) was transferred to a microtube containing Dowtherm A (20 μl) and was then removed at room temperature under a stream of nitrogen. The alkaline solution was re-extracted with a further 3 ml of cyclohexane. The cyclohexane (3 ml) from this final extraction was added to the liquid residue in the microtube and the cyclohexane removed as above. Dowtherm A prevented the solutes from solidifying and also imparted selective solution of the cyclizines. The resultant oily residue was injected into the gas chromatograph (4 μl per injection). Standards and unknowns were analysed in duplicate.

Determination of urinary cyclizine and norcyclizine

Combined standards of cyclizine and norcyclizine covering the range 0—200 ng/ml were prepared by dilution of the aqueous stock solution (1 mg/ml) in urine. A 1-ml aliquot of standard or unknown was placed in an extraction tube followed by chlorcyclizine (internal standard, 50 μl; 10 μg/ml), sodium hydroxide (2 ml; 2 *M*) and cyclohexane (4 ml). The contents were extracted twice as in the final alkaline extraction of the plasma analysis. The acid back extraction step and addition of Dowtherm A were unnecessary as cyclizine and norcyclizine were in high yield and well resolved from all the other urinary con-

stituents. However, the resultant dried residue in the microtube was redissolved in 50 μl of isopropanol just prior to injection into the gas chromatograph (4-μl injections). The urine samples and unknowns were analysed in duplicate.

Chromatographic conditions

The gas chromatograph used was a Model F30 Perkin-Elmer (Beaconsfield, Great Britain) with an AFID detector. A glass column (1.8 m × 4 mm I.D.) was hand packed with 5% OV-17 on Chromosorb W HP (100—120 mesh) and conditioned at 310°C with a helium carrier gas flow-rate of 50 ml/min for at least 24 h before use. The AFID detector was used in the nitrogen mode with hydrogen (8.5 ml/min), air (92 ml/min) and helium carrier gas (50 ml/min). Manifold, oven and injection port temperatures were 321, 246 and 310°C, respectively. With a fully conditioned column cyclizine, norcyclizine and chlorcyclizine were well resolved with retention times of 190, 242 and 329 sec, respectively. Tailing of the norcyclizine peak indicated poor column condition; cyclizine and chlorcyclizine were less affected. The limit of detection was approximately 200 pg for cyclizine and chlorcyclizine and 2 ng for norcyclizine injected on column.

RESULTS

Calculation of results

A data system (Hewlett-Packard Model 3352) was used to calculate the peak

TABLE I

ASSAY PRECISION FOR CYCLIZINE (PLASMA AND URINE) AND NORCYCLIZINE (URINE) ESTIMATIONS

n = 6 at each concentration.

Sample drug and concentration (ng/ml)	Mean drug to internal standard ratio	Mean standard deviation of ratios (± S.D.)	± % S.D. of mean value
Plasma cyclizine*			
50	0.1032	0.0104	10.1
100	0.1877	0.0184	9.8
200	0.3772	0.0142	3.8
500	0.9233	0.0387	4.2
Urine cyclizine**			
50	0.0747	0.0026	3.5
100	0.1635	0.0140	8.5
200	0.3302	0.0243	7.4
500	0.9378	0.0419	4.5
2000	3.9152	0.1953	5.0
Urine norcyclizine***			
50	0.051	0.010	19.2
100	0.110	0.014	12.6
200	0.256	0.020	7.7
500	0.645	0.050	7.7
2000	2.835	0.045	1.6

*y = 0.001830 x + 0.006; r = 0.9999, where y = ratio of cyclizine or norcyclizine to chlorcyclizine (peak areas), and x = plasma concentrations of cyclizine or norcyclizine.
**y = 0.001980 x — 0.043; r = 0.9983.
***y = 0.001426 x — 0.028; r = 0.9994.

areas of cyclizine, norcyclizine and their ratios to the internal standard peak area. The calibration curve was a plot of the cyclizine or norcyclizine concentration (abcissa) against its peak area ratio to that of the internal standard (ordinate). Since the same known mass of internal standard (500 ng of chlorcyclizine) was added to the unknown samples (plasma or urine) the amount of cyclizine or norcyclizine in the sample could be determined from its peak area ratio using the calculated (Texas T151 [III] calculator) least squares regression line derived from the calibration curve.

Validation

Methods were validated by adding known amounts of cyclizine to plasma and urine and known amounts of norcyclizine to urine; six determinations were made at each sample concentration. Table I shows the precision of cyclizine determinations at all concentrations tested in plasma and urine and for norcyclizine in urine. Recovery was linear relative to the drug standards (see Table I). Mean percentage deviations of duplicates were calculated and mean values for unknowns and standards compared using a *t*-test (with appropriate modifications if suggested by inequality of variance). No significant differences were seen.

By comparing peak areas with those given by injection of the cyclizine and

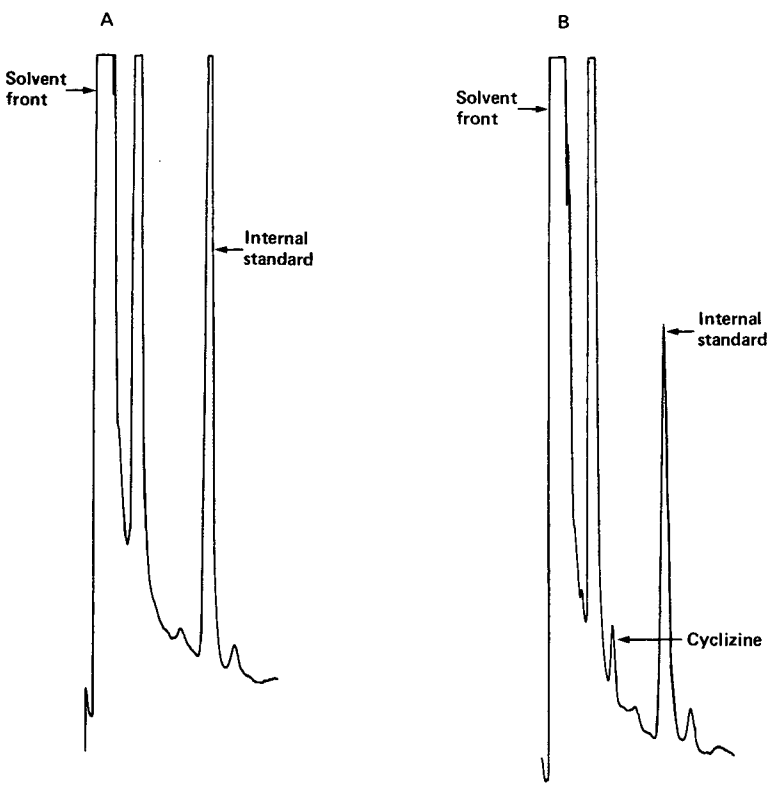

Fig. 2. Chromatograms from the analysis of (A) blank plasma and (B) plasma to which cyclizine (50 ng/ml) was added. Internal standard, chlorcyclizine, 10 μg/ml.

norcyclizine bases (in isopropranol) the total extraction efficiencies were about 45% for cyclizine in plasma and greater than 80% for cyclizine and norcyclizine in urine. This reflects the greater number of transfer steps and the final selective solution into Dowtherm A required for the plasma determinations. A small contaminant in the plasma extracts elutes near norcyclizine and as norcyclizine is poorly recovered from plasma, quantitation below 500 ng/ml in plasma is unreliable.

