VOL. 223 NO. 2 MAY 8, 1981
(Biomedical Applications, Vol. 12, No. 2)
THIS ISSUE COMPLETES VOL. 223

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
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Printed in The Netherlands

(Biomedical Applications, Vol. 12, No. 2)

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7162 A

Journal of Chromatography, 223 (1981) 253—265
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 814

SIMPLE COMPUTER PROGRAM FOR A LOW-COST DESK-TOP CALCULATOR APPLIED TO THE EVALUATION OF GAS—LIQUID CHROMATOGRAPHIC ANALYSES OF 17-KETOSTEROIDS AND PREGNANES*

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(First received September 10th, 1980; revised manuscript received December 16th, 1980)

SUMMARY

A simple computer program consisting of 445 steps for a low-cost desk-top calculator (Hewlett-Packard 97) to be applied in chromatographic analyses of 17-ketosteroids and pregnanes from human urine samples is described. This program permits the calculation of peak factors following the chromatographic separation of an external standard mixture containing up to ten different fractions, the subsequent printout of the constants and their transcription to magnetic data cards for later retrieval when making calculations for unknown samples. After manual input of sample constants (such as total and aliquot volumes, recovery as determined by addition of a radioactive tracer steroid, internal standard) and peak factors via the data card, the individual peak heights of the samples are automatically converted to milligrams of steroid in 24-h urine and each is stored separately. All fractions can be recalled later and printed out in the order of detection or can be transformed into several diagnostically valuable parameters such as the total sum of 17-ketosteroids and pregnanes excreted, the group sums of androgens, of 11-substituted steroids and of pregnanes, the individual percentages of both the fractions and the group sums, and the ratios of aetiocholanolone—androsterone and of pregnanetriol—pregnanediol. Finally, an extension subprogram can automatically generate a plot to illustrate the steroid excretion pattern in a comprehensive fashion.

INTRODUCTION

Gas—liquid chromatography (GLC) of 17-ketosteroids (K) and pregnanes (P) is a valuable approach to the diagnosis of certain congenital enzyme deficien-

*Parts of this work were presented at the 3rd European Congress of Clinical Chemistry, Brighton, June 3—8, 1979.

0378-4347/81/0000—0000/$02.50

cies, especially with respect to adrenal disorders, e.g., those involving the 11β-, the 17α- and the 21-hydroxylases which generally manifest themselves in early childhood as so-called adrenogenital syndrome with or without salt-losing syndrome. Despite the availability of specific radioimmunoassays for measuring steroid hormones and their precursors in human plasma [1—4], GLC analysis provides the important advantage of allowing the assessment of a complete steroid profile from a single sample of urine. In general, the overall procedural errors are approximately equal for all fractions analysed.

Data evaluation in GLC, however, is tedious, time consuming and prone to error, therefore necessitating the use of electronic integrators. For those laboratories which do not have access to such expensive ancillaries, we have developed a computer program that allows the rapid conversion of hand-measured peak heights into final results, expressed as milligrams of steroid in 24-h urine, as well as the output of various additional diagnostically valuable parameters (total and group sums, percentages, ratios). The hardware required is a low-cost desk-top calculator.

EXPERIMENTAL

The GLC procedure employed in our laboratory is similar to that reported by other investigators [5—8]. It involves enzymatic hydrolysis of usually one tenth of a 24-h urine sample, acidified to pH 5, using combined β-glucuronidase—steroid sulphatase (from *Helix pomatia*), diethyl ether extraction, alkaline and neutral washing, pre-purification on aluminium oxide columns, stabilization as trimethylsilyl ether derivatives, isothermal GLC fractionation on Gas-Chrom Q coated with 3% OV-225 (Supelco, Bellefonte, PA, U.S.A.) using a 2-m all-glass column in a Perkin-Elmer Model F 22 instrument and final measurement by flame ionization detection. Paper chart speed was 10 mm/min. Measurement of peak heights (h) was performed by hand.

Generally, ten different steroid fractions can clearly be separated and identified by comparison with an external standard mixture, the order of detection in our procedure being as follows: 1, pregnanediol (P2)*; 2, androsterone (A); 3, aetiocholanolone (AE); 4, dehydroepiandrosterone (D); 5, pregnanetriol (P3); 6, 11-ketoandrosterone (KA); 7, 11-ketoaetiocholanolone (KAE); 8, 11-hydroxyandrosterone (HA); 9, 11-hydroxyaetiocholanolone (HAE); and 10, pregnanetriolone (P3ON).

PROGRAM ANALYSIS

The program is written in an operational language applicable to the Hewlett-Packard 97 calculator. Algebraic formulae are expressed in Reverse Polish Nota-

*The abbreviations used represent the following systematic names: P2, 3α,20α-dihydroxy-5β-pregnane; A, 3α-hydroxy-5α-androstan-17-one; AE, 3α-hydroxy-5β-androstan-17-one; D, 3β-hydroxy-5-androsten-17-one; P3, 3α,17α,20α-trihydroxy-5β-pregnane; KA, 3α-hydroxy-5α-androstane-11,17-dione; KAE, 3α-hydroxy-5β-androstane-11,17-dione; HA, 3α,11β-dihydroxy-5α-androstan-17-one; HAE, 3α,11β-dihydroxy-5β-androstan-17-one; P3ON, 3α,17α,20α-trihydroxy-5β-pregnan-11-one; IntSt (internal standard), 5α-androstane-3,17-dione.

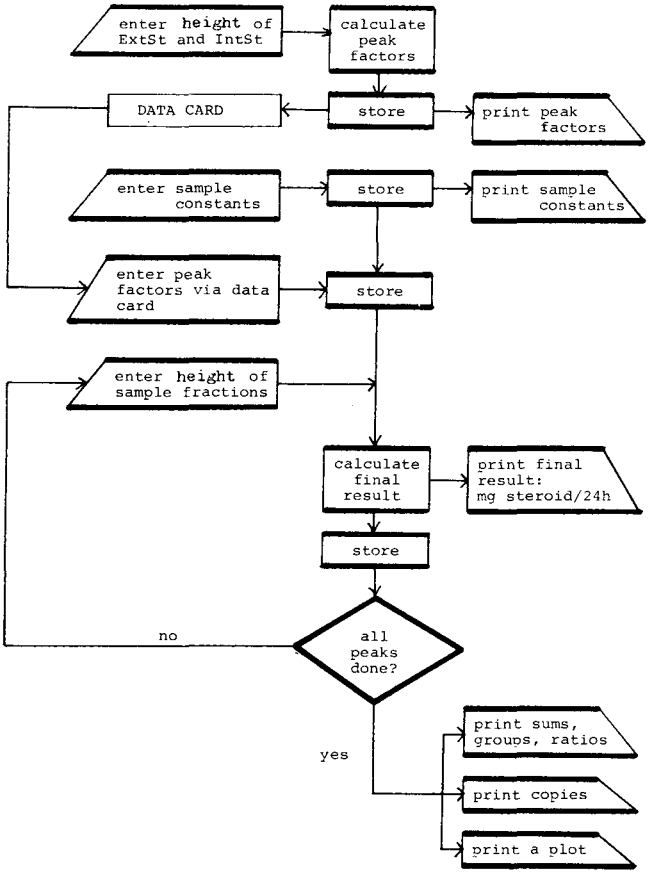

Fig. 1. Simplified flow chart representing the whole program (for abbreviations, see text).

tion. This calculator (cost less than US$ 1000) offers a 224-step memory and 25 data registers. The total program, which consists of 27 subprograms, is divided into 3 parts, each of which can be stored on a magnetic card (I, II and III). A simplified flow diagram is shown in Fig. 1 and the subprograms are listed in Table I.

Mathematics

1. The peak area (a) of a steroid fraction (S) is the product of peak height (h) and the width at half-height (w):

$$a_S = h_S w_S$$

2. The peak factor (f) of a steroid fraction contained in an external standard (ExtSt) mixture is

$$f_S = \frac{(hw)_{IntSt^*}}{(hw)_S}$$

*Internal standard within the external standard mixture. Equal amounts of each steroid fraction were injected (in our method, 1 μg).

TABLE I

LIST OF ALL SUBPROGRAMS

Letters represent those programs which are directly addressable by the operator via the corresponding keystroke; numbers stand for non-directly addressable subroutines.

Card	Label	Line	Comment
I	a	001—008	Clear all registers
	b	003—008	Clear secondary registers only
	A	009—022	Enter sample constants manually and peak factors via data card
	7	012—016	Waiting subroutine
	B	023—054	Enter sample constants (manually)
	2	025—046	Print peak factors
	4	047—054	Space formatting (4 times)
	5	049—054	Space formatting (3 times)
	1	055—098	Enter sample constants, store and print
	C	098—127	Enter *h* of steroids in external standard mixture to calculate and store on data card peak factors
	6	128—137	Print *h* of ExtSt, calculate peak factor
	D	138—166	Enter peak number, *h* of sample peaks, calculate amount excreted, sum up
	d	167—170	Space formatting (2 times)
	e	171—175	Clear sum register
	E	176—182	Print sum of steroids excreted as evaluated under subprogram D of card I
II	A	001—056	Enter *h* of all 10 sample peaks, store amount of fraction excreted in separate register
	8	057—085	Print peak number, calculate amount of steroid excreted, print
	4	086—101	Print peak number of fraction being recalled
	6	092—101	Calculate percent of steroid fraction of total ketosteroids and pregnanes
	1	102—108	Space formatting (4 times)
	3	109—181	Print total sum of steroids evaluated, sum of androgens, of 11-substituted steroids and of pregnanes; AE/A ratio, P3/P2 ratio; print percentages of fractions and groups
	5	159—181	Subroutine for register recalling
	2	182—188	Calculate percentages of fractions and groups
	B	189—200	Print copy for patient's report
	9	197—209	Subroutine for register recalling
III	C	001—035	Enter lowest and highest value of fraction excreted
	0	036—054	Plot excretion profile

3. The final amount of a steroid fraction excreted per day (mg_S/24-h urine) is:

$$\frac{(hw)_S \cdot f_S \cdot \text{aliquot factor}^{\star} \cdot 100^{\star\star}}{(hw)_{\text{IntSt}}^{\star\star\star} \cdot \dfrac{\text{recovery (\%)}}{100} \cdot 1000^{\S}}$$

*Aliquot factor = total 24-h urine volume divided by the extracted urine volume.

**If a 1/100 aliquot of total extract volume taken up in the final volume is injected (in our method generally 1/10 of total 24-h urine is extracted and, after silylation, taken up in 200 μl, of which 2 μl are injected).

***Internal standard within the unknown sample; as in the external standard, 1 μg is injected.

§ To convert μg into mg.

4. As a simplification, the product *hw* in this program is replaced by *h* alone in eqns. 2 and 3.

Algorithms

For recalling 10 different data register contents in a desired printout format, a special (program steps saving) algorithm was developed: by use of the *i*-register ("*i*" for *i*ndirect register addressing) a considerable reduction of program space was achieved. This iterative subroutine is shown in Fig. 2. The plotting algorithm has been proposed by other workers recently [9].

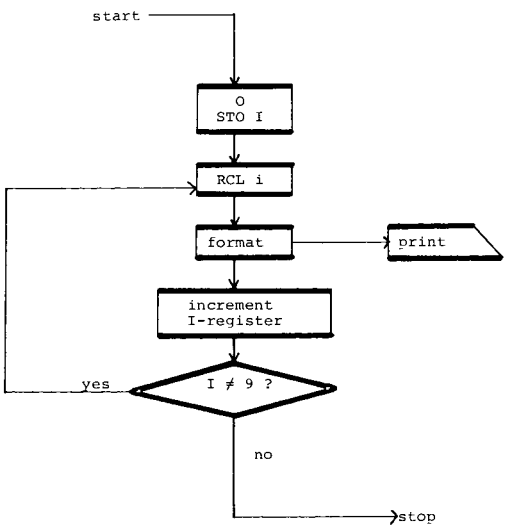

Fig. 2. Flow chart for an algorithm for recalling (RCL) primary registers No. 0 to No. 9, which contain in this program the peak factors 1—10.

PROGRAM OPERATION

The program allows the performance of the following operations in GLC data reduction.

1. Calculation of peak factors for ten different fractions from an ExtSt mixture run. After manual input of *h* of IntSt and all fractions within the ExtSt, peak factors are automatically calculated, transcribed to the magnetic data card for storage and finally printed out (see upper part of Fig. 1).
2. Calculation of selected sample fractions.
   2.1. Sample constants (24-h urine volume, extracted urine volume, radioactivity of added tracer, radioactivity recovered, *h* of IntSt within the sample) are entered manually. The previously determined peak factors are then entered via the data card and subsequently printed out for control purposes.
   2.2. The number of the peak to be evaluated (which corresponds to the order of appearance at the detector) is entered, followed by input of *h* for that peak. These data are automatically converted into the final result (mg steroid/24-h urine), printed out and summed for subsequently recalling the total sum of steroids analysed (for comparison with a

colorimetric group determination of 17-ketosteroids, e.g., the Zimmermann [10] reaction).

3. Calculation of all ten steroid fractions. Part II of the program provides an alternating order of "communication", where the calculator prints the peak number and the operator enters *h*, resulting in output of the final concentration and the number of the following peak. This sequence continues for up

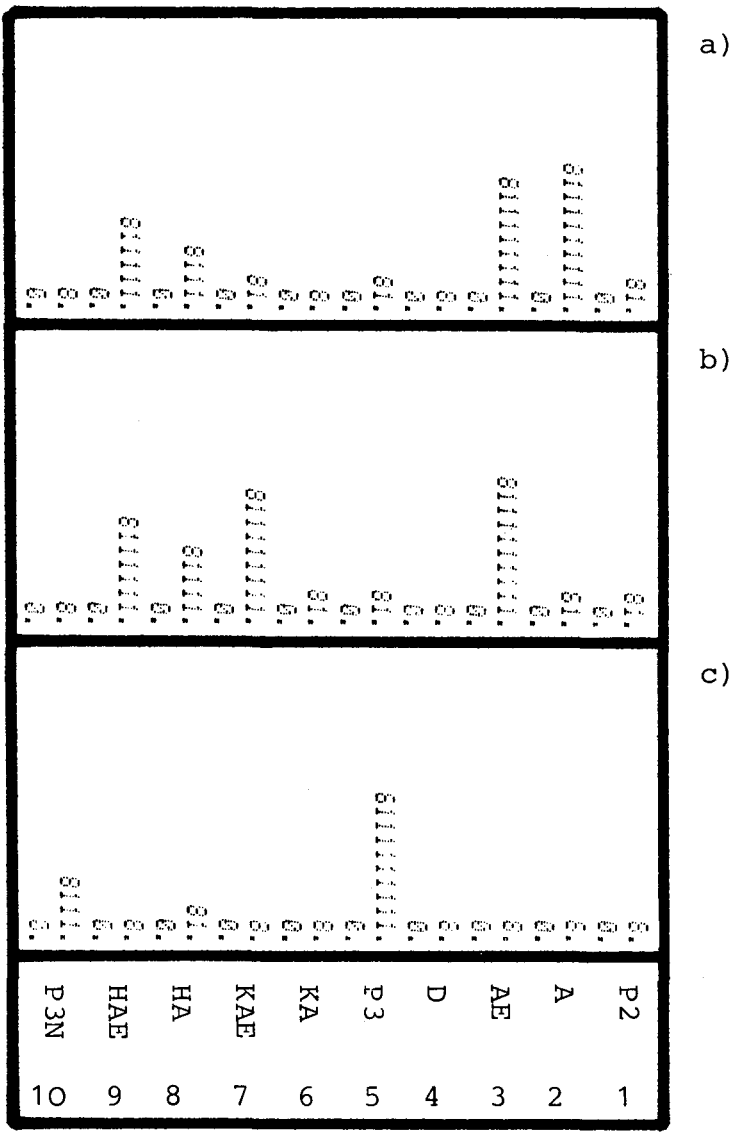

Fig. 3. Simplified computer plot to depict a steroid excretion pattern. The difference between the highest and the lowest absolute fraction value is divided into nine equal parts, the fractions being printed as multiples thereof. (a) Normal profile of a healthy 25-year-old woman in follicular phase; (b) adrenal hyperplasia due to ectopic ACTH syndrome of an adult man; (c) patient with 21-hydroxylase deficiency before treatment with dexamethasone (7-year-old girl).

to ten peaks and terminates with the printout of the following parameters:
total sum of 17-ketosteroids and pregnanes;
group sums of androgens (A, AE and D);
of 11-substituted steroids (KA, KAE, HA and HAE);
of pregnanes (P2, P3 and P3N);
the ratios of AE/A and of P3/P2; and
the percentages of all ten fractions and groups.
Further, a single keystroke produces a copy of the results to attach to the patient's report and any number of further copies thereof. Finally, a summarizing plot of the excretion pattern can be obtained by a single keystroke, which brings Part III of this program into operation (Fig. 3).

A step-by-step description of the program operation together with a listing of the program steps is given in the Appendix.

DISCUSSION

Hormone analyses require specialized methods (e.g., saturation analysis, radioimmunoassay, GLC, high-performance liquid chromatography) and, consequently, a specialized mathematical treatment of the raw data. As most endocrinological laboratories do not have extensive computer facilities, we have attempted to facilitate data reduction for a low-cost desk-top calculator (Hewlett-Packard 97) for a variety of problems, such as Scatchard plot analysis, calculation of kinetic and thermodynamic parameters of macromolecule—ligand interactions, radioimmunoassay evaluation and quality control [11—13].

The mathematics of the program presented here are based on the assumption that the signal recorded is linearly proportional to the amount of steroid injected. The range for which this is valid must be determined in each laboratory before applying this evaluation method. Otherwise, calibration graphs are necessary, from which unknown peak heights can be interpolated. Measurement of peak heights and widths at half-height is generally done visually with the aid of a magnifying lens. Provided that the peaks are approximately symmetrical, the equation for the area of a triangle (product of h and w) can be used to calculate the peak area. Alternatively, the peak height alone may be taken to relate the signal with the amount of analyte. The latter possibility is simple and straightforward and, provided the peaks are well separated from each other, very accurate. It was therefore implemented in our program, making it applicable to capillary column GLC analysis.