Chromatograms of the plasma and urine extracts are shown in Figs. 2 and 3. An example of the plasma concentration curve in a healthy adult male who was given 50 mg cyclizine hydrochloride intravenously is shown in Fig. 4. The delay in excretion seen in the plasma profile around 30 min is well known for basic drugs (e.g. amphetamine [4]).

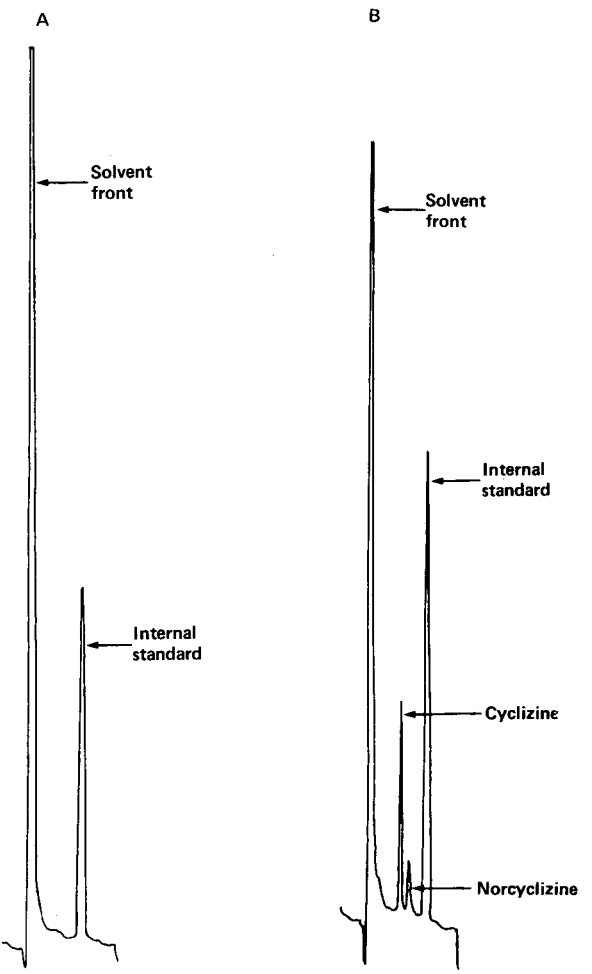

Fig. 3. Chromatograms from the analysis of (A) blank urine and (B) urine to which cyclizine (200 ng/ml) and norcyclizine (200 ng/ml) were added. Internal standard, chlorcyclizine, 10 μg/ml.

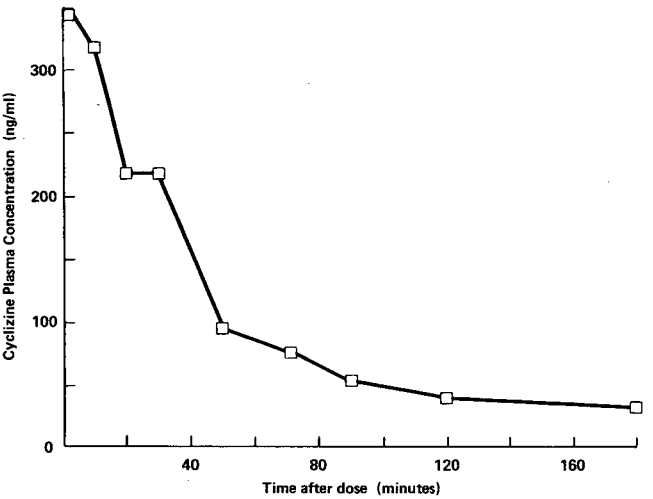

Fig. 4. Plasma cyclizine profile in healthy adult male subject after administration of 50 mg cyclizine intravenously.

DISCUSSION

The GLC technique described here was found to be specific for unchanged cyclizine in plasma and urine and for norcyclizine in urine. Dipipanone hydrochloride, morphine, ergotamine tartrate and caffeine are present in combination with cyclizine in some pharmaceutical preparations but were found not to interfere with cyclizine chromatography. Cyclizine determinations showed good precision at all concentrations tested in plasma and urine. For norcyclizine in urine good precision was achieved at the higher concentrations but at the lower concentrations (50 ng/ml) the precision was poor (% S.D. of mean was ±19%). Attempts to improve the extraction efficiences with solvents more polar than cyclohexane resulted in a loss of resolution of cyclizine from other substances eluting with the solvent front.

A chloroform syringe wash between injections minimised carryover caused by a low avidity adsorption of the compounds to glass. Without the wash low concentration samples could be erroneously high especially if they followed a high concentration sample. For the same reason all glassware was routinely acid washed. The use of an internal standard reduces the effects of error from transfer losses.

The methods described are suitable for the analysis of cyclizine in plasma and urine and norcyclizine in urine with sufficient sensitivity for human bioavailability and pharmacokinetic studies, the limit of sensitivity being 10 ng/ml for cyclizine in plasma and urine and 40 ng/ml for norcyclizine in urine.

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CHROMBIO. 699

Note

High-performance liquid chromatography determination of 4′-demethyl-epipodophyllotoxin-9-(4,6-O-ethylidene β-D-glucopyranoside) (VP 16-213) in human plasma

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VP 16-213 (VP16) or 4′-demethyl-epipodophyllotoxin-9-(4,6-O-ethylidene β-D-glucopyranoside) (NSC 141540) is a semisynthetic derivative of podophyllotoxin which has antitumour activity in several experimental and human malignancies [1,2]. In clinical use it is an effective drug in small cell lung cancer [3—5] and has also shown considerable activity in testicular teratomas [6,7], myeloid leukemia [8,9], teratocarcinoma of the ovary [6], choriocarcinoma [10] and in non small cell lung cancer [8,9].

The pharmacokinetics of VP16 have been studied in animals and man by radiochemical analytical assay using the tritium-labelled compound [11,12]. These procedures, however, lack specificity, among other reasons because the drug is metabolized in vivo [13]. In addition, they are not appropriate for clinical pharmacokinetic studies as they require the administration of radioactive material to patients. The need for knowledge of the pharmacokinetics of VP16 in animals and man prompted us to develop an analytical method with high-performance liquid chromatographic (HPLC) separation and UV absorbance detection which offer sufficient specificity, sensitivity and simplicity to be employed clinically.

EXPERIMENTAL

Patients

VP16 was assayed in plasma from two women, 28 and 35 years old, with choriocarcinoma who received the drug at a dose of 100 mg/m^2 as a 60-min intravenous infusion. They did not receive any other drugs in combination;

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both patients had normal kidney and liver function, as assessed by standard tests.

Blood was collected in heparinized tubes before the drug treatment, at the end of VP16 infusion and 5, 15, 30, 60, 120, 180, 240, 480, 720 and 1440 min after the end of the infusion. The blood was centrifuged at 400 *g* for 20 min and plasma was kept at −20°C until analyzed.

Standard and reagents

VP16 and 4-demethyl-β-epipodophyllotoxin-D-thenylidene glucoside (VM26) used as standards were generously provided by Dr. Lenaz of Bristol Myers (New York, NY, U.S.A.). VP16 and VM26 used as standards were dissolved in methanol (100 μg/ml) and stored at −20°C when not in use. VP16 administered to the patients was the commercial preparation for clinical use. The following reagents were used: diisopropyl ether, chloroform and methanol (Carlo Erba, Milan, Italy). Water and methanol were filtered through 0.40-μm nucleopore polycarbonate membranes (BDH, Milan, Italy) prior to use.

Extraction procedure

Heparin-treated plasma (1 ml) was washed with 5 ml diisopropyl ether, shaking for 5 min. VM 26 was then added as internal standard and 8 ml of chloroform were used for extraction. The test-tubes were mechanically shaken for 20 min, centrifuged at 600 *g* for 5 min, then the aqueous phase was discarded and the organic phase was transferred to a second test-tube and brought to dryness at room temperature in a rotary evaporator under vacuum. The drug residue was redissolved in 100 μl of water—methanol (45:55) and 5—20 μl of this solution were injected into the chromatograph.

High-performance liquid chromatography

HPLC separation was performed on a Waters Model 440 instrument equipped with a 254-nm absorbance detector. Separation was achieved with an isocratic solvent system of water—methanol (45:55) at a flow-rate of 1 ml/min using a LiChrosorb RP-8 (5 μm) column from Merck (Darmstadt, G.F.R.).

Stability of VP16 in plasma

The stability of VP16 in plasma (20 μg/ml) was determined at several incubation times (0, 1, 2, 3, 4, 5, 6, 12 and 24 h), during which samples were maintained at 37°C. All analyses were performed in triplicate.

Calibration curve

Pools of plasma containing 0.5, 1.0 and 5.0 μg/ml of VP16 were divided into 1-ml samples which were extracted for VP16 determination as described. Experiments were run in quadruplicate for each concentration and replicated three times. After chromatographic analysis the peak area ratios of VP16 to the internal standard were plotted for the linear regression analysis against the theoretical concentration in the samples.

Recovery

In order to estimate the recovery, various amounts of VP16 (0.5, 1.0, 5.0

and 10 μg) were added to 1 ml of human plasma and VM26 (50 μg) was added after extraction. The VP16:VM26 peak area values were compared with those obtained when injecting corresponding external samples at the same concentrations.

Pharmacokinetic calculations

The results were processed using a two-compartment open model after intravenous infusion described by the equation $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ where C_p is the plasma concentration at time t, A and B are the intercepts on the ordinate at zero time and α and β represent the slopes of the respective exponential segments.

The elimination half-life ($T_{1/2}$) as calculated by the "peeling" method [14] ($T_{1/2} = 0.693/\beta$) using a Hewlett-Packard Model 9810 computer.

RESULTS AND DISCUSSION

Typical HPLC chromatograms of extracts from human plasma before and after VP16 treatment are shown in Figs. 1 and 2. VM26 was used as internal standard for the quantitative determination of VP16 because of its structural similarity. The extraction recovery of VP16 was 79±3% and of VM26 81±2%.

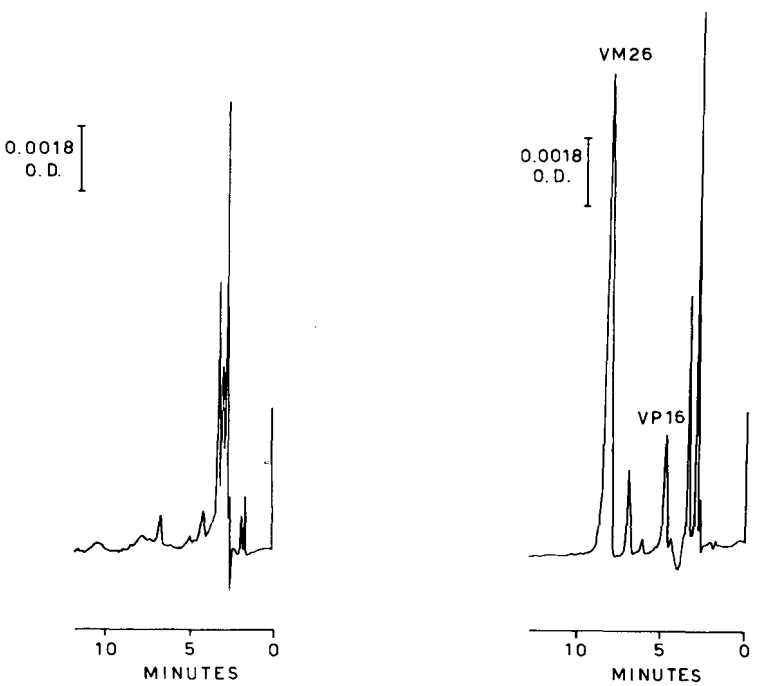

Fig. 1. Chromatogram of human plasma extract before VP16 administration. The equivalent of 100 μl of plasma was injected at 0.02 a.u.f.s.