The automatic calculation of group sums, percentages and ratios should allow for a more efficient, clinically oriented interpretation of steroid excretion profiles. Analysis of literature data [8] and also data obtained in our laboratory suggests that, for example, the AE/A ratio is approximately 1 throughout life except in early childhood. Similarly, the P3/P2 ratio is close to 1, typical deviations occurring only during the luteal phase and pregnancy (low ratio) and in patients with 21-hydroxylase deficiency (high ratio). Androgens (A, AE and D) and 11-substituted steroids (KA, KAE, HA, HAE) generally represent together approximately 80% of the total ketosteroids and pregnanes, thus giving K : P = 4 : 1. Inverse relationships may be found in adrenogenital syndrome where, additionally, a high P3/P2 ratio and the presence of P3ON (pregnanetriolone)

is pathognomonic for 21-hydroxylase deficiency, whereas low levels or the absence of 11-substituted steroids are indicative of the 11β-type enzyme deficiency. High levels of androgens can be observed in some patients with idiopathic hirsutism [14], sometimes associated with an atypically high absolute or percentage value for dehydroepiandrosterone.

Hence, the calculation subprogram described here produces a number of diagnostically valuable parameters, facilitating interpretation and allowing immediate recognition of abnormal or pathological levels in a gas–liquid chromatogram of fractionated 17-ketosteroids and pregnanes. Further, this program could easily be modified for a variety of other chromatographic problems. After 2 years' experience, we are able to present this program as a realistic alternative to an expensive chromatographic profile integrator module.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Tyrolean Raiffeisenkasse, 1978. The authors thank the Head of the Institute, Prof.Dr. G. Wick, for his continuous encouragement and support.

APPENDIX

Description of program operation

General

(a) Switch the calculator into operation mode "PRGM" (for program editing). Key in the program steps of Part I, as listed in Table V. After having entered all steps, insert an empty magnetic card for permanent storage of the program. Clip the card on both edges to protect of erasing. Parts II and III are programmed in the same way.
(b) Switch the calculator into operation mode "RUN" (for program operation).

1. Calculation of peak factors

1.1. Use card I, calculator being in the "RUN" position. Press /a/ for initializing (= for clearing all register contents).
1.2. Enter height (h) of IntSt in external standard mixture, press /C/.
1.3. Enter h of $ExtSt_1$, press /R/S/.
1.4. Repeat step 1.3 for $ExtSt_{2-10}$.
1.5. After having entered h of $ExtSt_{10}$ and having pressed /R/S/, "Crd" will appear on the display. Following the insertion of an empty magnetic data card reserved for that purpose only, peak factors will be permanently stored on this data carrier. An automatic printout of peak factors thus calculated terminates the program.
1.6. Attach outprint as proposed in Table II.

2. Calculation of selected sample steroid fractions

2.1. When starting the calculations, repeat step 1.1.
2.2.1. Enter 24-h urine volume (ml), press /A/.
2.2.2. Enter extracted urine volume (ml), press /R/S/.
2.2.3. Enter total counts of recovery tracer added, press /R/S/.
2.2.4. Enter counts of recovery tracer found, press /R/S/.
2.2.5. Enter *h* of IntSt in sample, press /R/S/.
2.2.6. Press /R/S/ and immediately insert data card (holding the previously determined peak factors). After a short pause (effected by a special waiting routine within the program), the data card will automatically pass the reading station; 9.000000000 will appear in the display for a few seconds and then the printout of peak factors will signal to the operator the end of this program.
2.3. For calculation of a further sample on the basis of the same peak factors, step 2.2.6 can be omitted and step 2.2.1 is changed by pressing /B/ instead of /A/. In this case, peak factors will be printed out for control automatically without inserting the data card.
2.4. Attach printout.
2.5.1. Enter number of the peak to be evaluated (the number corresponds to the order of appearance at the detection unit as assessed by the ExtSt mixture), press /D/.
2.5.2. Enter *h* of this peak, press /R/S/: mg steroid/24-h urine will be printed out.
2.6. Repeat steps 2.5.1 and 2.5.2 for all further peaks; press /d/ once when omitting one peak (correspondingly, press /d/ twice when leaving out 2 peaks).
2.7. If a sum of all peaks calculated is desired, press /E/.
2.8. For clearing this sum register, press /e/.

TABLE II

PROTOCOL AND PRINTOUT FOR CALCULATING PEAK FACTORS FROM AN EXTERNAL STANDARD MIXTURE GLC RUN, AND PROTOCOL OF SAMPLE CONSTANTS

Sample constants: 24-h urine volume, 900 ml; incubated volume, 100 ml; recovery, 65% (total cpm added, 10.000; cpm recovered, 6.500); internal standard *h*, 23.0

Peak No.	Fractionated 17-ketosteroids and pregnanes		Height (mm)	Peak factor
Internal standard		—	37.5	—
1	P2	Pregnanediol	182.0	0.206
2	A	Androsterone	189.0	0.198
3	AE	Aetiocholanolone	143.5	0.261
4	D	Dehydroepiandrosterone	211.0	0.178
5	P3	Pregnanetriol	106.0	0.354
6	KA	11-Ketoandrosterone	83.0	0.452
7	KAE	11-Ketoaetiocholanolone	82.0	0.457
8	HA	11-Hydroxyandrosterone	59.0	0.636
9	HAE	11-Hydroxyaetiocholanolone	51.5	0.728
10	P3ON	Pregnanetriolone	43.1	0.870

TABLE III

PROTOCOL AND PRINTOUT OF SAMPLE FRACTIONS, TOGETHER WITH SUMS, PERCENTAGES OF FRACTIONS AND OF GROUPS, AND RATIOS APPLYING THE SAMPLE CONSTANTS AND PEAK FACTORS OF TABLE II

Peak No.	Sample peak	Peak height (mm)	mg/24 h	%
1	P2	22	0.273	3.5
2	A	202	2.413	31.3
3	AE	152	2.391	31.1
4	D	2	0.021	0.3
5	P3	19	0.405	5.3
6	KA	4	0.109	1.4
7	KAE	11	0.303	3.9
8	HA	18	0.689	8.9
9	HAE	25	1.096	14.2
10	P3ON	0	0.000	0.0
Total sum of K and P			7.70	
Androgens			4.83	62.7
11-Substituted K			2.20	28.5
Pregnanes			0.68	8.8
AE/A ratio			0.99	
P3/P2 ratio			1.48	

TABLE IV

PATIENT'S REPORT PRINTOUT AS CALCULATED IN THE PREVIOUS PROGRAM SECTION (SEE TABLE III)

Peak No.	Component	mg/24 h
1	P2	0.273
2	A	2.413
3	AE	2.391
4	D	0.021
5	P3	0.405
6	KA	0.109
7	KAE	0.303
8	HA	0.689
9	HAE	1.096
10	P3ON	0.000

3. Calculation of all ten steroid fractions

3.1. Instead of doing step 2.5.1, insert program card II and press /A/; number "1" will be printed out to ask for h of the 1st peak.

3.2. Enter h_1, press /R/S/: result in mg steroid/24-h urine will be printed out together with number "2".

3.3. Enter h_2 and so on for all ten peaks.

3.4. This sequence will, after the printout of the last peak's result, automatically produce the following further outputs: total sum, groups sums and ratios. Cut off printout and press /R/S/: the percentage of all fractions and groups will be printed out. Cut off printout and attach to protocol sheet as proposed in Table III.

TABLE V

INDIVIDUAL PROGRAM STEPS OF PARTS I AND III

Card I

001	*LBLa	060	PRTX	119	STO8	178	RCLE
002	CLRG	061	STOB	120	R/S	179	DSP2
003	*LBLb	062	RCLA	121	GSB6	180	PRTX
004	P≠S	063	RCLB	122	STO9	181	GSB4
005	CLRG	064	÷	123	SPC	182	RTN
006	P≠S	065	STOA	124	SPC		
007	CLX	066	SPC	125	WDTA		
008	RTN	067	R/S	126	GSB2		
009	*LBLA	068	STOB	127	RTN		
010	GSB1	069	R/S	128	*LBL6		
011	R/S	070	RCLB	129	DSP1		
012	5	071	÷	130	STOI		
013	STOI	072	EEX	131	PRTX		
014	*LBL7	073	2	132	RCLA		
015	DSZI	074	x	133	RCLI		
016	GTO7	075	DSP1	134	÷		
017	9	076	PRTX	135	DSP3		
018	STOI	077	STOB	136	RTN		
019	MRG	078	SPC	137	*LBLD		
020	PSE	079	R/S	138	DSPØ		
021	GSB2	080	PRTX	139	PRTX		
022	RTN	081	STOC	140	1		
023	*LBLB	082	SPC	141	-		
024	GSB1	083	SPC	142	STOI		
025	*LBL2	084	CLX	143	R/S		
026	DSP3	085	DSP9	144	DSP1		
027	RCLØ	086	RTN	145	STOD		
028	PRTX	087	*LBLC	146	RCLC		
029	RCL1	088	DSP1	147	÷		
030	PRTX	089	PRTX	148	RCLi		
031	RCL2	090	STOA	149	x		
032	PRTX	091	SPC	150	RCLA		
033	RCL3	092	SPC	151	x		
034	PRTX	093	R/S	152	RCLB		
035	RCL4	094	GSB6	153	EEX		
036	PRTX	095	STOØ	154	1		
037	RCL5	096	R/S	155	÷		
038	PRTX	097	GSB6	156	÷		
039	RCL6	098	STO1	157	DSP2		
040	PRTX	099	R/S	158	PRTX		
041	RCL7	100	GSB6	159	STOI		
042	PRTX	101	STO2	160	RCLE		
043	RCL8	102	R/S	161	RCLI		
044	PRTX	103	GSB6	162	+		
045	RCL9	104	STO3	163	STOE		
046	PRTX	105	R/S	164	RCLI		
047	*LBL4	106	GSB6	165	DSP1		
048	SPC	107	STO4	166	RTN		
049	*LBL5	108	R/S	167	*LBLd		
050	SPC	109	GSB6	168	SPC		
051	SPC	110	STO5	169	SPC		
052	SPC	111	R/S	170	RTN		
053	CLX	112	GSB6	171	*LBLe		
054	RTN	113	STO6	172	Ø		
055	*LBL1	114	R/S	173	STOE		
056	DSPØ	115	GSB6	174	RCLE		
057	PRTX	116	STO7	175	RTN		
058	STOA	117	R/S	176	*LBLE		
059	R/S	118	GSB6	177	GSB5		

Card III

001	*LBLC
002	STOA
003	X≠Y
004	-
005	9
006	÷
007	STOB
008	1
009	Ø
010	STOI
011	RCLi
012	GSBØ
013	*LBL7
014	ISZI
015	RCLi
016	GSBØ
017	1
018	9
019	ENT↑
020	RCLI
021	X≠Y?
022	GTO7
023	Ø
024	STOA
025	STOB
026	STOC
027	STOD
028	STOE
029	SPC
030	SPC
031	SPC
032	SPC
033	CLX
034	DSP9
035	RTN
036	*LBLØ
037	RCLA
038	-
039	DSPØ
040	RCLB
041	÷
042	9
043	+
044	RND
045	$1Ø^x$
046	9
047	1/X
048	8
049	+
050	x
051	PRTX
052	Ø
053	PRTX
054	RTN

TABLE VI

INDIVIDUAL PROGRAM STEPS OF PART II

Card II

001	*LBLA	060	1	119	RCL2	178	GSB2
002	Ø	061	-	120	+	179	PRTX
003	STOE	062	STOI	121	RCL3	180	GSB1
004	1	063	R/S	122	+	181	RTN
005	GSB8	064	DSP1	123	STOA	182	*LBL2
006	P≠S	065	STOD	124	PRTX	183	RCLE
007	STOØ	066	RCLC	125	RCL5	184	÷
008	P≠S	067	+	126	RCL6	185	EEX
009	2	068	RCLi	127	+	186	2
010	GSB8	069	x	128	RCL7	187	x
011	P≠S	070	RCLA	129	+	188	RTN
012	STO1	071	x	130	RCL8	189	*LBLB
013	P≠S	072	RCLB	131	+	190	1
014	3	073	EEX	132	STOB	191	Ø
015	GSB8	074	1	133	PRTX	192	STOI
016	P≠S	075	÷	134	RCLØ	193	SPC
017	STO2	076	+	135	RCL4	194	RCLi
018	P≠S	077	DSP3	136	+	195	DSP3
019	4	078	PRTX	137	RCL9	196	PRTX
020	GSB8	079	STOI	138	+	197	*LBL9
021	P≠S	080	RCLE	139	STOC	198	ISZI
022	STO3	081	RCLI	140	PRTX	199	SPC
023	P≠S	082	+	141	SPC	200	RCLi
024	5	083	STOE	142	RCL2	201	PRTX
025	GSB8	084	RCLI	143	RCL1	202	1
026	P≠S	085	RTN	144	+	203	9
027	STO4	086	*LBL4	145	DSP2	204	ENT↑
028	P≠S	087	RCLI	146	PRTX	205	RCLI
029	6	088	9	147	SPC	206	X≠Y?
030	GSB8	089	-	148	RCL4	207	GTO9
031	P≠S	090	DSPØ	149	RCLØ	208	GSB1
032	STO5	091	PRTX	150	P=S	209	RTN
033	P≠S	092	*LBL6	151	÷		
034	7	093	RCLi	152	PRTX		
035	GSB8	094	RCLE	153	GSB1		
036	P≠S	095	+	154	R/S		
037	STO6	096	EEX	155	1		
038	P≠S	097	2	156	Ø		
039	8	098	x	157	STOI		
040	GSB8	099	DSP1	158	GSB4		
041	P≠S	100	PRTX	159	*LBL5		
042	STO7	101	RTN	160	ISZI		
043	P≠S	102	*LBL1	161	GSB4		
044	9	103	SPC	162	1		
045	GSB8	104	SPC	163	9		
046	P≠S	105	SPC	164	ENT↑		
047	STO8	106	SPC	165	RCLI		
048	P≠S	107	CLX	166	X≠Y?		
049	1	108	RTN	167	GTO5		
050	Ø	109	*LBL3	168	GSB1		
051	GSB8	110	SPC	169	SPC		
052	P≠S	111	SPC	170	RCLA		
053	STO9	112	SPC	171	GSB2		
054	P≠S	113	RCLE	172	DSP1		
055	GSB3	114	DSP2	173	PRTX		
056	RTN	115	PRTX	174	RCLB		
057	*LBL8	116	SPC	175	GSB2		
058	DSPØ	117	P≠S	176	PRTX		
059	PRTX	118	RCL1	177	RCLC		

3.5. If a copy of the result is desired, press /B/. Repeat this step if necessary.
3.6. If a plot of the excretion pattern is desired, insert program card III, enter the lowest value (mg/24 h), press /ENTER↑/, enter highest one, press /C/ and the plot will be automatically generated.

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Journal of Chromatography, 223 (1981) 267—275
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 827

A NEW METHOD FOR DETECTION OF DRUG-BINDING PROTEINS USING A PARALLEL-FLOW DIALYSIS TECHNIQUE★

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(Received November 20th, 1980)

SUMMARY

A parallel-flow dialysis technique utilizing a Technicon dialyzer and a constant-flow system has been described for the detection of drug-binding proteins. The effect of temperature, flow-rate and drug concentration was investigated by measuring the efficiency of dialysis and detecting the binding of methyl orange to bovine serum albumin. The larger response was shown to be achieved by increasing the efficiency of dialysis or the drug concentration. The present method will enable the continuous monitoring of drug-binding proteins.

INTRODUCTION

Drug binding to various blood proteins and tissue proteins affects the pharmacological activities and blood distribution of drugs. Albumin is the major drug-binding protein in the blood plasma. Binding of albumin to various drugs has been studied by equilibrium dialysis, ultrafiltration, and many other methods [1—4]. Proteins binding organic anions have been found in the 110,000 *g* supernatant of liver homogenate by Levi et al. [5]. They were named Y-protein and Z-protein according to the elution pattern of the organic-anion—protein complex from a Sephadex G-75 column. Some other unknown drug-binding proteins are expected to be detected by a similar chromatography of the drug—tissue homogenate. However, the method is inappropriate when the drug—protein complex dissociates in the column.

To search for unknown drug-binding proteins by column chromatography, detection must be done after elution from the column. However, application of the traditional equilibrium dialysis or ultrafiltration methods to chromatographic fractions is time-consuming and laborious. In such cases, a post-

★This work was presented at the 100th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1980.

column flow detection system is desirable. This paper deals with a parallel-flow dialysis technique which is applicable to the post-column detection of drug-binding proteins.

Concept of the present method

The principle of "parallel-flow dialysis" (PFD) is shown schematically in Fig. 1. Two flow channels in parallel on a dialysis membrane are utilized. The column effluent is directed into one channel, "protein channel", and the drug solution of fixed concentration is directed into the other channel, "drug channel", at the same flow-rate. The concentration of drug in the drug channel will decrease when a drug-binding protein appears in the protein channel. Thus by continuously monitoring the drug concentration at the exit of the drug channel, drug-binding protein can be detected as a decrease in the concentration. The degree of this change may depend upon the concentration of the drug—protein complex and the dialysis rate of the drug.

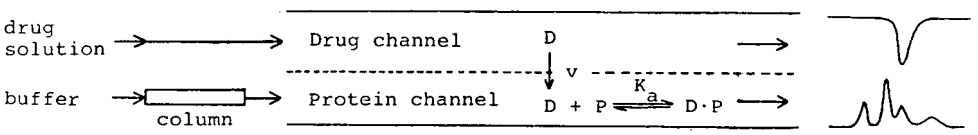

Fig. 1. The principle of the parallel flow dialysis. D = drug; P = protein; D·P = drug—protein complex; K_a = affinity constant; v = dialysis rate.

EXPERIMENTAL

Materials

Crystallized and lyophilized bovine serum albumin (BSA, Product No. A-4378) was purchased from Sigma (St. Louis, MO, U.S.A.). Phenobarbital (PB, Iwaki Seiyaku, Tokyo, Japan), methyl orange (MO), bromphenol blue (BPB, Kanto Chemicals, Tokyo, Japan), phenol red (PR, Tokyo Kasei, Tokyo, Japan) and eosin yellowish (EY, E. Merck, Darmstadt, G.F.R.) were used. Phosphate-buffered saline (PBS) (pH 7.4) was prepared by dissolving 4.03 g of sodium chloride in one liter of 50 m*M* NaH_2PO_4—K_2HPO_4 buffer (pH 7.5). For the dialysis a Technicon Autoanalyzer dialyzer (basic module) fitted with a cellulose membrane (Cuprophan membrane, Technicon Chemicals Co., Belgium) was used. The absorbance of the solutions was measured by a Uvidec-505 spectrophotometer (Jasco, Tokyo, Japan) at 254 nm.