Fig. 2. Chromatogram of human plasma showing VP16 concentration 24 h after intravenous administration of 100 mg/m^2. The final calculation indicates a concentration of 0.54 μg of VP16 per ml of plasma.

As can be seen in Table I, the calibration curve of VP16 is linear ($r = 0.99$) and the coefficient of variation (C.V.) is less than 10%. No interference from endogenous compounds was observed. VP16 appears to be stable in plasma at 37°C; in fact no significant degradation was found under these conditions even after 24 h of incubation. The suitability of the analytical procedure for clinical pharmacokinetic studies was tested by determining VP16 plasma levels in two cancer patients who had received a single dose of 100 mg/m^2 as a 60-min intravenous infusion. Fig. 3 shows the plasma disappearance of VP16 in the two

TABLE I

REPRODUCIBILITY OF VP16 ASSAY

$r = 0.99$ ($p < 0.05$) for a line with a slope of 0.29 and an intercept of —0.01, not significantly different from zero.

	Plasma concentration (μg/ml)		
	0.5	1.0	5.0
Peak area ratio	0.132	0.283	1.456
S.D.	0.0119	0.0232	0.1455
C.V.	9.0	8.2	9.9
n	12	12	12

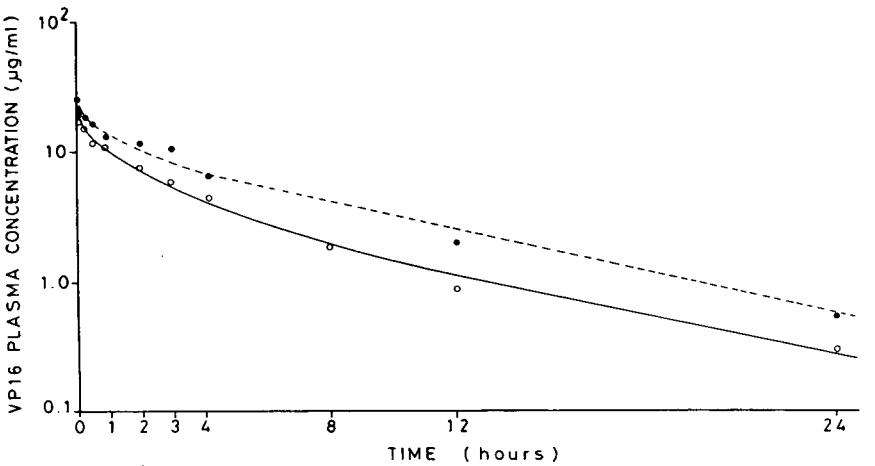

Fig. 3. Plasma concentration—time curves from two patients after an intravenous infusion dose of 100 mg/m^2 of VP16.

patients. Applying a two-compartment open model, the elimination half-lives ($T_{½}$) were 4 and 5 h, whereas previous studies, in which the ^{3}H-labelled compound was measured reported a half-life of 6—16 h. The slightly shorter half-life we found, might be explained by a lack of specificity of the radiochemical method which could not perhaps distinguish the drug from metabolites.

In conclusion the method described in the present study appears to possess a sufficient degree of specificity, sensitivity and reliability to be employed for experimental and clinical pharmacological studies.

The preliminary pharmacokinetic data obtained in the cancer patients studied show that the method is suitable to be routinely used in clinical situations. This method can also be applied for the determination of VM26 which is also clinically employed as an antitumour agent [2]. The optimal conditions of sample preparation before HPLC analysis of this drug are under investigation in our laboratory at the moment.

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CHROMBIO. 711

Note

Liquid chromatographic determination of an antineoplastic aziridinylbenzoquinone in human and murine serum

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In recent comparisons of the activity in vivo of a series of aziridinylbenzoquinones [1—3] one compound, 1,4-cyclohexadiene-1,4-dicarbamic acid 2,5-bis(1-aziridinyl)-3,6-dioxo diethyl ester (AZQ), demonstrated superior antitumor activity in rodents especially with respect to intracerebrally injected L1210 and P388 leukemias as well as several other tumor lines including ependymoblastoma, B-16 melanoma and C26 colon tumor. AZQ therefore represents a promising new lipid-soluble antineoplastic drug that may have significant activity against CNS tumors in humans. This drug is currently under Phase I investigation in patients with cancer at the National Cancer Institute, the Vermont Regional Cancer Center and elsewhere.

This paper presents the first report of a simple analytical method for determination of AZQ in serum samples that is sensitive to drug concentrations as low as 20 ng/ml. We describe a reversed-phase ion-pair isocratic chromatographic system that utilizes a variable-wavelength UV detector. The proposed method is also applicable for the analytical study of certain other aziridinylbenzoquinones which also have demonstrated significant antitumor activity in experimental animal tumor systems.

EXPERIMENTAL

Chromatographic apparatus

A Spectra Physics Model 8000 microprocessor-controlled high-performance liquid chromatograph equipped with a data system was used. The chromatograph was equipped with a Schoeffel Model 770 variable-wavelength UV detector set at 340 nm. The column was an Altex (Berkeley, CA, U.S.A.) Ultra-

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sphere I.P. 5-μm column (25 cm × 4.6 mm I.D.). A guard column (7 cm × 0.2 cm I.D.), packed with Co:Pell ODS, 25—37 μm (Whatman, Clifton, NJ, U.S.A.) was installed to protect the 5-μm column. Samples were injected onto the column through a 10-μl loop, using a manual injector.

Chemicals

High-performance liquid chromatography (HPLC) grade water and methanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). AZQ (NSC 182986) and appropriate diluents for clinical preparation were obtained from the Investigational Drug Branch, National Cancer Institute, National Institutes of Health. The AZQ analogs investigated were provided by Dr. John S. Driscoll (Laboratory of Medicinal Chemistry, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD, U.S.A.). PIC reagent A was purchased from Waters Assoc. (Milford, MA, U.S.A.).

Stock solutions of AZQ were made by solubilizing the drug in dimethylacetamide, and diluting with 0.1 *M* phosphate buffer, pH 6.5. Further dilutions were carried out in HPLC-grade water when necessary. Stock solutions of the water-soluble AZQ analogs were made by placing the compound in HPLC-grade water followed by sonication to break up residual particles. Analogs which were not water soluble were solubilized in dimethylsulfoxide and diluted to the desired concentration with HPLC-grade water. All solutions were filtered through a 0.45-μm Millipore filter before injection onto the column. The final concentration of AZQ was estimated using the extinction coefficient of the compound in methanol at 340 nm [1].

Drug administration and serum collection

Human. AZQ was administered in the wards and clinics of the Medical Center Hospital of Vermont and the University Health Center. The patients all had far advanced cancer unresponsive to conventional therapy. Written informed consent was obtained from all patients. AZQ was administered to patients over a 5-min period by injection into a running intravenous line containing normal saline. Doses up to 10 mg per square meter of body surface area with three or four patients per dose were examined in this Phase I study. Ten ml of whole blood were obtained from the arm opposite to that used for the drug injection. Serum was obtained by centrifugation and stored at −10°C for less than 48 h prior to analysis for AZQ content.

Mouse. AZQ was injected into the tail vein of female Swiss Webster mice (20—25 g) at a high dose (5—10 mg/kg) over a period of 10—20 sec. Blood samples were obtained by retro-orbital puncture at various times following injection. Serum samples were prepared by centrifugation of blood at 7000 *g* for 5 min in a bench top centrifuge and were either assayed immediately or frozen at −15°C for subsequent analysis (within 24 h).

Preparation of serum samples for analysis by HPLC

Serum samples were prepared for injection onto the column using Sep-Pak cartridges (Waters Assoc.). The use of the Sep-Paks affords both sample purification and drug concentration. Cartridges were prepared for use as described in the brochure supplied by Waters Assoc. A measured volume (0.5—4.0 ml) of

serum or stock drug solution was loaded onto the Sep-Pak, which was then washed with 1.0 ml HPLC-grade water containing PIC reagent A. AZQ was then eluted by washing the Sep-Pak with 3.0 ml HPLC-grade methanol containing PIC reagent A. The methanol was removed under nitrogen and the residue was taken up in a minimum volume (100—200 μl) of HPLC-grade water. The sample was vortexed, and then centrifuged to remove the insoluble material. The resulting solution was used directly for injection onto the column.

HPLC procedure

AZQ was eluted from the column using an isocratic mobile phase of water—methanol (65:35, v/v) containing 0.005 *M* tetrabutylammonium phosphate (PIC reagent A). A flow-rate of 1.0 ml/min was used. The concentration of AZQ in the eluate was measured by UV detection at 340 nm. Quantitative analysis was based on peak areas, and was computed using pre-set integration programs in the data system of the Spectra Physics HPLC instrument. Prior to sample analysis, the chromatograph was calibrated daily using AZQ solutions of known concentration.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatogram obtained when AZQ was injected either as a stock solution (Fig. 1A) or as an extract of human serum (Fig. 1B). Drug elution occurred at a retention time that was well separated from the solvent front. A relatively minor increase or decrease in the percent of methanol in the described mobile phase permitted shortening or lengthening, respectively, of AZQ retention time as desired. The elution of AZQ using either isocratic or gradient systems of water and methanol without the PIC reagent A resulted in peaks which were very broad and poorly defined making accurate quantitation of peak area difficult. A standard calibration curve for AZQ was generated daily by plotting peak areas (as computed by the data system) against the concentration of the drug injected onto the column. The relationship was linear over a ten-fold concentration range, with an excellent correlation coefficient of 0.998 calculated using the least squares regression line. The correlation between measured peak height and drug concentration was also linear with an excellent correlation coefficient.

The minimum detectable level of AZQ was 7.0 ng injected, at a detector setting of 0.01 a.u.f.s. and a recorder attenuation of zero. The signal-to-noise ratio was 6 or less under these conditions.

The reproducibility of the described method was checked by sequentially injecting four samples of a stock AZQ solution (3.05 μg/ml) and then measuring retention times and areas of the eluted peaks. The standard error of the calculated concentration as peak area was less than 5% of the mean value (2.99 ± 0.14 μg/ml). The standard error of the mean retention time was less than 1% of the mean value (10.33 ± 0.06 min).

Several analogs of AZQ which had previously been shown to possess significant antitumor activity [1—3] were investigated using the described chromatographic procedure. The structures and retention times for these compounds

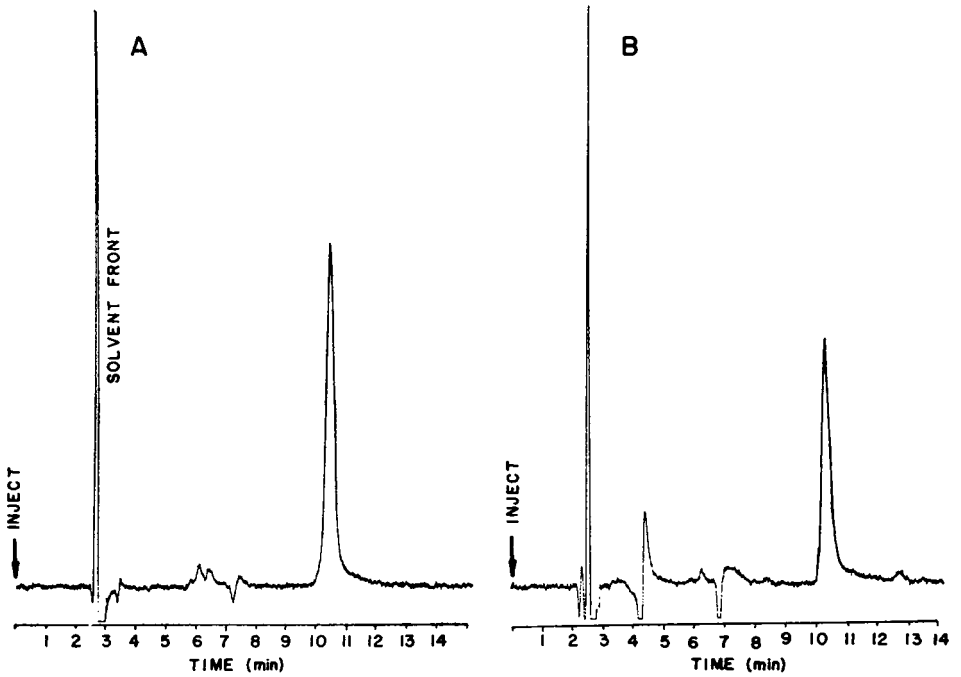


Fig. 1. Typical chromatograms of AZQ eluted from an Altex I.P. C_{18}-column. Conditions: column, 25 cm × 4.6 mm I.D.; mobile phase, water—methanol (65:35, v/v) with PIC A; flow-rate, 1.0 ml/min; column temperature, ambient; detector, Schoeffel 770 variable-wavelength UV (340 nm) at 0.1 a.u.f.s. (A) AZQ stock solution, 35 ng injected; (B) AZQ extract from human serum, the sample was drawn 5 min after the patient had received AZQ (10 mg/m^2).