Flow dialysis system

The flow diagram of the system is illustrated in Fig. 2. Continuous flow in the protein and drug channels was produced by two plunger-type pumps, KSD-16 and KHD-16 (Kyowa Seimitsu, Tokyo, Japan). In place of the column, a 240-μl loop injector (Toyo Soda, Tokyo, Japan) was used for in-stream injection of protein samples. The dialyzer and the buffer reservoir were placed in a water bath to maintain a constant temperature. Concentrations of BSA and drugs were monitored by measuring absorbance at 254 nm with a Uvigraph

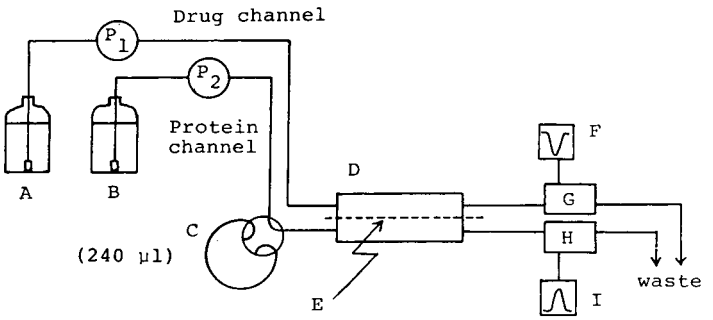

Fig. 2. A schematic diagram of the parallel-flow dialysis system. A = reservoir of PBS; B = reservoir of the drug solution in PBS; C = loop injector; D = dialyzer of the Technicon Autoanalyzer basic module; E = cellulose Cuprophan membrane; F = TOA EPR-100A recorder; G = LDC SF-1205 detector; H = Uvigraph LC-1 detector; I = Shimadzu R-101 recorder; P_1 = KHD-16 plunger pump; P_2 = KSD-16 plunger pump. Teflon tubes of outer diameter 2 mm and inner diameter 1 mm were used for connection with the partial aid of silicone tubes of outer diameter 3 mm and inner diameter 1 mm.

LC-1 monitor (Iatron Laboratories, Tokyo, Japan) and an LDC SF-1205 UV monitor (Atto Corporation, Tokyo, Japan), respectively.

Efficiency of dialysis

Efficiency of dialysis was adopted as an index for the dialysis rate of the drug from the drug channel into the protein channel. The effluents from both channels were collected under steady-state conditions. Then the concentration of the drug in the protein channel (D_p) and in the drug channel (D_d) was estimated by measuring the absorbance of each solution at 254 nm. Efficiency of dialysis was defined as

$$\frac{2D_p}{D_d + D_p} \times 100 \quad (\%)$$

Under this definition, the efficiency is 100% when the equilibrium is completed.

RESULTS

Response of the PFD system

In order to verify the concept of the PFD system, detection of the binding of MO to BSA was tested by the system shown in Fig. 2. A 100-μM MO solution was pumped through the drug channel at 0.35 ml/min while the solvent (PBS) was pumped through the protein channel at the same flow-rate. By maintaining these conditions a steady-state baseline was obtained in each channel. Then a BSA solution (10 mg/ml) was injected into the protein channel. The response in each channel is shown in Fig. 3. As expected, a transient decrease in the recorder response of the drug channel appeared as an inverse peak corresponding to the elution of BSA.

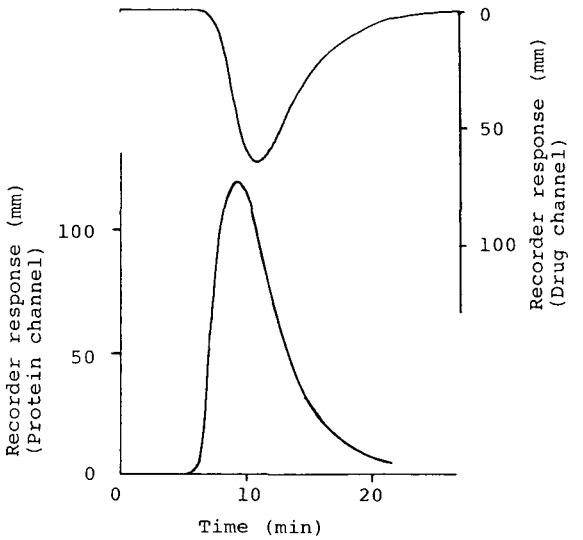

Fig. 3. The recorder responses of the system caused by injection of 2.4 mg of BSA. Recorder range of the protein channel was 0.64 a.u.f.s. (250 mm full scale) and that of the drug channel was 0.04 a.u.f.s. (150 mm full scale).

Factors affecting response

Various factors such as temperature, flow-rate and drug concentration affect the response of the drug channel. As shown in Fig. 4, the peak height of the response and the efficiency of dialysis increased with increasing temperature. However, when the temperature was higher than 32°C, the appearance of small bubbles in the dialyzer interfered with detection. Therefore, a temperature of 27°C was selected for further experiments. As shown in Fig. 5, increasing the flow-rate resulted in a decrease in peak height and ef-

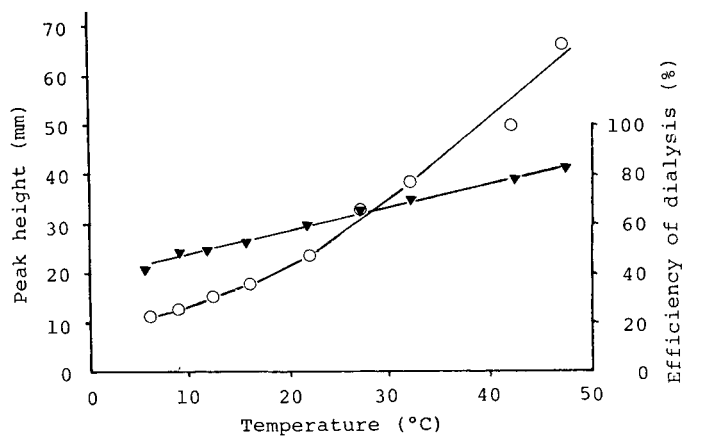

Fig. 4. Effects of the temperature of the dialyzer on the response of the drug channel (○) and on the efficiency of dialysis (▼). A 100-μM MO solution was passed through the drug channel at a flow-rate of 0.35 ml/min. A 240-μl volume BSA solution (5 mg/ml) was injected through the loop injector.

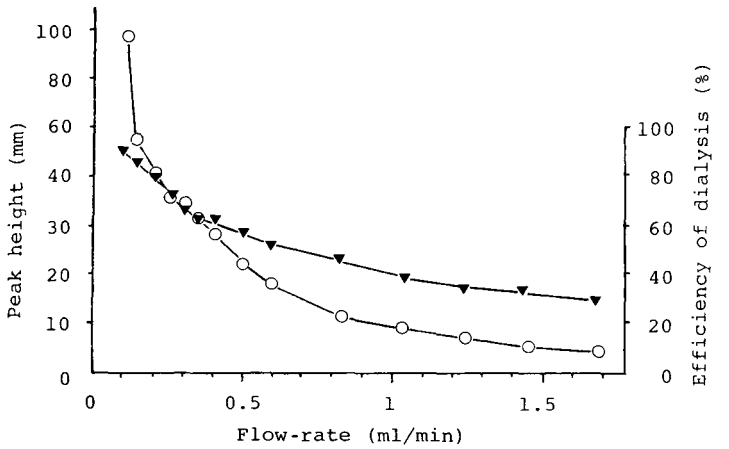

Fig. 5. Effects of the flow-rate on the response of the drug channel (○) and on the efficiency of dialysis (▼). A 100-μM MO solution was passed through the drug channel, the temperature of the dialyzer being kept constant at 27°C. A 240-μl volume of BSA solution (5 mg/ml) was injected through the loop injector.

ficiency of dialysis. In the concentration range $2 \cdot 10^{-5}$ M to $2 \cdot 10^{-4}$ M of MO solution applied to the drug channel, the peak height of the drug channel was almost proportional to MO concentration (Fig. 6) even if the efficiency of dialysis was constant.

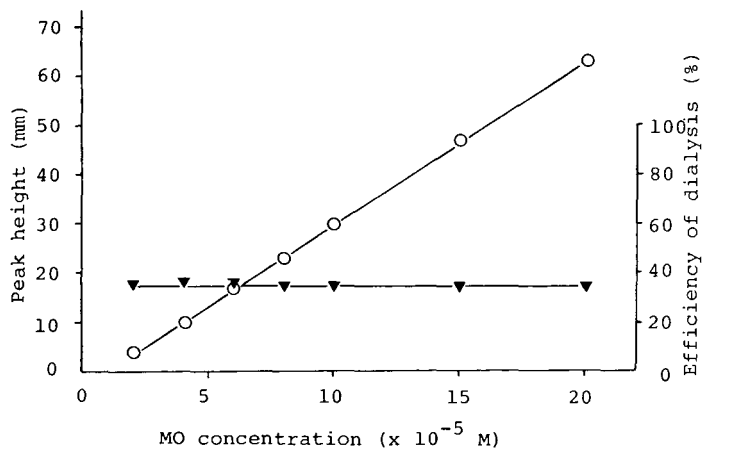

Fig. 6. Effects of MO concentration on the response of the drug channel (○) and on the efficiency of dialysis (▼). Various concentrations of MO were applied to the drug channel. The temperature of the dialyzer was 27°C and the flow-rate was 0.35 ml/min. A 240-μl volume of BSA solution (5 mg/ml) was injected through the loop injector.

Application of various compounds to PFD

A further experiment was planned to clarify the limit and scope of the PFD system by using various compounds of different affinity constants to BSA (Table I). In order to compare the results of different compounds, the peak height of the drug channel was converted to absorbance change by using

TABLE I

AFFINITY CONSTANTS OF VARIOUS COMPOUNDS TO BSA

Compound	n_1	K_1	n_2	K_2	Method	Reference
Phenobarbital	1	2500	—	—	Dynamic dialysis	6
Methyl orange	22	2080	—	—	Dynamic dialysis	7
Phenol red	1	174,000	6	1970	Dynamic dialysis	7
Bromphenol blue	5	242,000	—	—	Equilibrium dialysis	8
Eosine yellowish	3.9	5,620,000	5.8	231,000	Ultracentrifugation	9

working curves for the protein channel (Fig. 7) and the drug channel (Fig. 8). Zero adjustment of the protein channel recorder was made against solutions of 0.21 and 0.43 absorbance, which represent the actual experimental conditions. Also the drug channel detector response was studied by introducing solutions of 0.34 and 1.16 absorbance into the reference cell. The ratio of

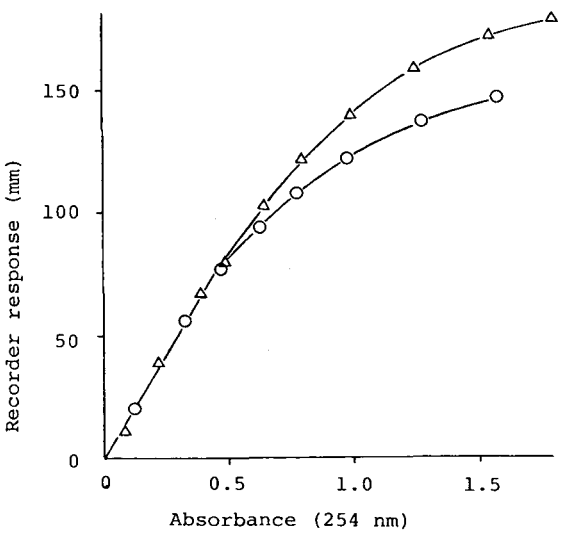

Fig. 7. Working curves of the Uvigraph LC-1 detector (protein channel). Recorder range was set at 0.64 a.u.f.s. (250 mm full scale). Zero adjustment was made against MO solutions with absorbances of 0.21 (△) and 0.43 (○) at 254 nm.

the response to the absorbance change was constant under 1.5 absorbance by the LDC SF-1205 detector. For experimental convenience, the concentrations of the five compounds were chosen so that their absorbance at 254 nm was below 1.5. From the working curves of the drug channel (Fig. 8) and the molar extinction coefficient, the concentration change corresponding to a 100-mm recorder response was calculated (Table II). The relative change of the concentration in the drug channel was used to represent the response of the drug channel. This was obtained by first converting the peak height to the concentration change (ΔD_d) using the conversion coefficient in Table

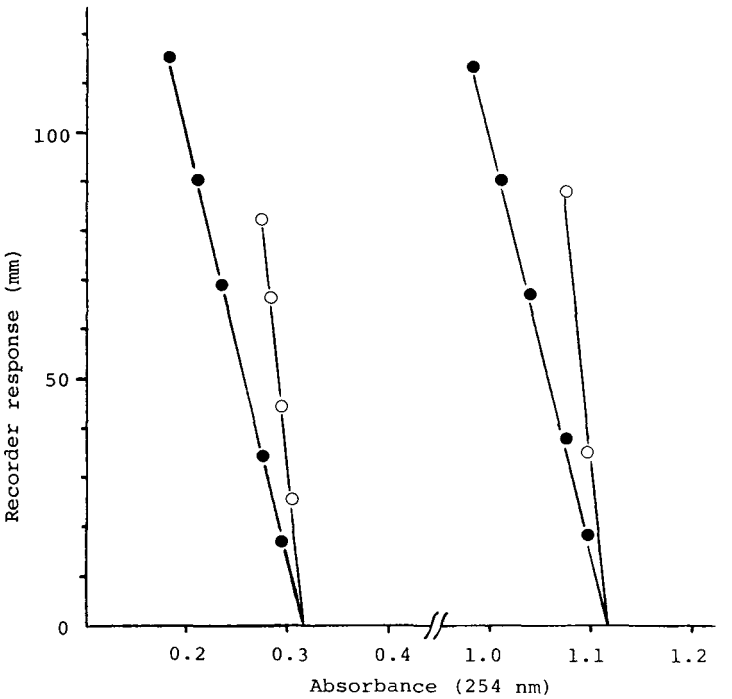

Fig. 8. Working curves of the LDC SF-1205 detector (drug channel). Recorder range was 0.04 a.u.f.s. (•) and 0.01 a.u.f.s. (○) at 150 mm full scale. Zero adjustment was made against a PR solution with an absorbance of 0.34 at 254 nm or an EY solution of absorbance 1.16 at 254 nm.

TABLE II

RELATION OF CONCENTRATION, ABSORBANCE OF DRUG AND RECORDER RESPONSE

Compound	ϵ (254 nm)	Conc. (M)	Absorbance (254 nm)	Conc. corresponding to a 100-mm recorder response (M)
Phenobarbital	$2.6 \cdot 10^3$	$5 \cdot 10^{-4}$	1.3	$2.0 \cdot 10^{-5}$
Methyl orange	$7.7 \cdot 10^2$	$1 \cdot 10^{-4}$	0.76	$7.0 \cdot 10^{-6}$
Phenol red	$8.1 \cdot 10^3$	$1 \cdot 10^{-4}$	0.81	$6.7 \cdot 10^{-6}$
Bromphenol blue	$6.4 \cdot 10^3$	$1 \cdot 10^{-4}$	0.64	$8.4 \cdot 10^{-6}$
Eosin yellowish	$3.0 \cdot 10^4$	$2 \cdot 10^{-5}$	0.59	$1.8 \cdot 10^{-6}$

II, and then dividing by the steady-state concentration (D_d) in the drug channel. The relative change was defined as

$$\frac{\Delta D_d}{D_d} \times 100 \quad (\%)$$

As shown in Fig. 9, the responses of PB, PR and MO were in proportion to

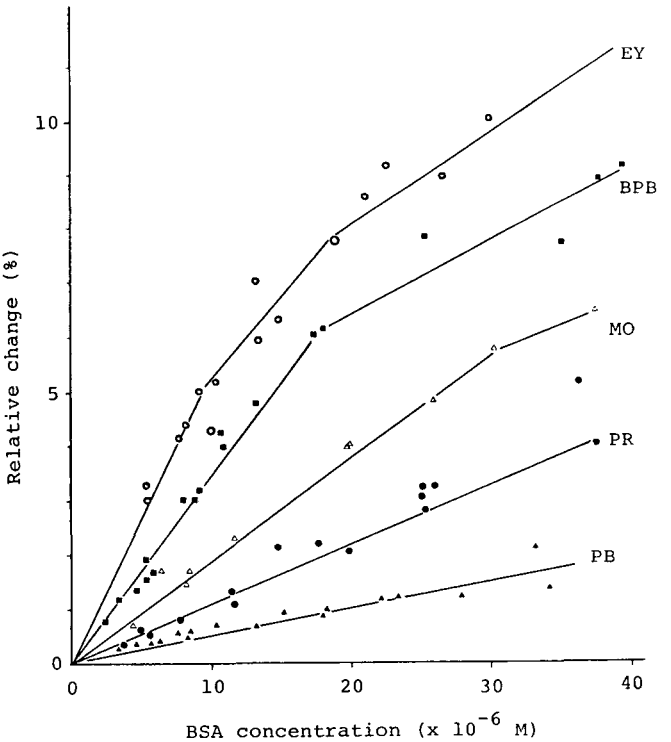

Fig. 9. Correlation between relative change in drug concentration and the concentration of BSA. The temperature of the dialyzer was 27°C and the flow-rate was 0.35 ml/min.

BSA concentration up to $30 \cdot 10^{-6}$ *M*, while BPB and EY gave saturation curves. At the same concentration of BSA, compounds with higher affinity constants tended to show a higher relative change. The efficiency of dialysis for each compound was: PB, 66%; PR, 52%; MO, 60%; BPB, 44%; and EY, 48%.

DISCUSSION

A PFD technique was developed by which binding of BSA to five compounds was detected. In this system, a larger response can be obtained with higher dialysis efficiency (higher temperature or lower flow-rate). In the case of MO, the efficiency of dialysis reaches 90% by 22 minutes of residence in the dialyzer at 27°C (Fig. 5), indicating effective dialysis of the drug in the present co-current flow system compared to a static equilibrium dialysis system. The use of a higher drug concentration in the PFD system also resulted in a larger response. However, the detector response lost linearity at higher concentrations of drug that gave an absorbance of more than 1.5 at 254 nm with the LDC SF-1205 detector. To overcome this defect, the use of a variable-wavelength detector is recommended.