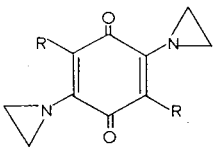
are shown in Table I. All of the alkyl-substituted analogs investigated showed a distinct peak, with retention times varying from 4.3 to 27.2 min. These retention times can be altered by minor changes in the percent of methanol in the mobile phase. Neither the piperidine nor the pyrrolidine analogs showed a peak within 30 min after injection. The piperazine-substituted compound, which also contained a substituted alkyl side-chain, showed a distinct peak at 4.8 min after injection. None of the analogs investigated interfered with the peak for AZQ. In addition, when the compounds were cochromatographed, each drug retained its individually determined retention time. The dimethylacetamide and phosphate buffer used to dissolve the AZQ did not interfere with the detection of the drug in this system.

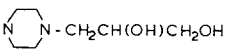
In order to determine the recovery of AZQ from plasma, samples of human plasma were spiked with known concentrations of AZQ, and then extracted using the Sep-Pak cartridges. Even when the plasma samples were spiked with relatively low concentrations of AZQ (e.g., 1—3 μg/ml), the recovery of the drug from the plasma was 90% or greater as calculated by the data system of the chromatograph. Recovery of AZQ from human serum samples was identical to that obtained in human plasma samples.

As can be seen in Fig. 2, administration of AZQ to humans results in a rapid elimination of this drug from serum. There appears to be an initial rapid phase of

TABLE I

RETENTION TIMES OF AZQ ANALOGS



R group	Retention time (min)
$NHCOOC_2H_5$ (AZQ)	10.3
NH_2	4.3
$NHCH_3$	6.9
$N(CH_3)_2$	27.2
$NHCH_2CH_2OH$	5.6
$NHCH_2CH(OH)CH_2OH$	4.8
	4.8
	>30.0
	>30.0

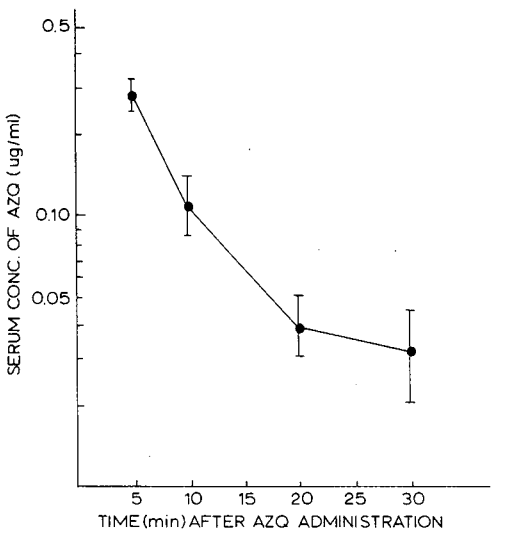


Fig. 2. Serum AZQ concentration in four patients given 10 mg/m^2 AZQ intravenously. Sample drug concentrations were determined by the described HPLC method. Data are presented as mean ± S.E.

drug distribution, followed by a rapid elimination phase with a half-life ($t_{1/2}$) of approximately 8 min. Serum drug levels were detectable through at least 30 min in patients who received 7 or 10 mg/m^2 AZQ. Drug levels in patients receiving less than 7.0 mg/m^2 AZQ were not always detectable at other than the 5-min time period. This phenomenon appears to be a function of the rapid

drug elimination kinetics and necessitates early and frequent serum drug level determinations if an accurate pharmacokinetic profile of this compound is to be done.

AZQ serum levels were also measured in Swiss Webster mice administered 10 mg/kg (30 mg/m^2) AZQ by tail vein injection. Our studies demonstrated that serum AZQ distribution and elimination phases were extremely rapid and closely resembled those observed in humans. Peak serum levels in the mice were much higher (2.0 μg/ml) than those determined in humans (0.3 μg/ml), an expected observation in view of the higher dose administered to mice and the shorter injection time. The serum $t_{1/2}$ in mice was estimated to be approximately 5 min. We currently are applying this method for analysis of AZQ to more extensive studies of the pharmacokinetics of this new drug in human patients and in experimental animals.

ACKNOWLEDGEMENTS

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CHROMBIO. 714

Note

Method for rapid determination of urinary tetracycline by high-performance liquid chromatography

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Measurement of the amount of tetracycline excreted unchanged in the urine offers a convenient means of investigating the pharmacokinetics of this antibiotic in man [1, 2]. Several analytical techniques have been developed for the separation and quantitation of tetracycline by high-performance liquid chromatography (HPLC) [3—7]. However, most of these methods are suitable only for the quality control of tetracycline in pharmaceutical preparations and are subject to interference from UV-absorbing components of biological fluids [8]. Sharma et al. [8] have developed a sensitive and precise method to measure tetracycline in plasma and urine; however the procedure is time-consuming and requires the formation of a tetracycline—calcium complex followed by two extraction steps. Similarly, gradient elution methods [9, 10] which have been developed for tetracycline are unsuitable for processing of large numbers of samples necessary for pharmacokinetic studies. Mack and Ashworth [7] have investigated the utility of reversed-phase chromatography of tetracyclines for quality control purposes. We report a rapid, sensitive and precise method for measuring urinary concentrations of tetracycline for pharmacokinetic studies.

EXPERIMENTAL

Materials

Isopropanol and diethanolamine were supplied by Ajax Chemicals (Sydney, Australia). Both compounds were used without further purification. Tetra-ammonium EDTA was synthesized [7] from ammonia solution (BDH Chem-

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icals, Port Fairy, Australia). Tetracycline hydrochloride reference compound was kindly supplied by Commonwealth Serum Labs. (Melbourne, Australia).

Chromatographic system

All HPLC analyses were carried out using an M-6000A solvent delivery system connected to a Model 440 absorbance detector (Waters Assoc., Milford, MA, U.S.A.) set at 365 nm. Chromatographic separations were achieved on a 25 cm × 4.6 mm I.D. Brownlee RP-10A column maintained at 40°C in a water bath. A 3 cm × 4.6 mm I.D. Brownlee guard column (RP-GU) was used to protect the analytical column. The sorbent in the analytical column and the guard column consisted of 10-μm totally-porous particles of LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.). The mobile phase consisted of isopropanol—diethanolamine buffer—tetraammonium EDTA—distilled water (11:5:1:83). The diethanolamine buffer was prepared by adjusting a 1 *M* aqueous solution of diethanolamine to pH 7.3 with orthophosphoric acid [7]. The flow-rate of the mobile phase was maintained at 2 ml/min. The samples were centrifuged (Microfuge B; Beckman, Fullerton, CA, U.S.A.) and 20 μl of the supernatant phase were injected onto the column through a syringe loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). Chromatograms were obtained on a pen recorder having a 10-mV input and run at a chart speed of 0.25 cm/min.

Calibration graphs

A master stock solution (5 mg/ml) of tetracycline hydrochloride was prepared in 0.03 *N* hydrochloric acid and used for the preparation of primary stock solutions containing 0.1, 0.5, 1, 2, 3 and 4 mg/ml in 0.03 *N* hydrochloric acid. The favourable stability of tetracycline in 0.03 *N* hydrochloric acid had been established previously [11]. Working standards of tetracycline hydrochloride were prepared by appropriate dilution of the stock solutions with tetracycline-free urine to give antibiotic concentrations of 1, 5, 10, 20, 30, 40 and 50 μg/ml. These solutions were submitted to HPLC analysis and acted as external standards in the assay. Calibration graphs were constructed by plotting the peak height of tetracycline against concentration of the antibiotic. Tetracycline standards were chromatographed routinely at the beginning, middle and end of the day's assays and the results averaged.

RESULTS AND DISCUSSION

Fig. 1 shows liquid chromatograms obtained from the assay of blank urine, tetracycline hydrochloride (30 μg/ml) in urine and urine from a subject who had taken 500 mg of tetracycline hydrochloride orally, 2 h previously. Tetracycline had an average retention time of 6 min when 11% (v/v) isopropanol in diethanolamine buffer was used as the mobile phase. Between 10 and 12% of isopropanol could be used with this buffer depending on the age and condition of the column. This solvent system gave minimal tailing of the tetracycline peak on the RP-8 column. Various combinations of methanol, acetonitrile and inorganic buffers were tried and found to be unsatisfactory in this regard.

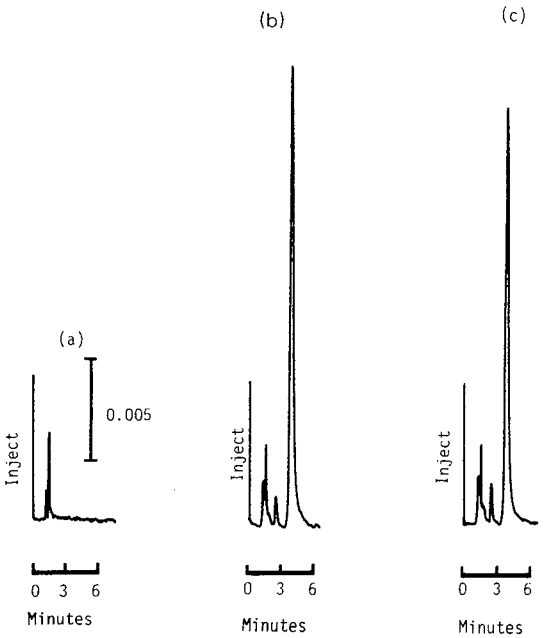

Fig. 1. Representative chromatograms of (a) tetracycline-free urine, (b) urine containing 30 μg/ml of tetracycline hydrochloride and (c) urine from a patient taking a 500-mg dose of tetracycline hydrochloride. In each case samples were diluted 1:10 in 0.03 *N* hydrochloric acid before assay. A detector response of 0.005 a.u. is indicated by the range bar.

The choice of 365 nm as the detector wavelength was important as this offered high sensitivity with minimal interference from other UV-absorbing constituents of urine. Calibration graphs of tetracycline hydrochloride concentration versus peak height were linear from 1 to at least 50 μg/ml ($r > 0.99$) and passed through the origin. The assay can detect 0.4 μg/ml of tetracycline hydrochloride in urine although 0.1 μg/ml of the antibiotic could be detected using an injection volume of 100 μl. To determine the between-day precision of the assay, solutions of tetracycline hydrochloride in urine containing 1, 5, 20 and 50 μg/ml were assayed on ten different days. The results

TABLE I

BETWEEN-DAY PRECISION OF HPLC ASSAY FOR URINARY TETRACYCLINE

The data were obtained from 10 repeat assays of tetracycline HCl in urine

Tetracycline HCl concentration (μg/ml)	Mean peak height (mm)	Standard deviation	Coefficient of variation (%)
1	18.1	0.70	3.9
5	79.5	3.88	4.9
20	345.5	15.27	4.4
50	903.8	28.96	3.2

in Table I indicate the precision of the assay over this range of tetracycline concentrations.

The minimal pre-treatment of the urine samples does not demand the use of an internal standard for the assay provided that a constant volume of sample is injected onto the column. This simplifies the procedure and permits at least 50 to 60 samples to be assayed during a normal working day.

The HPLC method described is convenient, rapid and precise and would be valuable for measuring urinary concentrations of tetracycline when the bioavailability and pharmacokinetics of the antibiotic are under investigation.

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CHROMBIO. 718

Note

Rapid method for the high-performance liquid chromatographic determination of bredinin in human serum

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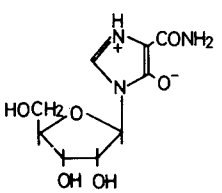
(Received June 26th, 1980)

Bredinin® (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) has been isolated from the culture filtrate of *Eupenicillium brefeldianum* M-2166 [1] and has a potent immunosuppressive activity [2]. However, adequate



information on dosages of bredinin in patients undergoing renal transplantation is now required. The best way to overcome this problem is to monitor the drug in the serum of patients. However, until now no specific method for

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the determination of bredinin has been described in the literature. The animal study concerning its absorption, distribution, metabolism and excretion using the ^{14}C-labelled compound shows that bredinin is excreted mainly unchanged in the urine (81% of the unchanged radioactivity was found in the 24-h rat urine) [3].