As illustrated in Fig. 9, the response of PB, PR and MO were almost proportional to the concentration of BSA. On the other hand, the responses show a curved line in the cases of EY and BPB which have strong affinity to

BSA. This phenomenon suggests that the concentration of the compound to BSA is saturated in the protein channel. Furthermore, Fig. 9 conversely suggests that proteins with larger affinity constants to the drug show a larger response. Thus, the PFD system will be useful for the continuous detection of unknown drug-binding proteins in a column effluent. The following paper [10] will describe the detection of drug-binding proteins of human serum or rat liver homogenate in column effluent.

ACKNOWLEDGEMENT

The authors thank Dr. Kojiro Matsumoto for kindly providing the dialyzer.

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Journal of Chromatography, 223 (1981) 277—284
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 806

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE LECITHIN/SPHINGOMYELIN RATIO IN AMNIOTIC FLUID

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(Received October 28th, 1980)

SUMMARY

A high-performance liquid chromatographic (HPLC) procedure has been developed for the separation of phospholipids commonly found in amniotic fluid. The chromatographic separation was achieved on a 25-cm column packed with LiChrosorb DIOL (10 μm). A 3-cm column packed with silica was fitted between the injector and the DIOL column to provide complete separation of lecithin (L) and sphingomyelin (S) from the remaining amniotic fluid phospholipids. The eluted phospholipids were quantitated employing an ultraviolet absorption detector set at 203 nm. The new HPLC separation described herein has improved the resolution and peak sharpness of L and S. Furthermore, phosphatidyl glycerol and phosphatidyl inositol were completely separated and quantitated. Amniotic fluid L/S ratios determined by this technique have been compared to those of an established thin-layer chromatographic procedure.

INTRODUCTION

Since Gluck et al. [1] first introduced the thin-layer chromatographic (TLC) separation of lecithin (L) and sphingomyelin (S) in amniotic fluid, the L/S ratio procedure has been widely used and modified for the evaluation of fetal lung maturity [2—4].

In addition to L and S there is a group of minor phospholipids in amniotic fluid which include phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), and lysolecithin (LL) [5]. Recently, several groups of researchers have recognized the importance of these minor phospholipids, particularly PG and PI. Analyses of these phospholipids in amniotic fluid have been accepted to be of value as additional indices of fetal lung maturity [5—9].

In an attempt to improve upon the conventional TLC procedures, separations have been carried out on TLC rods precoated with silica gel, followed by quantitation using a hydrogen flame ionization detector [10]. Phospholipids have also been separated by high-performance liquid chromatography (HPLC) [11—13]. Though HPLC separations have the potential to provide more accurate and precise quantitations of L and S as a result of advances made in column technology, pumping systems and detectors, to date procedures have not been reported for the separation of the minor amniotic fluid phospholipids.

In this paper all the amniotic fluid phospholipids have been separated by HPLC and quantitated by ultraviolet absorption. A preliminary patient study has been conducted to correlate the L/S ratio in amniotic fluid determined by this technique with that of an established TLC procedure.

MATERIALS AND METHODS

Apparatus

The HPLC separations were carried out on a Hewlett-Packard (Avondale, PA, U.S.A.) Model 1084B high-performance liquid chromatograph equipped with an automatic sampler and a variable-wavelength detector (190—600 nm). The chromatographic column was 25 cm × 4.6 mm I.D. from Hewlett-Packard. The column was packed with LiChrosorb DIOL (10 μm) supplied by Merck (Darmstadt, G.F.R.). A Brownlee (Santa Clara, CA, U.S.A.) MPLC guard column, 3 cm × 4.6 mm I.D. packed with 10 μm silica (SI 60, Merck), was fitted between the injector and the DIOL analytical column. The thin-layer chromatograms were scanned by reflectance densitometry on a Joyce Loebl (Team Valley, Great Britain) Chromoscan 200 equipped with a Scan 201 TLC scanning accessory.

Reagents and samples

Water used in the HPLC solvent was distilled in the presence of $KMnO_4$ in an all glass still, and stored in a borosilicate glass bottle. HPLC grade acetonitrile, glass-distilled chloroform and Merck silica gel 60H were purchased from BDH Chemicals (Vancouver, Canada). Certified ACS grades of ammonium sulfate, methanol, and acetone were purchased from Fisher Scientific (Winnipeg, Canada). The following phospholipids were obtained from Sigma (St. Louis, MO, U.S.A.): lysolecithin (Type I, egg yolk), sphingomyelin (bovine brain), lecithin (Type III-E, egg yolk), phosphatidyl ethanolamine (Type III, egg yolk), phosphatidyl serine (bovine brain), phosphatidyl inositol (Grade III, soybean) and phosphatidyl glycerol (Grade I, egg yolk). Commercially prepared lecithin/sphingomyelin standard solutions were also obtained from Sigma.

Amniotic fluid samples were collected by amniocentesis.

Chromatographic analysis

The chromatographic mobile phase was composed of Solvent A: acetonitrile—water (80:20, v/v) and Solvent B: acetonitrile. Both solvents were maintained at 40°C. A linear solvent gradient was run from 87.5 to 25.0% B between 4.5 and 11.0 min which produced a linear gradient of water running from 2.5 to 15.0%. The flow-rate was constant at 2.0 ml/min and the column oven temperature was 35°C. The column effluent was monitored at 203 nm [11] and the detector response was set to 0.0128 a.u./cm. Quantitation was by integration of peak areas using a software integrator.

Aliquots of a commercially prepared 1:1 L/S standard solution were diluted with chloroform—methanol (2:1, v/v) to prepare a series of standards containing L and S each at concentrations of 1.0, 0.75, 0.50, 0.25 and 0.125 mg/ml of solution.

Standard solutions of each of the following were prepared at similar concentrations: LL, PE, PS, PI and PG. Twenty μl of each standard solution were injected in triplicate and the areas of the peaks averaged to produce calibration curves for each of the phospholipids.

The TLC procedure used was that described by Gluck et al. [14]. Phospholipids were extracted from 5.0 ml of amniotic fluid. Following the cold acetone precipitation, the precipitate was dissolved in 30 μl of chloroform, of which 5—10 μl were applied to the TLC plate. An additional 40 μl of chloroform—methanol (2:1, v/v) was added to the sample and a 10—30-μl aliquot was injected into the HPLC instrument.

RESULTS AND DISCUSSION

A chromatogram illustrating the separation of a mixture of standard phospholipids is depicted in Fig. 1A. When PS was chromatographed alone, a broad tailing peak was observed. The tailing of the PS is the primary reason for the baseline deviation. The baseline was much improved when PS was not included in the standard mixture, as illustrated in Fig. 1B.

In the preliminary studies, separations were performed using only a DIOL column. Sphingomyelin appeared as a split peak in the majority of chromatograms. Similar splitting of the S peak has been shown for procedures employing a silica column [11—13]. However, the primary difficulty with the preliminary separation was the appearance of PE between L and S. PE was often difficult to distinguish from S. Inclusion of a short 3-cm silica column in tandem with the DIOL column, resulted in PE eluting prior to L and S. Furthermore, this improved the resolution between the peaks and S was no longer observed to split. The elution order of the remaining 6 phospholipids in the standard mixture was unchanged. When a 25-cm silica column was connected in series with the DIOL column in place of the shorter 3-cm column, however, the separation was unsatisfactory with most of the phospholipids appearing as broad split peaks.

Solvent A was composed of acetonitrile—water (80:20, v/v) in order that the solvent delivery system would accurately deliver a relatively shallow solvent gradient running from 2.5 to 15.0% water.

Calibration curves for L and S are illustrated in Fig. 2. Linearity was ob-

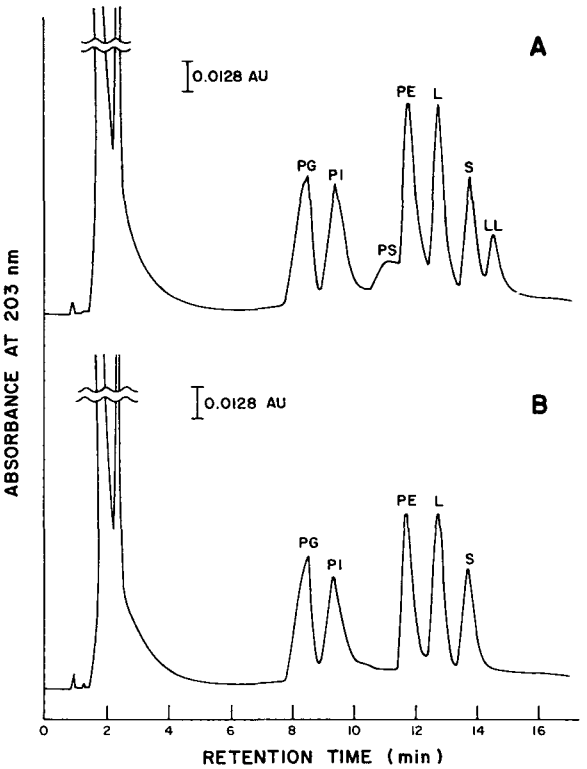

Fig. 1. (A) High-performance liquid chromatogram of a mixture of 7 standard phospholipid samples each at a concentration of 5 μg. Peaks: PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PS = phosphatidyl serine; PE = phosphatidyl ethanolamine; L = lecithin; S = sphingomyelin; LL = lysolecithin. (B) High-performance liquid chromatogram of the standard phospholipids mixture excluding PS and LL.

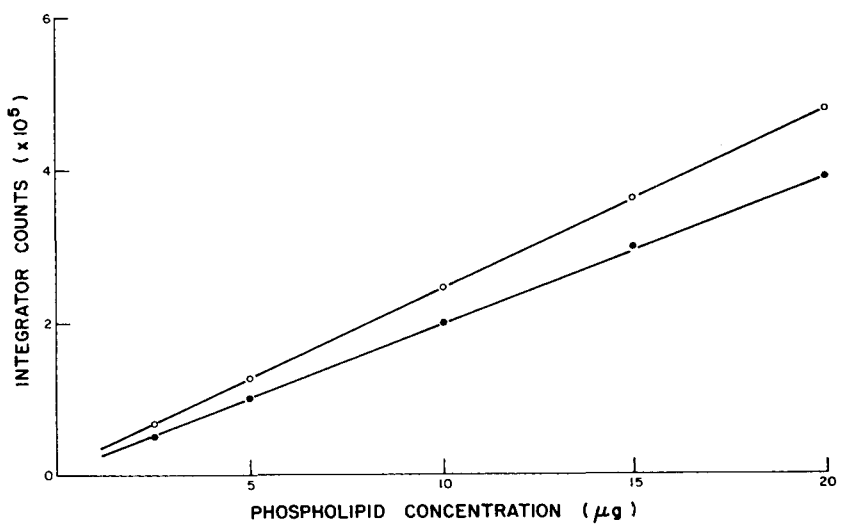

Fig. 2. Standard calibration curves for lecithin (○) and sphingomyelin (●).

served between 2.5 and 20.0 μg, which was the range of values expected for L and S in the majority of amniotic fluid samples as performed by this procedure. Of note, the linearity of the calibration curve for L was observed to extend to at least 100 μg. The calibration curves for each of the minor amniotic fluid phospholipids are depicted in Fig. 3. The linearity of these curves

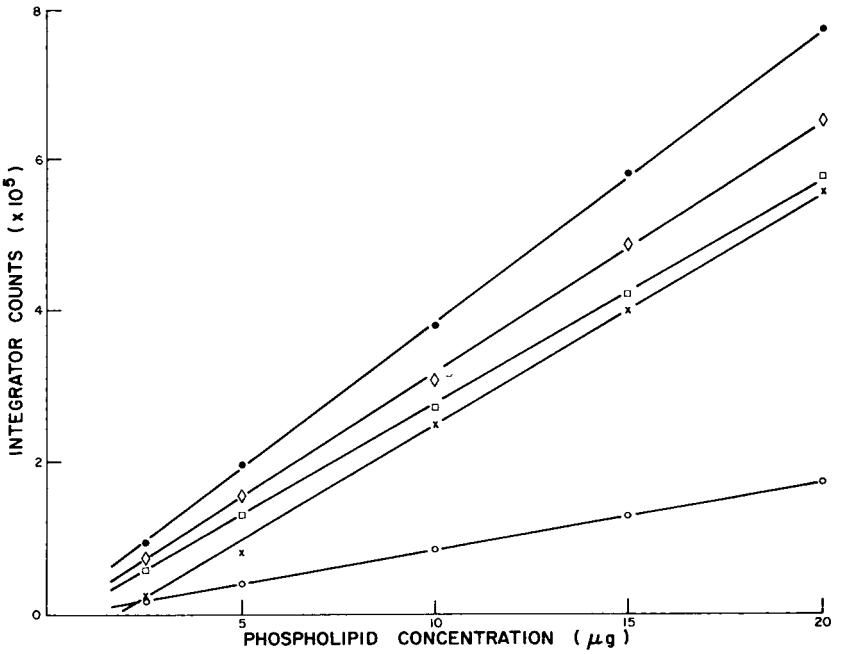

Fig. 3. Standard calibration curves for phosphatidyl glycerol (•), phosphatidyl ethanolamine (◇), phosphatidyl inositol (□), phosphatidyl serine (×) and lysolecithin (○).

was also well maintained between 2.5 and 20.0 μg. Each point on all of the phospholipid standard curves was the mean of 3 measurements, each with a coefficient of variation of less than 2.5%, with the exception of the 2.5- and 5.0-μg points on the PS standard curve which had coefficients of variation of 17.6% and 6.7%, respectively. The linear regression correlation coefficients for each of the phospholipid calibration curves was better than 0.999. The non-zero intercepts of the curves may be attributed to peak tailing. This is especially pronounced for the PS calibration curve which exhibited the greatest peak tailing.

Representative chromatographic separations of phospholipids from amniotic fluid specimens collected before and after fetal lung maturation are depicted in Figs. 4 and 5, respectively. Fifty amniotic fluid samples were analyzed by a conventional TLC procedure [14] and by the new HPLC method. The correlation between these two methods of analysis is illustrated in Fig. 6. A commercially prepared standard solution with an L/S ratio of 3.0 was tested repeatedly throughout the study to monitor the precision of the L/S ratio determination. For a total of 14 test results, the mean and coefficient of variation were 2.93 and 3.74%, respectively. The same commercial standard was also tested 14 times in succession, which produced a mean value of 2.79 and a coefficient of variation of 1.0%.

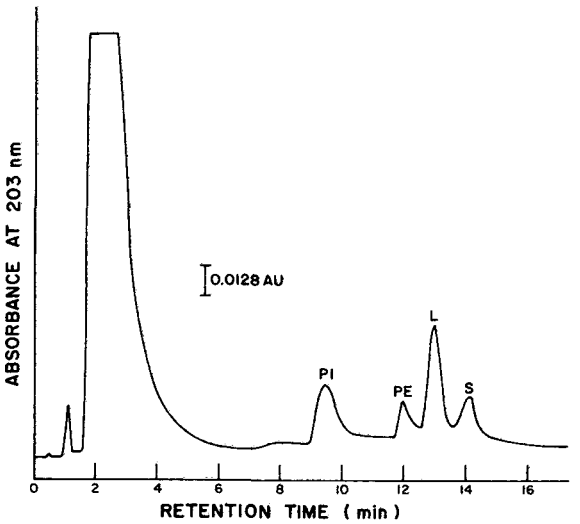

Fig. 4. High-performance liquid chromatogram of an amniotic fluid sample taken before fetal lung maturity.

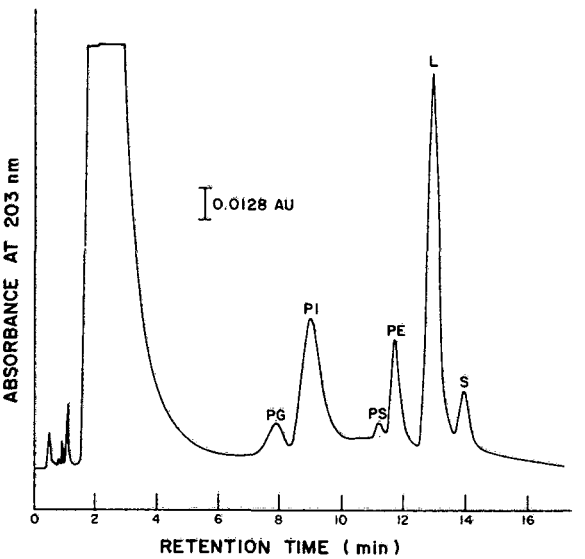

Fig. 5. High-performance liquid chromatogram of an amniotic fluid sample taken after fetal lung maturity.

Gluck et al. [15] have stated that "An L/S ratio of 4.0 by the gravimetric technique corresponds to one of 2.0 by reflectance densitometry, representing maturity of the lung". From the preliminary patient study described herein, employing only 50 patient samples, it would appear that an L/S ratio of 3.0 as determined by HPLC would correspond most closely to an L/S ratio of 2.0 by TLC and reflectance densitometry. To establish a reliable range of values for the interpretation of fetal lung maturity, a larger number of amniotic fluid

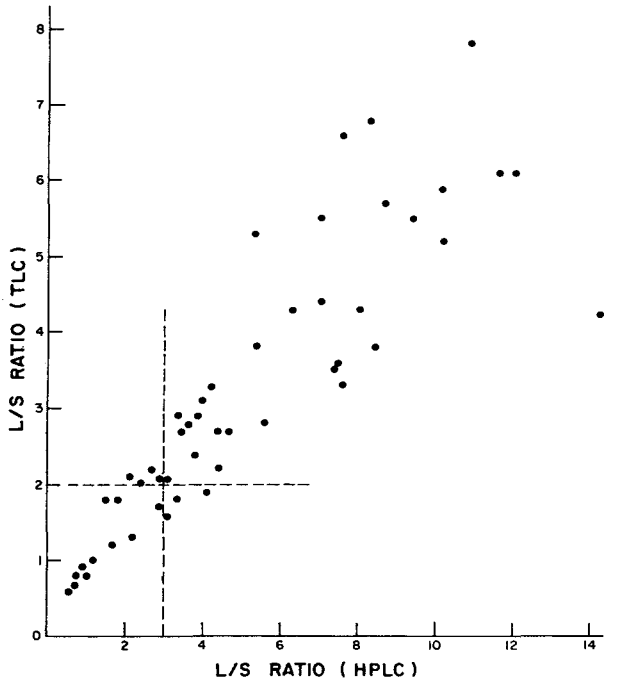

Fig. 6. Correlation of amniotic fluid L/S ratio results established by TLC and HPLC analyses.

specimens should be collected at parturition, analyzed, and the results carefully correlated with fetal lung maturity.

Jungalwala et al. [11] have shown that phospholipids containing unsaturated fatty acids gave much greater ultraviolet absorption at 203 nm than phospholipids with saturated fatty acids. These researchers suggested that if the degree of unsaturation in the phospholipids varied, quantitation by direct ultraviolet absorption would not be accurate, and that an alternative detection system would be required. However, Geurts van Kessel et al. [13] stated that the ultraviolet absorption was due not only to the presence of unsaturated centres, but also due to functional groups such as carbonyl, carboxyl, phosphate, amino, and quaternary ammonium. They went on to state, that if there were a variety of fatty acid constituents, there would be problems with quantitation by direct ultraviolet absorption. Several researchers [16—19] have determined the fatty acid composition of L. Though differences have been reported, a correlation coefficient of 0.860 was obtained by linear regression analysis between the HPLC method employing ultraviolet absorption detection and the TLC procedure using charring and reflectance densitometry (Fig. 6).

The presence and relative concentration of PG and PI in amniotic fluid have been reported to be of clinical significance for the evaluation of fetal lung maturity [5—9]. False positive L/S ratio results have been reported in pregnancies with complications, e.g. diabetes, placenta previa, and maternal fever [7,8,20]. In these circumstances analysis of the minor amniotic fluid

phospholipids is believed to be of particular importance. It has been suggested that a lung phospholipid profile would be valuable and possibly essential in institutions caring for patients with high risk pregnancies [21].

The present HPLC separation provides not only an improved separation and precise quantitation of L and S, but also the possibility of a complete lung phospholipid profile.

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Journal of Chromatography, 223 (1981) 285—293
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 804

HIGH-PERFORMANCE AQUEOUS GEL PERMEATION CHROMATOGRAPHY OF SERUM LIPOPROTEINS: SELECTIVE DETECTION OF CHOLESTEROL BY ENZYMATIC REACTION

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(First received September 30th, 1980; revised manuscript received December 2nd, 1980)

SUMMARY

A rapid method for the quantitation of cholesterol in each lipoprotein fraction has been developed which utilizes high-performance aqueous gel permeation chromatography followed by enzymatic reaction using reaction-type high-performance chromatography.

Cholesterol in serum lipoproteins eluted from the column could be sensitively and selectively detected by the absorbance at 550 nm following the enzymatic reaction. The sensitivity of the detection for cholesterol measured by A_{550} was compared with that for protein measured by A_{280} using the standard lipoprotein fractions: low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). The effects of changing the flow-rate and lengthening the column on the resolution of LDL and HDL were examined. Analyses of serum protein and cholesterol were performed with this method for human and animal subjects.

INTRODUCTION

A new application of high-performance gel permeation chromatography (GPC) for analysis of human serum lipoproteins has been developed using a combination of TSK GEL columns [1, 2]. In a previous paper [1] the best combination of the column for the separation of human serum lipoproteins and the effects of pH or salt concentration of the eluent on their separation were examined by monitoring the peaks of lipoproteins by A_{280} using the total lipoprotein mixture prepared by ultracentrifugation.

The protein levels in each lipoprotein fraction could be analyzed by monitoring A_{280} with the total lipoprotein fraction ($d < 1.210$) prepared from

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individual sera by ultracentrifugation [1]. On the other hand, the cholesterol in each lipoprotein fraction could be directly measured by the enzymatic reaction after separation by high-performance GPC with whole serum [3, 4]. The study of the optimum conditions for the enzymatic reaction given in the flow diagram (Fig. 1) using the reaction-type high speed liquid chromatograph (HLC 805, Toyo Soda Manufacturing Co., Tokyo, Japan) will be reported in another paper [5].

In this paper, the sensitivity of the detection for cholesterol by A_{550} was compared with that for protein by A_{280} with use of the standard lipoprotein fractions: low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). The effects of changing the flow-rate and lengthening the column on the resolution of LDL and HDL subfractions were examined. The elution patterns of protein and cholesterol for individual subjects including human and animal sera were examined using this flow system.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography (HPLC) was carried out using the high-speed chemical derivatization chromatograph (HLC 805, Toyo Soda) equipped with a reactor (20 m × 0.25 mm I.D. stainless-steel tube) and detectors for A_{280} and A_{550} as shown in Fig. 1.

Ultracentrifugation for the separation of the standard lipoprotein fractions from human serum was performed using an RP55 rotor in an Hitachi 55 P-2 ultracentrifuge (Hitachi, Tokyo, Japan).

Materials and methods

Samples. Human sera used in this experiment were obtained from normal men and women or patients with various diseases after 12—16 h of fasting. Standard lipoprotein fractions for analysis by high-performance GPC were prepared from the sera by the sequential flotation method [6]. HDL_2 was isolated from serum as the d = 1.063—1.125 fraction. The d > 1.063 fraction obtained after centrifugation at 105,000 g for 24 h in the RP55 rotor was recentrifuged under the same conditions. After density adjustment to 1.125 with solid sodium bromide and 24 h of centrifugation at 105,000 g, the top fraction was recentrifuged at the same density. HDL_3 and LDL were prepared as the d = 1.125—1.210 and the d = 1.006—1.063 fractions, respectively, in the same way.

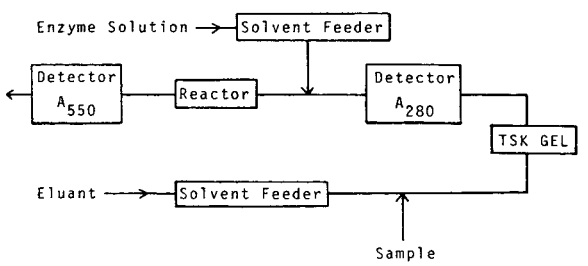

Fig. 1. Flow diagram for the enzymatic detection of cholesterol by high-performance GPC.

Reagents. The concentration of total cholesterol in the samples subjected to high-performance GPC or in the fraction separated by high-performance GPC was enzymatically determined using commercially available reagent kits (Determiner TC"555", Kyowa Hakko Co., Tokyo, Japan). The reagent was supplied in premixed lyophilized vials, together with buffer solution. When reconstituted with (per vial) 80 ml of 0.025 *M* potassium hydrogen phthalate buffer (pH 6.0) containing detergents the individual components were present in the following concentrations: cholesterol esterase, 1 unit/ml; cholesterol oxidase, 1.8 units/ml; peroxidase, 6.7 units/ml; 4-aminoantipyrine, 1 m*M*; N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine, 0.3 m*M*. The reconstituted reagent has to be used within three days.

Separation of lipoproteins by high-performance GPC. The separation of lipoproteins was performed by HPLC with gel permeation columns (TSK GEL, G5000PW and G3000SW; Toyo Soda). Experimental conditions in this study were as follows. Columns: G5000PW + G3000SW (size of each column, 600 mm × 7.5 mm), G5000PW + G3000SW + G3000SW, G5000PW + G3000SW + G3000SW + G3000SW. Eluent, 0.15 *M* NaCl; flow-rate, 0.46—1.06 ml/min.

Detection of protein and cholesterol. The protein peaks were directly monitored by A_{280}. Cholesterol can be determined by measuring the A_{550} of the post-column effluent using the Determiner TC"555" kit after measurement of the A_{280}. The absorbance at 550 nm of the mixed eluate and enzyme solution (TC"555") was monitored after passage through the reactor (20 m × 0.25 mm I.D.) at 40°C using a high-speed chemical derivatization chromatograph (see Fig. 1).

RESULTS AND DISCUSSION

Recently, highly sensitive and selective methods have been developed for the quantification of cholesterol by the enzymatic reaction [7—10]. With these methods, the quantification of cholesterol in a very small amount of serum (10 μl) can be precisely performed in an aqueous system. In this experiment, the detection of cholesterol in the eluate from the column was achieved using the commercial kit (Determiner TC"555") with the following reaction schemes.

$$\text{cholesterol ester} + H_2O \xrightarrow[\text{cholesterol esterase}]{} \text{free cholesterol} + \text{fatty acid}$$

$$\text{free cholesterol} + O_2 \xrightarrow[\text{cholesterol oxidase}]{} \text{choleste-4-en-3-one} + H_2O_2$$

$$\text{4-aminoantipyrine} + \text{N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine} + 2H_2O_2 \xrightarrow[\text{peroxidase}]{} \text{quinone diimine dye } (\lambda_{max.}\ 555\ \text{nm}) + 4H_2O$$

Using this reagent, cholesterol can be measured by the absorbance at 550 nm of the quinone diimine dye which is produced by the enzymatic reaction.

The absorbance at 550 nm of the mixed eluate and enzyme solution (TC''555'') was determined after passage through the reactor at constant temperature. The optimum conditions for cholesterol measurement used in this experiment are as follows: temperature of the reactor, 40°C; dimensions of the reactor, 20 m × 0.25 mm I.D.; flow-rate of the main path (i.e. the pathway of the eluate from the column), 1.0 ml/min; flow-rate of the enzyme solution (TC''555''), 0.35 ml/min. Under these conditions, the concentration of the enzyme solution in the reaction solution and the reaction time were experimentally confirmed to be sufficient for completion of the reaction [5].

The elution profiles of cholesterol and protein are shown in Fig. 2—4 for the standard lipoprotein fractions (LDL, HDL_2 and HDL_3) which were prepared by the repeated ultracentrifugation described in Experimental.

The sensitivity of cholesterol detection by measuring A_{550} was compared with that for protein by A_{280} using the standard lipoprotein fractions, LDL, HDL_2 and HDL_3. The elution curves were monitored by A_{280} and A_{550} by applying various amounts of each lipoprotein fraction to the GPC apparatus. With LDL fraction, the sensitivity for cholesterol was fifteen times higher than that for protein, as shown in Fig. 2. For example, LDL fraction containing 2.6 μg of cholesterol could be detected by A_{550} but not by A_{280} (Fig. 2d). The detection limit for cholesterol was found to be 0.5 μg per one sepa-

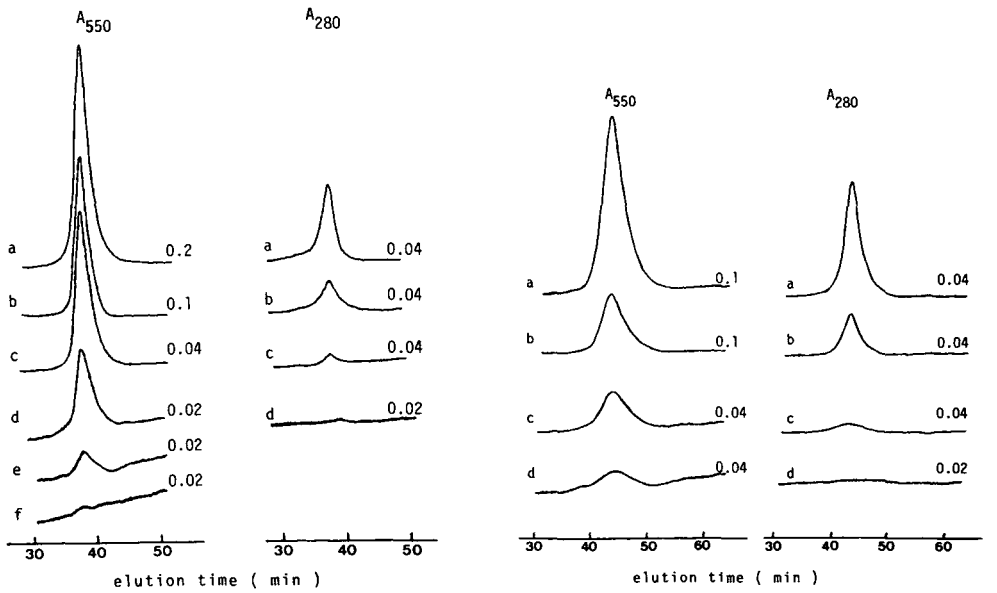

Fig. 2. Elution patterns of cholesterol (A_{550}) and protein (A_{280}) for LDL fraction. Column, G5000PW + G3000SW + G3000SW; eluent, 0.15 *M* NaCl; flow-rate, 1.0 ml/min (main path), 0.35 ml/min (enzyme solution); temperature of the reactor, 40°C; sample, LDL fraction (d = 1.006—1.063). Cholesterol contents of loaded samples: a, 27.0 μg; b, 10.8 μg; c, 4.9 μg; d, 2.6 μg; e, 1.0 μg; f, 0.5 μg. Numbers on the curves are the ranges of absorbance in the detector (10 mV).

Fig. 3. Elution patterns of cholesterol (A_{550}) and protein (A_{280}) for HDL_2 fraction. Sample, HDL_2 fraction (d = 1.063—1.125). Cholesterol contents of loaded samples: a, 23.5 μg; b, 9.0 μg; c, 2.5 μg; d, 1.5 μg. Conditions as in Fig. 2.

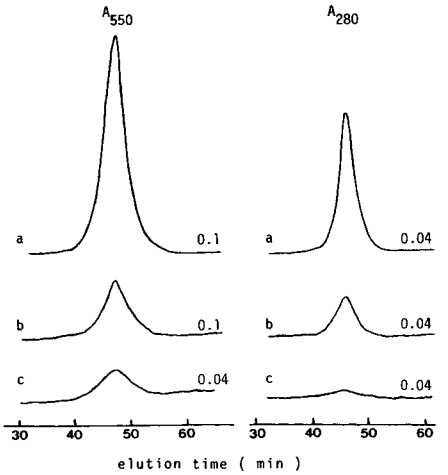

Fig. 4. Elution patterns of cholesterol (A_{550}) and protein (A_{280}) for HDL_3 fraction. Sample, HDL_3 fraction (d = 1.125—1.210). Cholesterol contents of loaded samples: a, 32.0 μg; b, 9.6 μg; c, 3.0 μg. Conditions as in Fig. 2.

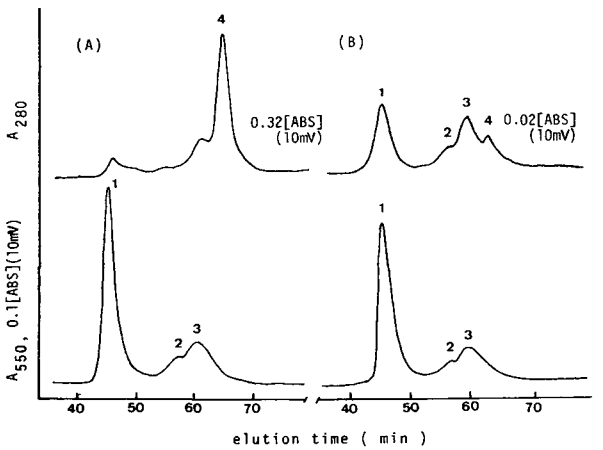

Fig. 5. Elution patterns of protein (A_{280}) and cholesterol (A_{550}) for human serum. Sample (normal male subjects): (A) 20 μl of whole serum; (B) 20 μl of total lipoprotein fraction (d < 1.210). Column: G5000PW + G3000SW + G3000SW + G3000SW. Peaks: 1, LDL; 2, HDL_2; 3, HDL_3; 4, albumin. Other conditions as in Fig. 2.

rated peak. Therefore, 25 μg of cholesterol per ml of serum can be detected by this method using 20 μl of serum for analysis. The respective sensitivities of detection for cholesterol were four times higher than that for protein in the case of HDL_2 (Fig. 3) and three times in the case of HDL_3 (Fig. 4). Therefore, even with HDL fractions, detection by A_{550} was superior to that by A_{280}.

The reproducibility of this method was ± 0.06 ml for the elution volume and ± 0.10 μg for cholesterol quantification [3].

The elution patterns of protein and cholesterol for whole serum and total lipoprotein fraction (d < 1.210) are shown in Fig. 5. In the case of the total

lipoprotein fraction (Fig. 5B), the peaks of lipoproteins can be monitored both by A_{280} and A_{550}. The elution curves of A_{280} for whole serum show that a large amount of serum proteins are eluted in the same fraction as the lipoproteins. Therefore, the protein level in each lipoprotein fraction can only be analyzed using the total lipoprotein fraction prepared from each individual serum by the ultracentrifugation as described in the previous paper [1]. On the other hand, the lipoprotein peaks can be monitored by the selective detection of cholesterol by A_{550} using whole serum. As presented in Fig. 5, the elution patterns of A_{550} with whole serum was not quite different from those with the total lipoprotein fraction. This suggests that the presence of serum proteins does not affect the separation of lipoproteins by GPC and the quantitation of cholesterol by the enzymatic reaction.

The effects of lengthening the column and reducing the flow-rate on the separation of lipoprotein by GPC were examined with use of whole serum (school children, female). Fig. 6 shows the elution patterns of protein and cholesterol by various combined column systems while keeping the flow-rate of the main path constant (1.0 ml/min). In the case of G5000PW + G3000SW, the LDL and HDL fractions could be separated but the HDL subfractions (HDL_2 and HDL_3) eluted as one peak. In the best working combined column system (G5000PW + G3000SW + G3000SW), the separation of LDL and HDL fractions was complete and the subfractions of HDL could be analyzed in less than 50 min. By adding one more G3000SW column to this system, the resolution of each lipoprotein fraction is improved, as shown in Fig. 6C.