As bedinin has a characteristic UV absorption spectrum (Fig. 1), a high-performance liquid chromatographic (HPLC) procedure for the determination of

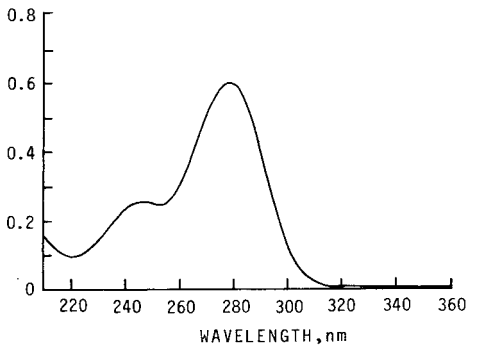

Fig. 1. The UV absorption spectrum of bredinin at a concentration of 10 μg/ml in 30% (v/v) 0.1 *M* imidazole · HCl buffer (pH 7.0) in acetonitrile.

bredinin to study its absorption and pharmacokinetics in serum has been developed. Proteins are removed by precipitation with perchloric acid. The supernatant is neutralized by potassium carbonate. Excess perchloric acid is thus precipitated as potassium perchlorate.

PROCEDURE

A 0.5-ml serum sample was pipetted into a tube containing 50 μl of 70% perchloric acid. After mixing for 30 sec on a Vortex mixer, the tube was centrifuged to precipitate the denatured proteins. This was followed by the addition of 100 μl of a saturated potassium carbonate solution and further mixing. The potassium perchlorate was precipitated by centrifuging at 8000 *g* for 2 min using an Eppendorf centrifuge Model 3200. Suitable volumes, usually 25 μl, of the clear supernatant were injected on to the column.

Analyses were performed using a constant-volume high-pressure liquid chromatograph (Hitachi, Tokyo, Japan) containing a Model 635A pump, a sample injection valve for high pressure and Model 635M UV detector. The column (25 cm × 4 mm I.D., stainless steel) was packed with nominal 10-μm ODS-silica gel with NH_2 groups (LiChrosorb NH_2, manufactured by E. Merck, Darmstadt, G.F.R.) using a balanced density slurry packing procedure similar to that described by Majors [4]. The mobile phase, consisting of 30% (v/v) 0.1 *M* imidazole · HCl buffer (pH 7.0) in acetonitrile, was prepared fresh daily. A flow-rate of 1 ml/min (20 kg/cm^2) was used. Absorbance was monitored at 280 nm. The detector was operated at a sensitivity of 0.01 a.u.f.s. Peak heights were used for quantitation.

RESULTS AND DISCUSSION

Chromatograms of serum samples (Fig. 2) demonstrate that the bredinin peak is sharp and well resolved with no interference from endogenous compounds. The retention time of bredinin is 8 min and a sample could be injected every 18 min. Decreasing this time by increasing the acetonitrile concentration in the eluent causes the serum and bredinin peaks to merge. The standard curve of bredinin added to serum was linear over the range 0.25—10 μg/ml and passed through the origin. The correlation coefficient was $r = 0.968$, and the standard deviation was 3.2% ($n = 6$ at 2.5 μg/ml). The lower detection limit was 0.25 μg/ml of serum. The standard curves were reproducible even though no internal standard was used in this assay. This can probably be attributed to the stability of the column and the simplicity of the procedures.

This HPLC method is easy to perform, involves no extraction or derivatization, and can be successfully performed with 25 μl of serum. The standards were prepared in serum, so the recovery was 100%.

Using this assay, an absorption and disposition kinetics study of bredinin is now in progress with patients undergoing renal transplantation.

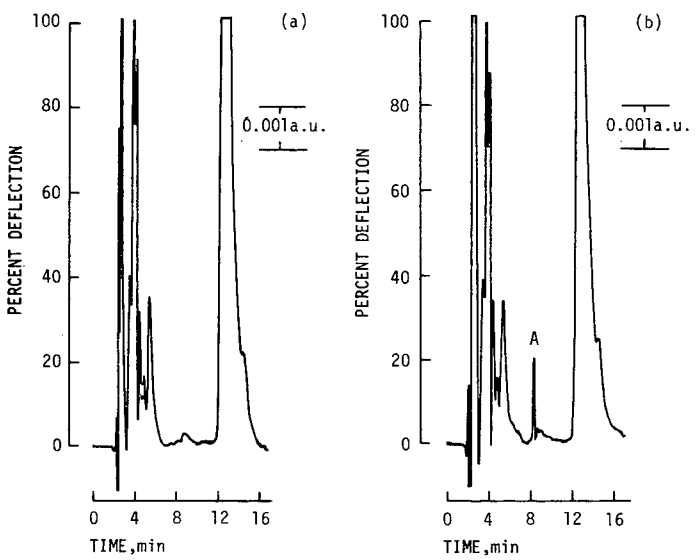

Fig. 2. (a) Chromatogram of a blank human serum. (b) Representative HPLC chromatogram of bredinin in human serum (1 μg/ml). Peak A = bredinin.

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We are much indebted to Toyo Jozo Co. Ltd., Ohito, Shizuoka, Japan, for providing us with bredinin.

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Book Review

Quantitative mass spectrometry in life sciences II, edited by A.P. De Leenheer, R.R. Roncucci and C. Van Peteghem, Elsevier Scientific Publishing Company, Amsterdam, Oxford, New York, 1978, X + 501 pp., price Dfl. 109.00, US $ 53.25, ISBN 0-444-41760-5.

The volume represents the proceedings of the second International Symposium on Quantitative Mass Spectrometry in Life Sciences held in June 1978 in Ghent, Belgium. It contains forty-six articles grouped into five sections: plenary lectures, papers of general interest, quantitation of endogenous substances, quantitation of drugs and drug metabolites, quantitation of exogenous substances. Some of the papers deal with general concepts and general applications of quantitative mass spectrometry, such as definitive and reference methods, selection of standards, correlation with radioimmunological methods for steroid determinations, measurements of gas partial pressures and the role of field desorption mass spectrometry in quantitative investigations. The majority of the papers, however, are concerned with determinations of specific substances.

Quantitative mass spectrometry in life sciences is applied to drugs, drug metabolites and other exogenous compounds more than to endogenous substances. Nevertheless, the articles on measurements of exogenous constituents appear to be overemphasized.

The contributions provide valuable information on mass fragmentography in its various forms, ionization methods, direct quantification by mass spectrometry, sample preparation procedures, precision, linearity and sensitivity of the methods, concentration and excretion characteristics of the substances. Illustrations of the mass spectra and discussions of the fragmentations are generally short.

The book has been produced from camera-ready manuscripts. The text, figures and tables are very well presented. The volume can be recommended to researchers in all branches of life sciences working with mass spectrometry, especially to scientists investigating concentration, metabolization and excretion of endogenous and exogenous substances.

Tübingen (G.F.R.) H.M. LIEBICH

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		The publication schedule for further issues will be published later.								
Chromatographic Reviews														
Biomedical Applications	221/1	221/2	222/1	222/2	222/3									

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

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