It has been demonstrated in this study that the resolution of LDL, HDL_2 and HDL_3 can be improved by lengthening the G3000SW column. The effect of the flow-rate in the main path on the resolution of LDL and HDL_2 was examined for each column system using the same subjects as indicated in Fig. 6. The plots of the resolution values against the flow-rate are presented

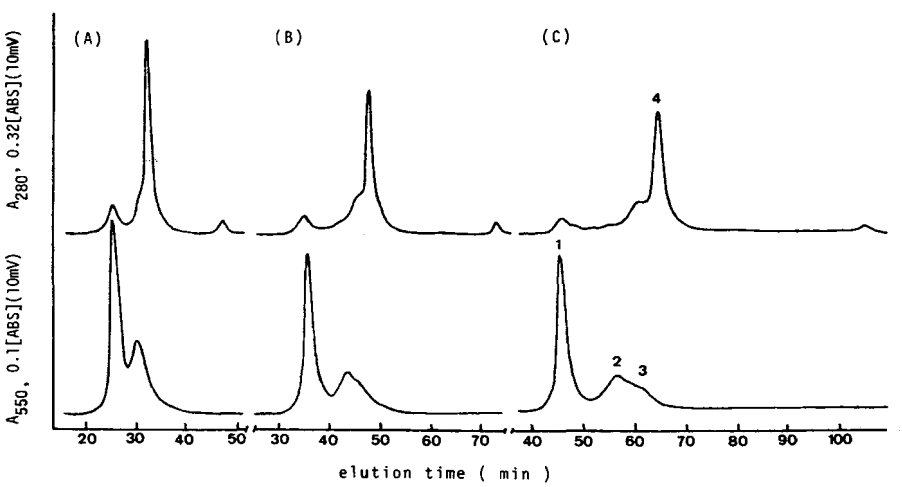

Fig. 6. Elution patterns of protein (A_{280}) and cholesterol (A_{550}) for human serum by various combined column systems. Sample: 20 μl of whole serum (school children, female). Columns: (A) G5000PW + G3000SW; (B) G5000PW + G3000SW + G3000SW; (C) G5000PW + G3000SW + G3000SW + G3000SW. Peaks as in Fig. 5. Other conditions as in Fig. 2.

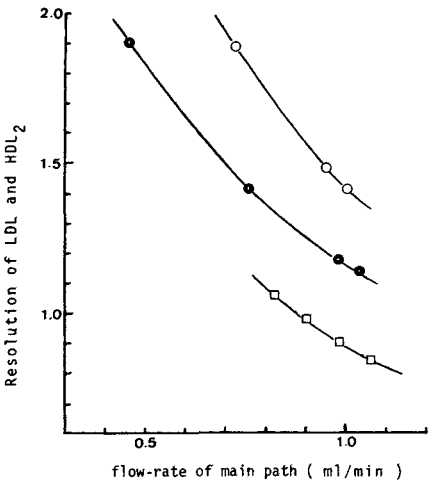

Fig. 7. Effects of flow-rate and lengthening of column on the resolution of LDL and HDL_2 fractions. Columns: (□), G5000PW + G3000SW; (•), G5000PW + G3000SW + G3000SW; (○), G5000PW + G3000SW + G3000SW + G3000SW. Sample as in Fig. 6. Other conditions as in Fig. 2.

in Fig. 7, from which it is clear that with the decrease in the flow-rate the resolution of LDL and HDL_2 increased significantly. Improvement in the separation can be achieved not only by lengthening the column but also by reducing the flow-rate of the main path. Until now the best conditions for analysis of lipoproteins, including the separation of the HDL subfractions, have been found to be: column system, G5000PW + G3000SW + G3000SW; flow-rate of main path, 1.0 ml/min.

Analyses of lipoproteins in individual human and animal sera were performed using the flow system (Fig. 1) under the optimum experimental conditions described above. In Fig. 8 typical elution patterns of protein and cholesterol are presented for three examples of human sera: a normal subject (young female, A) and two pathological cases (liver cirrhosis, B; and hyperlipidemia, C). Six distinct peaks of A_{550} are observed. Using standard lipoprotein fractions they were identified as: peak 1, VLDL; peak 3, LDL; peak 4, HDL_2; peak 5, HDL_3. Peak 2, observed between VLDL and LDL, and peak 6, seen after HDL_3, are assumed to be those of IDL (intermediate-density lipoprotein) and VHDL (very-high-density lipoprotein), respectively. Many serum protein peaks were observed. Two of them were identified as: peak 7, γ-globulin; peak 8, albumin. The levels of both serum protein and serum lipoprotein were found to vary with individual subjects. The pathological subjects in particular presented quite different patterns from those of normal subjects. In the case of liver cirrhosis, total lipoprotein levels were low, HDL fractions were mainly composed of HDL_2, the level of γ-globulin increased and that of albumin decreased compared with those of normal subjects. The hyperlipidemia serum (Fig. 8C) had a large amount of the high molecular weight lipoprotein fraction between VLDL and LDL.

Fig. 9 shows the elution patterns shown by animal sera under the same experimental conditions as in Fig. 8. Although the characteristic patterns

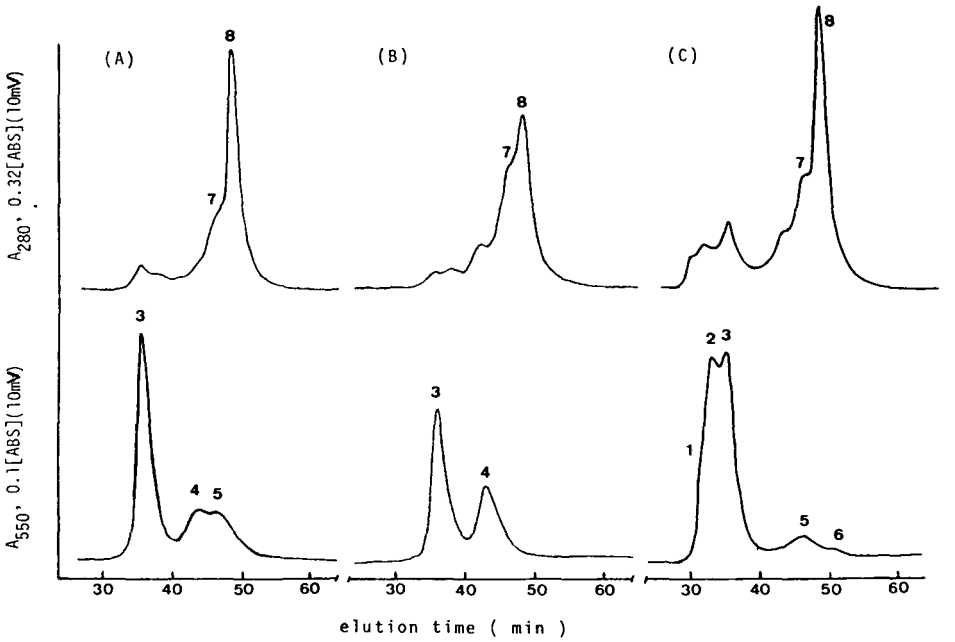

Fig. 8. Analyses of protein and cholesterol for human sera. Samples: (A) normal female subject; (B) liver cirrhosis subject; (C) hyperlipidemic (type III) subject. Loaded volume: 20 μl of whole serum. Peaks: 1, VLDL; 2, IDL; 3, LDL; 4, HDL_2; 5, HDL_3; 6, VHDL; 7, γ-globulin; 8, albumin. Conditions as in Fig. 2.

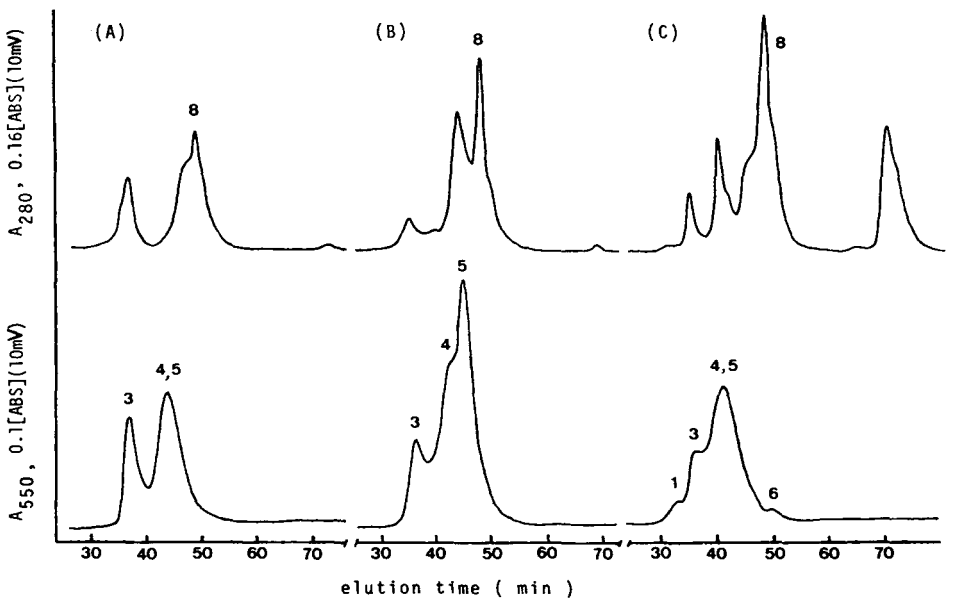

Fig. 9. Analyses of protein and cholesterol for animal sera. Samples: (A) 20 μl of whole serum (bovine); (B) 10 μl of whole serum (dog); (C) 50 μl of whole serum (rat). Peaks as in Fig. 8. Conditions as in Fig. 2.

of serum proteins are observed according to each species, the cholesterol peaks are separated into three major lipoprotein classes: VLDL, LDL, and HDL. With dog serum, HDL is separated into subfractions. In the case of rat serum, a small peak of cholesterol probably due to the VHDL fraction is always observed.

From these experiments, it is found that analyses of protein and lipoprotein of not only human but animal serum can be carried out with a very small amount of serum (10—50 μl) using the described flow system for high-performance GPC. The detection of cholesterol by enzymatic reaction is useful for studying the lipoprotein distribution in both human and animal sera. This analytical method is available for the investigation of lipoprotein metabolism and related diseases because of its short experimental time, high resolution and high sensitivity of detection. The quantitation of glyceride in serum lipoprotein is now being studied in our laboratory using this flow system for high-performance GPC.

ACKNOWLEDGEMENTS

The authors deeply appreciate the great help of Mr. K. Sakane, Toyo Soda Manufacturing Co., in the experiments and the serum gifts offered by Associate Professor J. Hosaki and Dr. A Tanaka, Tokyo Medical and Dental University.

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Journal of Chromatography, 223 (1981) 295—303
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 805

DETERMINATION OF THE PRIMARY METABOLITE OF CENTRAL NERVOUS SYSTEM NOREPINEPHRINE, 3-METHOXY-4-HYDROXY-PHENETHYLENEGLYCOL, IN MOUSE BRAIN AND BRAIN PERFUSATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received August 26th, 1980; revised manuscript received December 3rd, 1980)

SUMMARY

Assays are described for the determination of picomole levels of 3-methoxy-4-hydroxy-phenethyleneglycol (MHPG) in mouse brain and in the perfusate of an intact mouse brain. High-performance liquid chromatography with electrochemical detection yielded a MHPG detection limit of 0.37 pmol. This technique offers a sensitive and inexpensive alternative to gas chromatography with mass spectrometry.

INTRODUCTION

Neuronal noradrenergic systems in brain have been implicated in a variety of behavioral and psychopharmacological phenomena [1—3]. It has been proposed that the rate of production of 3-methoxy-4-hydroxyphenethyleneglycol (MHPG), the principal metabolite of norepinephrine in mammalian brain, is an indicator of the rate of norepinephrine turnover [4, 5]. Therefore, new methods of MHPG isolation and detection are important in furthering our knowledge of the role of norepinephrine in normal and dysfunctional mammalian brain.

Among the methods available for MHPG detection, gas chromatography with electron-capture or mass spectrometric detection is certainly the most definitive technique currently in use [6, 7]. The disadvantages of these methods are that derivatization of MHPG is required to produce a volatile compound and that the mass spectrometric instrumentation is expensive. High-performance liquid chromatography with electrochemical detection (HPLC—EC) offers a relatively inexpensive, simple, and highly sensitive technique

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for the routine assay of MHPG. This paper describes the methodology we have developed for measuring MHPG in mouse brain and in artificial blood which had perfused an intact mouse brain [8—10].

EXPERIMENTAL

Reagents

DL-4-Hydroxy-3-methoxyphenylglycol, piperazine salt (No. 454205, Calbiochem-Behring Corp., Los Angeles, CA, U.S.A.; No. 14,879-2, Aldrich Chemical Co., Milwaukee, WI, U.S.A.); 3-hydroxybenzyl alcohol (No. H2,060-1, Aldrich Chemical Co.); hydroquinone (No. 374387, Matheson, Coleman and Bell, East Rutherford, NJ, U.S.A.); spectral grade ethyl acetate, redistilled (J.T. Baker, Phillipsburg, NJ, U.S.A.); HPLC mobile phase, 0.01 *M* potassium phosphate buffer (pH 7.0); mice, C3H/Ibg males (45—65 days old); artificial blood, washed bovine erythrocytes in an artificial serum [11].

MHPG purity and stability

The molar extinction coefficients of newly synthesized and dried MHPG (piperazine salt, $C_{22}H_{34}N_2O_8$, with the following elemental analysis: theoretical C = 58.1%, H = 7.54%, N = 6.17%; found C = 58.2%, H = 7.62%, N = 6.14%) are $\epsilon_{280} = 6.26 \cdot 10^3\ M^{-1}\ cm^{-1}$ and $\epsilon_{230} = 1.45 \cdot 10^4\ M^{-1}\ cm^{-1}$ in 100% ethanol [12]. MHPG ordered from Calbiochem-Behring Corp. and Aldrich Chemical Co. was assayed within a week after it had been received and stored at —20°C. The ϵ_{280} molar extinction coefficients were $5.75 \cdot 10^3\ M^{-1}\ cm^{-1}$ (Calbiochem-Behring) and $5.82 \cdot 10^3\ M^{-1}\ cm^{-1}$ (Aldrich) which indicated decreases of 8.2% and 7.0%, respectively, when compared to the theoretical ϵ_{280} noted above. These data suggested that the MHPG was slightly impure, which may have been due to possible hydration and/or contamination by products of chemical deterioration. The piperazine salt of MHPG is known to be hygroscopic and oxygen-labile [12], and thus aqueous solutions of the piperazine salt of MHPG, which are basic, should be buffered or neutralized to retard oxidation. Throughout most of the studies reported here, stock MHPG solutions were prepared weekly and stored at 5°C in 1 m*M* HCl. However, later on in our studies it was found that stock MHPG solutions can be stored for two months in 100% methanol at —20°C without significant deterioration. This was advantageous because it minimized the atmospheric exposure of the stock bottle of MHPG which accompanied weighing procedures.

Apparatus

Solvent metering pumps, Altex 110A (Altex Scientific); sample injection and window valves, Rheodyne 7010, RP-2 MPLC cartridge and holder, and RP-2 analytical column, 25 cm × 4.6 mm I.D., 10-μm LiChrosorb (Rheodyne, Berkeley, CA, U.S.A.); μBondapak C_{18} analytical column, 30 cm × 3.9 mm I.D., 10 μm (Waters Assoc., Milford, MA, U.S.A.); electrochemical detector (LC-4), CP-S carbon paste, and TL-4 cell (Bioanalytical Systems); recorder (Houston Instruments Omniscribe); sample filters (Millipore Corp. No. GSWP 01300; Schleicher and Schuell No. RC55; Bio-Rad Labs. No. 313-5009); filter holders (Millipore Corp., Swinnex No. SX00 01300; Bio-Rad Labs., Liqui-Holder, 13 mm, No. 342-0001).

Procedure

The chromatographic flow-rate was 1 ml/min and the detector potential was +0.8 V against a Ag|AgCl reference electrode. Because of extraneous components that eluted from the analytical column 60—75 min after the injection, we utilized the system shown in Fig. 1 to minimize the time between injections. The sample was injected onto an MPLC RP-2 column which had been precalibrated for the retention times of MHPG and the internal standard 3-hydroxybenzyl alcohol (3HBA). During that interval in which these compounds eluted from the pre-column, the window valve (Fig. 1) was opened, allowing them to flow on to the analytical column, after which the window valve was closed and the extraneous components were washed off the MPLC column into the waste container.

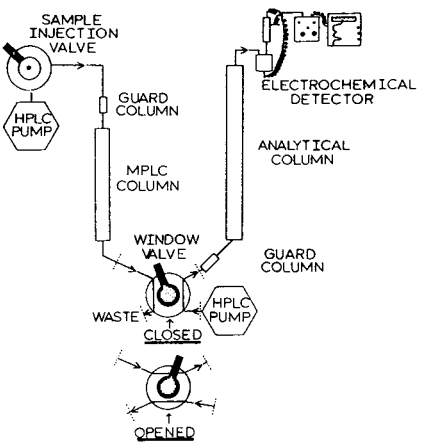


Fig. 1. Window-valve HPLC—EC system. Shown with valve in closed (upper) and opened (lower) positions. Equipment described in text.

Extraction of mouse brain tissue

C3H/Ibg male mice were sacrificed by decapitation; the heads were immediately frozen with liquid nitrogen. The frozen brains were removed, weighed, and pulverized with 1 ml of frozen 50 m*M* perchloric acid with a deeply cooled mortar and pestle. The mixture was then thawed on an ice bath after which 2 ml of cold 50 m*M* perchloric acid, along with the internal standard 3HBA (100 pmol) and MHPG (when appropriate), were added. The mixture was homogenized and then centrifuged for 15 min at 15,000 *g*. The supernatant was neutralized with 1 ml of 3 *M* tris(hydroxymethyl)aminomethane—HCl (Tris—Cl) buffer (pH 8.6) and extracted twice with 5 ml of ethyl acetate on an automatic shaker (5-min shaking intervals). The ethyl acetate extracts were maintained at 5°C until they were rotoevaporated to dryness, which was done as soon as possible following the extraction procedure. The remaining residues were dissolved in methanol, transferred to conical tubes, and evaporated under nitrogen. The warming baths for the evaporation steps were maintained below 40°C. The residues were then dissolved in 0.1 ml of the mobile phase, millipore-filtered, and injected onto the HPLC system.

Extraction of mouse brain perfusate

The perfusion fluid was a suspension of washed bovine erythrocytes in an artificial serum [11]. MHPG was extracted from the perfusate of a mouse brain by shaking the serum (10 ml), from which the erythrocytes had been separated by centrifugation, with ethyl acetate (10 ml) for three 10-min intervals. Again, the ethyl acetate extracts were maintained at 5°C until they were rotoevaporated to dryness. The residues were then dissolved in methanol, dried, dissolved in mobile phase, and filtered as described above in the mouse brain MHPG determination.

Chromatographic peaks obtained from extractions of brain perfusates were confirmed by gas chromatography—mass spectrometry (GC—MS). The peaks from twelve injections (approximately 72 pmol) were collected, pooled, and rotoevaporated to dryness. The residue was derivatized by adding bis(trimethylsilyl)trifluoroacetamide and heating at 60°C for 15 min. The GC column employed was 1.52 m × 2 mm I.D., packed with 3% SE-30 and operated at 150—200°C at 10°C/min. The mass spectrometer (Finnigan 3200 with a Model 6100 data system) was used to scan the mass range from *m/z* 50 to 500. The GC retention times and mass spectra obtained corresponded to those of authentic derivatized MHPG [13].

MHPG levels in the mouse brain were depleted by treatment with intraventricular injections of 6-hydroxydopamine [14, 15], which chemically lesioned the central noradrenergic systems. In other studies we have demonstrated that 99% of the brain MHPG was eliminated after this method of treatment [10].

RESULTS AND DISCUSSION

Chromatography

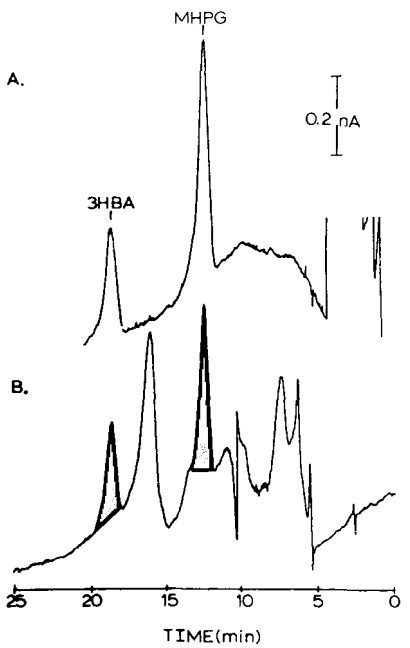
During the development of the MHPG assay it was found that filtration of the sample before injection on to the HPLC system introduced a component which eluted from the C-18 analytical column in a very wide band with a retention time of 60—75 min. This artifact complicated the analysis and prohibited further sample injections until the component had eluted from the column. This extraneous component was found in all the membrane filters cited in the experimental section. Through the use of the window-valve system (Fig. 1), injections on to the HPLC column were made at 20-min intervals and the subsequent detections of MHPG and 3HBA were accomplished without interference from extraneous components from the previous injection. Because the carbon-paste electrode of the electrochemical detector was spared the chemical perturbation that typically accompanies the injected solvent front in single-column systems, we found improved stability, sensitivity, and lifetime of the carbon-paste electrode. The detection limit (where the signal-to-noise ratio became less than 2.5) of MHPG in an aqueous (1 m*M* HCl) standard was 0.37 pmol.

Mouse brain MHPG determination

In Fig. 2 are shown HPLC—EC chromatograms of MHPG and 3HBA controls (Fig. 2A) and an extract of a mouse brain homogenate to which 100 pmol

of 3HBA have been added (Fig. 2B). The retention times for MHPG and 3HBA on the system shown in Fig. 1, when the RP-2 analytical column was employed, were 12.5 and 19.0 min, respectively.

The calibration curve for the determination of MHPG in homogenates of mouse brain tissue (Fig. 3) was generated by the following procedure. Brain homogenates from mice that had been treated with intraventricular injections of 6-hydroxydopamine were mixed with known amounts of MHPG (50—150 pmol) and 3HBA (100 pmol) and extracted as described above. The ratio of the MHPG and 3HBA detector responses (MHPG/3HBA) was plotted against the MHPG (pmol) that was added to the brain homogenates (open circles, Fig. 3). In the second type of experiment, 3HBA was added to the brain homogenates from normal mice which were then divided in half and a known amount of MHPG (25—100 pmol) was added to one half. Thus, the MHPG content of a half-brain homogenate contained either the endogenous MHPG level (approximately 60 pmol) or the endogenous level plus the amount of MHPG added. The endogenous level of each half brain was determined from the calibration curve generated from the 6-hydroxydopamine-treated animals. Then the numerical value of the endogenous level was combined with the amount of MHPG that had been added to the other half, and these data (closed



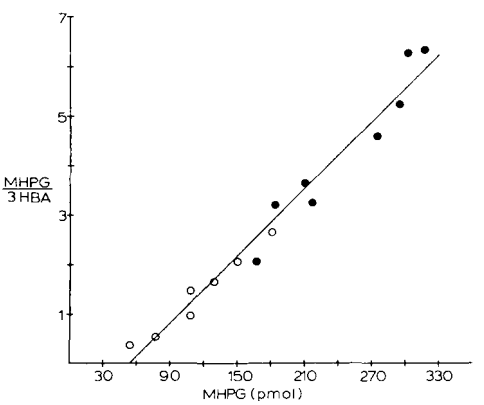


Fig. 2. HPLC—EC chromatograms. (A) MHPG (10 pmol) and 3HBA (5 pmol) standard solution. (B) Extract of brain homogenate. 100 pmol 3HBA added to homogenate before extraction. Flow-rate, 1 ml/min. Mobile phase, 10 m*M* potassium phosphate buffer (pH 7.0). Analytical column, Rheodyne RP-2.

Fig. 3. Calibration curve for determining MHPG in mouse brain homogenates. Ratio of detector responses for MHPG and internal standard 3HBA plotted against MHPG in 0.45 g of brain tissue. (○) 6-Hydroxydopamine-treated brains; (●) normal brains. 100 pmol 3HBA added to brain homogenate before extraction. Slope = $2.28 \cdot 10^{-2}$ $pmol^{-1}$; y intercept = −1.26.

circles, Fig. 3) were plotted with the data from the 6-hydroxydopamine-treated animals. The advantage of this method is the increased reliability in the measurement of brain MHPG levels that are lower than the endogenous level. Also, the inclusion of both methods ensured that the drug treatment did not affect the extraction efficiency.

The calibration curve shown in Fig. 3 has a slope of $2.28 \cdot 10^{-2}$ $pmol^{-1}$ and a y intercept of -1.26. The open and closed circles in Fig. 3 were generated from the extractions of 6-hydroxydopamine-treated and normal brains, as indicated. Because of the negative intercept in Fig. 3, the linear fit to the data is a good approximation only in the range of 54—317 pmol MHPG per 0.45 g of mouse brain.

The techniques described above were used to determine the MHPG levels in the brains of male C3H mice, the mean of which was 275 ± 12 pmol/g of brain (mean ± S.E.M., n = 9). This value is in agreement with data determined from other published methods using gas chromatography with electron-capture detection [16] and selected ion monitoring mass spectrometry [17]. The absolute recovery of MHPG, which was determined from that half of the mouse brain homogenate to which a known amount of MHPG had been added, was 23.8 ± 1.4% (mean ± S.E.M., n = 9). This was sufficient to produce amounts required for the HPLC—EC determination of MHPG levels in a whole mouse brain.

The mouse brain MHPG determination procedure described here can be used in conjunction with brain catecholamine determination. When the supernatant from a mouse brain homogenate is incubated with alumina in 3 *M* Tris—Cl buffer (pH 8.6) [18], the catecholamines are adsorbed by the alumina but the MHPG is not. Hence, the MHPG can be subsequently extracted from the Tris—Cl buffer [10] which is washed from the alumina. This is the reason for adding Tris—Cl buffer to the brain homogenate before the ethyl acetate extraction procedure as described in the Experimental section.

MHPG determination in mouse brain perfusate

The chromatogram of MHPG on the window-valve system with a C-18 analytical column is shown in Fig. 4A. Fig. 4C shows the chromatography of an extraction of perfusion fluid which had not passed through a mouse brain. The background levels of MHPG shown here were routinely subtracted from the levels determined in the perfusate of a mouse brain (Fig. 4B).

The C-18 analytical column was routinely used in the perfusate MHPG determinations because of occasional interferences by additional peaks (not shown in Fig. 4) which could not be resolved from the MHPG peak by the C-2 analytical column. However, the use of the C-18 column resulted in prohibitively long retention times for 3HBA which was used for the internal standard in the mouse brain MHPG determination. This is why the external standard hydroquinone was used in the brain perfusate MHPG determination. MHPG itself was not used as the external standard because of its instability and the relative ease of using another compound (such as hydroquinone) that does not require storage at low temperatures.

The MHPG calibration curve (Fig. 5) was normalized to 2.37 pmol of hydroquinone injected onto the column. The slope of the curve is $1.53 \cdot 10^{-1}$ $pmol^{-1}$

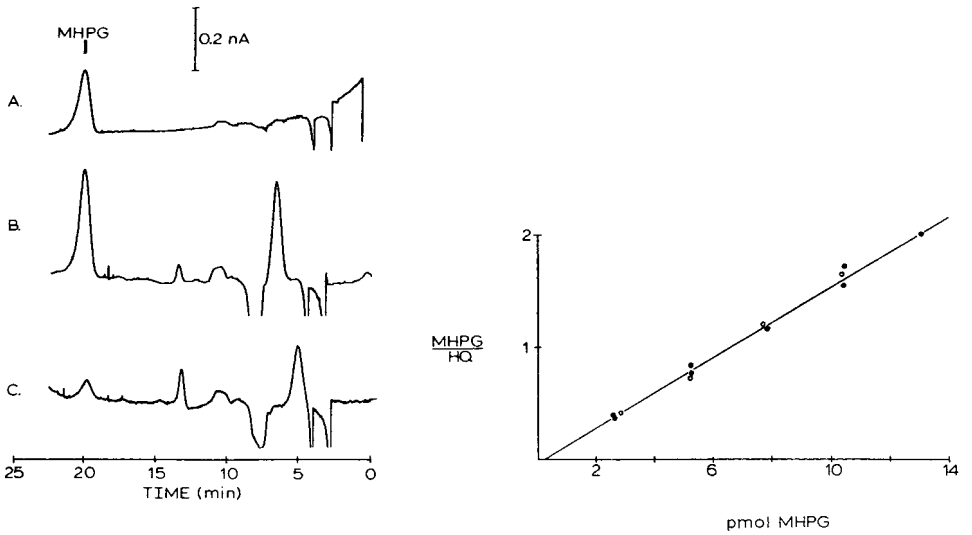


Fig. 4. HPLC—EC chromatograms. (A) MHPG (10 pmol) standard solution. (B) Extract of mouse brain perfusate. (C) Extract of control perfusion fluid. Flow-rate, 1 ml/min. Mobile phase, 10 m*M* potassium phosphate buffer (pH 7.0). Analytical column, μBondapak C_{18}.

Fig. 5. Calibration curve for determining MHPG in extract of mouse brain perfusate. Ratio of detector responses for MHPG and external standard hydroquinone (HQ) plotted against MHPG injected on to the column. Normalized to 2.97 pmol hydroquinone. Slope = $1.53 \cdot 10^{-1}$ $pmol^{-1}$ MHPG; y intercept = $-4.05 \cdot 10^{-2}$.

MHPG, the y intercept is $-4.05 \cdot 10^{-2}$, and it is linear within the range of 2—21 pmol MHPG. A graph of the amount of MHPG extracted from perfusion fluid vs. the amount of MHPG originally added is shown in Fig. 6. This graph has a slope of $1.99 \cdot 10^{-1}$ $pmol^{-1}$ MHPG and a y intercept of 6.75. The scatter in this calibration curve represents the variance in the extraction procedure over a period of three months and 34 separate extractions. It was demonstrated that MHPG rapidly equilibrated with the red blood cells which comprised 33% of the total volume of the artificial blood. The loss of MHPG in the hematocrit was accounted for in Fig. 6 because the MHPG was added to the blanks before the red cells were separated from the serum by centrifugation.

Recently Langlais et al. [19] demonstrated that MHPG and other neurotransmitter metabolites can be measured in human cerebrospinal fluid by direct injection of the fluid on to the HPLC column without prior extraction procedures. This technique demonstrates the power of HPLC—EC although direct injection of biological samples may contribute to column deterioration when performed on a routine basis. Their MHPG detection limit (0.27 pmol) is supported by the value reported here (0.37 pmol). We have determined that electrochemical detection is approximately 14 times more sensitive with the catecholamine norepinephrine than with the metabolite MHPG. This difference in sensitivity is supported by the electrochemical studies of Sternson et al. [20].

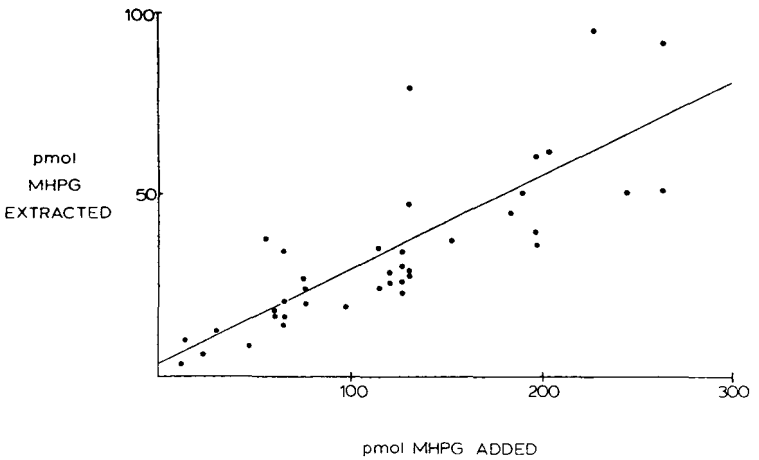


Fig. 6. Calibration curve for determining MHPG extraction efficiency from mouse brain perfusate. Amount of MHPG extracted plotted against MHPG added to control perfusion fluid samples. Slope = $1.99 \cdot 10^{-1}$ $pmol^{-1}$ MHPG; y intercept = 6.75.

In conclusion, we have developed assays for determining the primary metabolite of norepinephrine, MHPG, in mouse brain and in the perfusate of a mouse brain by HPLC—EC. These techniques offer a sensitive alternative to gas chromatography with electron-capture detection and mass spectrometry, and can be used in conjunction with brain catecholamine determinations.

ACKNOWLEDGEMENTS

The authors thank Keith Clay for helping us with the gas chromatography—mass spectrometric determination of MHPG, Laurie Coryell for her excellent technical assistance, and Mick Savage for sharing his HPLC expertise. We also thank John Thompson for his advice in the development of the analytical techniques described here and in the preparation of the manuscript. This work was supported in part by U.S. Public Health Service Grants AA 03527 and DA 07043 and by The Council for Tobacco Research — U.S.A., Inc., Grant 1251. John F. Towell was a National Institutes of Health Postdoctoral Drug Abuse Trainee while this research was being conducted.

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Journal of Chromatography, 223 (1981) 305—314
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 824

QUANTITATIVE DETERMINATION OF 3-METHOXY-4-HYDROXYPHENYLETHYLENEGLYCOL AND ITS SULFATE CONJUGATE IN HUMAN LUMBAR CEREBROSPINAL FLUID USING LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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(Received September 25th, 1980)

SUMMARY

A sensitive and direct reversed-phase liquid chromatographic method with amperometric detection was developed for the determination of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG). The concentrations of the free and sulfate conjugate of MHPG were measured in human lumbar cerebrospinal fluid. All samples were preconcentrated by extraction with ethyl acetate. Deconjugation of the sulfate form of MHPG was achieved by enzymatic hydrolysis with sulfatase.

Peaks were identified on the basis of chromatographic behavior, ratio of responses at several oxidation potentials and the stopped-flow UV spectra of the collected fractions.

The free MHPG content of 20 cerebrospinal fluid samples ranged between 0.720 and 19.51 ng/ml with the mean of 5.126 ± 4.652 (S.D.) ng/ml. The sulfate conjugate of MHPG in 12 samples of cerebrospinal fluid ranged between 0.08 and 0.850 ng/ml with the mean value of 0.2365 ± 0.2269 (S.D.) ng/ml. Although our results correlate well with the literature values, no attempt was made to interpret the quantitative data since samples were obtained from routine, diagnostic testing of patients admitted to the medical or neurologic services at the Mount Sinai Hospital.

INTRODUCTION

Since the biogenic amine hypothesis for affective disorders was proposed [1,2], considerable amount of research has been done in an effort to establish

the functional concentrations of catecholamines (CA) in the central nervous system (CNS), both in normal and certain disease states. At the present time there is considerable evidence that central noradrenergic neurons play an important role in cardiovascular regulation [3], hypertension [4], and depressive illnesses [1,2]. In the absence of non-invasive analytical techniques, clinical studies of norepinephrine (NE) turnover and the degree of intra-neural communication usually rely on studies of the products of metabolic disposition of this important catecholamine.

3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) is a principal metabolite of NE in the brain of various mammals [5—7], and it is found in urine [8—10], plasma [11, 12] and cerebrospinal fluid (CSF) [13, 14]. Measurement of urinary MHPG provides little information on the central turnover since NE is extensively metabolized before leaving the CNS. Urinary MHPG exists as the non-conjugated molecule (free MHPG), as the conjugate of sulfuric acid (MHPG·SO_4) and β-conjugate of glucuronic acid (MHPG·Glu). The conjugated forms predominate in the urine and it has been suggested that the sulfate conjugate reflects the central NE metabolism [15] while the β-glucuronide is derived from the metabolism of systemic NE [16]. The MHPG content in CSF is composed mainly of free MHPG and a small fraction of MHPG·SO_4 [11]. The fact that the sulfate conjugate of urinary MHPG is derived mainly from the central NE metabolism has aroused considerable interest in the analysis of the CSF levels of MHPG, where findings are not likely to be clouded by any peripheral contributions.

Quantitative analysis of MHPG in body fluids has been performed using gas—liquid chromatography (GLC) with electron-capture detection [17] or flame ionization detection [18], alone or in combination with mass spectrometry (MS) [19—21] and, more recently, by high-performance liquid chromatography (HPLC) [9,22]. Although the GLC methods are highly sensitive and lend themselves readily to coupling with MS, a technique of unsurpassed identification potential, poor volatility and thermal instability of MHPG necessitate the use of derivatization procedures for enhancement of volatility. This not only introduces a new step in the analysis but also poses additional problems due to the lack of stable derivatives and specific derivatizing agents.

The ability of HPLC, particularly in its reversed-phase mode, to resolve complex mixtures in body fluids is well documented in the literature. However, until the advent of electrochemical detection, this technique could not be fully exploited due to the inadequate sensitivity of the most commonly used LC detectors. Since organic functional groups such as phenols can be easily oxidized, the use of the oxidative mode of amperometric detection is ideally suited for the analysis of physiological levels of CA metabolites [9]. Furthermore, since the reversed-phase separations are usually performed using mixtures of aqueous buffers and organic modifiers (methanol or acetonitrile), the main operational requirement for electrochemical detection is thus satisfield. Therefore, we have investigated the use of the reversed-phase mode of HPLC, coupled with amperometric detection in the direct analysis of endogenous levels of free MHPG and its sulfate conjugate in samples of human lumbar CSF.

EXPERIMENTAL

Equipment

A Model 6000A solvent delivery system, Model 660 solvent programmer and Model U6K universal injector, all from Waters Assoc. (Milford, MA, U.S.A.) were used throughout this study. Chromatographic peaks in the HPLC effluents were detected using a Metrohm/Brinkmann voltametric/amperometric detector, Model E611 with an EA 1096 detector cell (Brinkmann Instruments, Westbury, NY, U.S.A.). The amperometric detector operates on a wall-jet principle and employs glassy carbon working and auxiliary electrodes and a Ag/AgCl reference electrode.

In addition, a Model SF 770 Spectroflow Monitor (Kratos, Schoeffel Instrument Div., Westwood, NJ, U.S.A.) with a deuterium lamp and an 8-μl cell equipped with a 330A wavelength drive and MM 700 memory module, was used for obtaining stopped-flow UV spectra.

Areas of chromatographic peaks were electronically integrated using a Hewlett-Packard Model 3380A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Stainless-steel columns (30 cm × 4.6 mm I.D.) were prepacked at the factory with 10-μm (average particle size) totally-porous support with a chemically-bonded octadecyl (C_{18}) moiety (Waters Assoc.).

Reagents

All reagents used were of highest purity (ACS Certified Grade). The MHPG reference compound and the enzyme sulfatase, Type V (β-glucuronidase activity: 2 units per mg of solid) were both purchased from Sigma (St. Louis, MO, U.S.A.); potassium dihydrogen phosphate from Mallinckrodt (St. Louis, MO, U.S.A.); methanol (distilled in glass) from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), and ethyl acetate (pesticide grade) from Fisher Scientific (Springfield, NJ, U.S.A.). Reference solutions of MHPG were prepared in distilled-deionized water and kept refrigerated when not in use.

Chromatographic conditions

For the determination of the free and sulfate conjugate of MHPG, a gradient elution mode of the reversed-phase HPLC was used. The low-strength eluent was 0.1 *M* KH_2PO_4, pH 2.50, and the high-strength eluent was a mixture of anhydrous methanol and distilled-deionized water (3:2, v/v). The low-strength eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, MA, U.S.A.), pore size 0.22 μm, and the high-strength eluent was regularly degassed under vacuum. A 45-min linear gradient from 0—60% of the high-strength eluent was used. The flow-rate was 1.2 ml/min and the temperature was ambient in all cases. Chromatographic peaks were detected amperometrically at oxidation potentials of +1.000 V and +0.700 V.

Sample preparation

Routine, unselected, diagnostic CSF specimens were obtained by lumbar puncture from 34 human subjects admitted to the medical and/or neurologic services at the Mount Sinai Hospital. For the analysis of free MHPG, 2—3-ml samples of CSF were acidified to a pH of 1.0 with 6 *M* HCl and extracted

three times with ethyl acetate (6 ml, 3 ml, 3 ml). The organic layers were pooled, evaporated to dryness under a stream of dry nitrogen, and reconstituted with water. The pH of the remaining aqueous layer was then adjusted to pH 5.2 and the samples were incubated for 16 h with 0.1 ml of sulfatase. Next, the pH was re-adjusted to 1 and the unconjugated compounds extracted three times with ethyl acetate, following the procedure analogous to the one used for the extraction of free MHPG. The protocol procedure is outlined in Fig. 1.

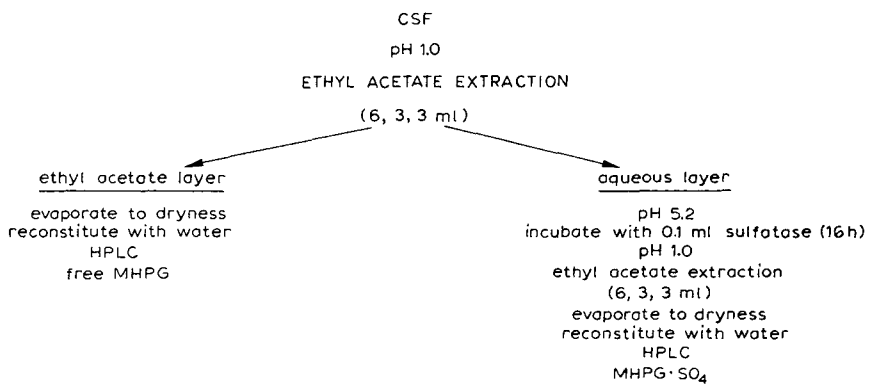

Fig. 1. Protocol procedure for the analysis of free MHPG and its sulfate conjugate.

Peak identification

Initial identification of MHPG in chromatograms of CSF was performed on the basis of retention behavior and co-chromatography with the MHPG reference solution. Since the oxidation potential of the amperometric detector can be varied, high sensitivity and selectivity can thus be achieved by careful selection of the detection potential. Furthermore, ratios of responses at several oxidation potentials were computed for the MHPG reference compound and compared with those for the peaks in CSF samples.

In addition, UV spectra of the MHPG reference compounds and the peaks with the same retention time in a pooled CSF sample were obtained. Due to the low levels of MHPG in the CSF samples, UV spectroscopy is not sufficiently sensitive for its detection. In order to circumvent this problem and gain further insight into the identity of the peak with the retention time of MHPG, several CSF samples were pooled, the sample re-chromatographed and the fraction collected. The corrected stopped-flow UV spectra were obtained on-line with the HPLC system and the details of this procedure are described in the literature [23]. Although these spectra characteristically lack in fine structure, they are nevertheless an important fingerprint of the absorber. The identity of the peaks was deduced on the basis of evidence accumulated from all identification steps.

Extraction efficiency

Prior to the analysis of CSF extracts, the efficiency of the extraction procedure was determined by adding known amounts (ng) of reference MHPG

to the CSF matrix, buffered to a pH of 1, 5 and 7 and extracting it with ethyl acetate. The highest recovery (96.5%) was obtained at the pH of 1.

Reproducibility of retention times and peak areas

CSF samples were kept frozen and their stability was tested periodically over a period of one month to ensure that no sample decomposition was taking place upon storage. The reproducibilities of peak areas and retention times were determined on a day-to-day and within-a-day basis from ten repeated injections. The percent standard deviations for the peak areas and retention times were 1.1% and 0.60%, respectively.

Detection limits and linearity of response

In order to optimize the sensitivity and selectivity of amperometric detection, samples were monitored at oxidation potentials between +0.400 V and +1.000 V. By monitoring the HPLC effluents at +0.700 V, adequate sensitivity is achieved with no loss of selectivity. In addition, the use of low oxidation potentials prevents the deterioration of the glassy carbon electrodes and extends their life-time. Although a 25% increase in the MHPG response can be obtained at an oxidation potential of +1.000 V, this is accompanied by a concomitant decrease in detection selectivity. However, MHPG is completely resolved from the remaining CSF constituents and can thus be detected free from interference at either potential. The detection limit from MHPG detected at +1.000 V was found to be approximately 50 pg.

Linearity of detector response was determined by adding ng amounts of the MHPG reference substance to the CSF matrix. The plot of peak area versus the amount injected was found to be linear over the concentration range of 1 ng to 1 μg. When the sample MHPG content was subtracted from the points on the calibration curve, the line intercepted the axes at the origin.

Interferences

Under the chromatographic conditions used, no interferences with other naturally-occurring CSF constituents were observed at oxidation potentials of +1.000 V and +0.700 V. The hydrolysis of conjugated MHPG can be carried out using several procedures: acid- or base-catalyzed cleavage and enzymatic hydrolysis using aryl sulfatase or glusulase (β-glucuronidase with aryl sulfatase). However, hydrolysis under acidic or basic conditions is not recommended, due to instability of MHPG [24]. Incubation with glusulase, which affords deconjugation of both the MHPG·SO_4 and MHPG·Glu, was not used due to insufficient purity of the enzyme preparation. Aryl sulfatase solutions were found to be of sufficient purity and did not give rise to any background interferences.

RESULTS AND DISCUSSION

Preliminary experiments were conducted in order to establish the best analytical conditions for the separation of MHPG from other naturally-occurring CSF constituents and to achieve sensitive and selective detection. The reversed-phase gradient elution mode of HPLC was adopted since it affords rapid analyses of MHPG and enables simultaneous detection of other CSF constituents.

Thus, the developed chromatographic method is general and yet selective for this important catabolite. Prior to the establishment of the protocol procedure, a study was conducted in order to determine the optimal oxidation potential which would afford high sensitivity without a concomitant loss in selectivity. Fig. 2 illustrates the change in response with detection potential. Although any

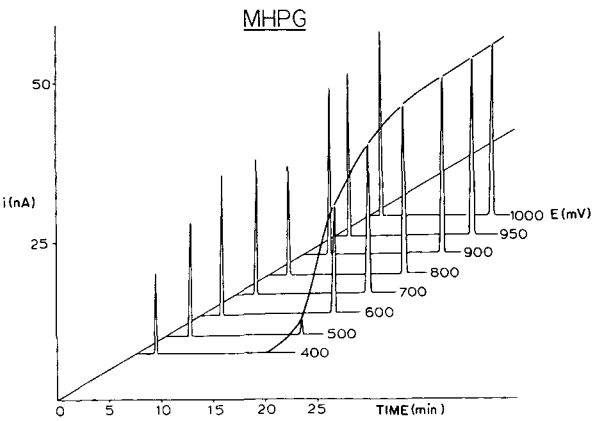

Fig. 2. Variation in the MHPG response with change in oxidation potential; amount injected: 3.0 μg; chromatographic conditions: column, μBondapak C_{18}; eluents: (low strength) 0.1 *M* KH_2PO_4, pH 2.50; (high strength) methanol—water (3:2, v/v); gradient: linear, from 0—60% of the high-concentration eluent in 45 min; flow-rate: 1.2 ml/min; temperature: ambient; detection: amperometric ; sensitivity: 2 μA f.s.; recorder attenuation: × 1024.

potential between +0.700 V and +1.000 V offers adequate sensitivity, the use of the former potential is advantageous since the detection selectivity is enhanced and the electrode life-time extended. The use of both potentials is illustrated with the analysis of free MHPG in CSF samples, shown in Figs. 3 and 4, respectively. Although no interferences with other naturally-occurring CSF constituents were observed at either potential in the course of this work, injection of large volumes of CSF may cause problems at high oxidation potentials (> +0.700 V), due to the relatively low levels of MHPG compared to other CSF constituents. In addition, extended use of high oxidation potentials might have a deleterious effect on the stability of glassy carbon electrodes.

The selectivity of the assay is further increased by means of a simple extraction procedure with ethyl acetate, since the phenolic acids are thus separated from the neutral and basic compounds. In order to avoid changes in the sample concentration due to the high volatility of ethyl acetate, the extracts were evaporated to dryness under a stream of dry nitrogen and reconstituted with water. Chromatographic peaks were identified on the basis of retention behavior, co-chromatography with the reference compound and ratios of responses at several oxidation potentials. Additional proof of the identity of the peak was obtained from the comparison of the UV spectra of the collected peak with the retention time of MHPG and the reference compound (Fig. 5).

For the analysis of conjugated MHPG, two enzymatic deconjugation pro-

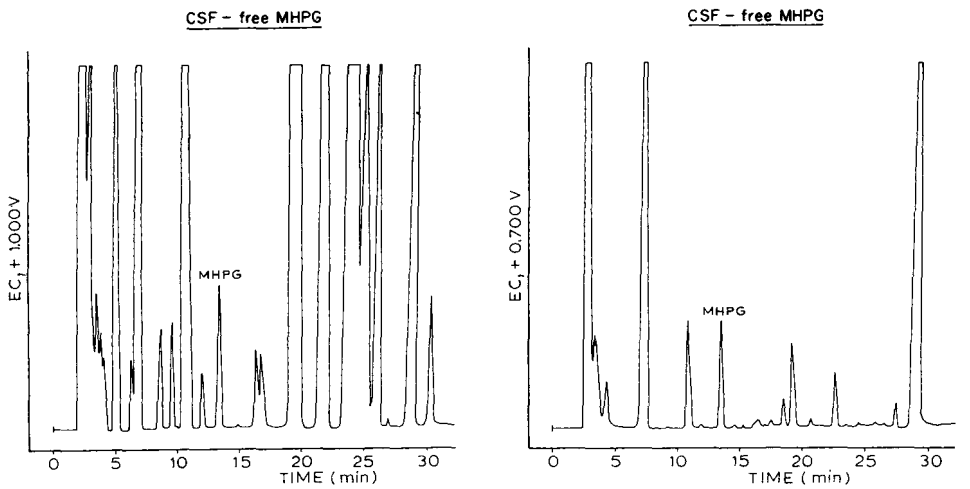

Fig. 3. Chromatogram of the ethyl acetate extract of CSF. Volume injected: 25 μl (corresponding to 0.52 ml of CSF); detection: amperometric, +1.000 V; recorder attenuation: × 256. Chromatographic conditions same as in Fig. 2.

Fig. 4. Chromatogram of the ethyl acetate extract of CSF shown in Fig. 3. Detection: amperometric at +0.7000 V; recorder attenuation: × 256. Chromatographic conditions same as in Fig. 2.

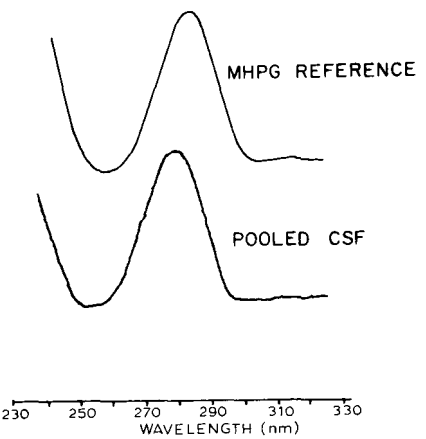

Fig. 5. Stopped-flow UV spectra of the MHPG reference compound and the peak with the same retention time in the pooled sample of CSF. Scanning rate: 100 nm/min; absorbance: 0.1 a.u.f.s. (reference MHPG), 0.02 a.u.f.s. (sample).

cedures were tried. The use of glusulase for the deconjugation of both the MHPG·SO_4 and MHPG·Glu gave rise to interferences due to insufficient purity of the enzyme preparation. In order to determine the enzyme blank, the pH of a sample of distilled-deionized water was adjusted to 5.2 and incubated with 0.1 ml of glusulase. The mixture was then extracted three times with ethyl acetate, the organic layers pooled, evaporated to dryness and reconstituted with water. A chromatogram of the enzyme blank is shown in Fig. 6. In addition, 9 ml of ethyl acetate were evaporated to dryness, reconstituted

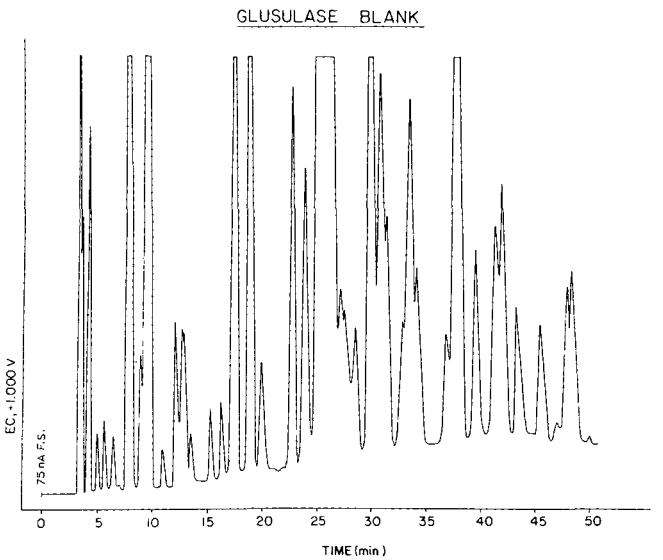

Fig. 6. Chromatogram of the ethyl acetate extract of a water sample incubated with glusulase under conditions indicated in Fig. 1. Volume injected: 100 μl; recorder attenuation: × 256. Chromatographic conditions same as in Fig. 2.

with water and subsequent chromatographing of the sample under identical conditions indicated that no peaks were present. Because of the presence of impurities in the glusulase preparation, this enzyme was not used for deconjugation. Since the literature reports indicate that the conjugated MHPG is present in CSF mostly as the sulfate form [19], the use of sulfatase was investigated. The enzyme preparation was found to be pure and no peaks were detected in the enzyme blank. A chromatogram of the ethyl acetate extract of a CSF sample incubated with sulfatase is shown in Fig. 7. Since the levels of MHPG·SO_4 are low, it is advantageous to use the oxidation potential of +1.000 V.

Prior to the quantitative analysis, a calibration plot was obtained by chromatographing a sample of CSF to which increasing amounts of MHPG were added. The relationship between the peak area and the amount of MHPG injected was found to be linear over the concentration range of interest (Fig. 8). The quantitative data for free MHPG and MHPG·SO_4 in human lumbar CSF samples, obtained by external calibration method, are shown in Table I. The mean value for free MHPG in 20 samples was 5.126 ± 4.652 (S.D.) ng per ml of CSF, and the range was 0.72—19.51 ng per ml of CSF. The mean values and the range for MHPG·SO_4 in 12 samples of CSF were 0.2365 ± 0.2269 (S.D.) and 0.08—0.850 ng per ml of CSF, respectively. Since unselected, diagnostic CSF specimens were used, it is difficult to interpret the quantitative data and correlate it with the medical and/or neurologic diagnosis. In addition, no effort was made to restrict medications. However, the quantitative results of this preliminary study are in agreement with the literature values [19]. The primary objective of this report was to demonstrate the usefulness of a direct HPLC method coupled with amperometric detection, in the analysis of en-

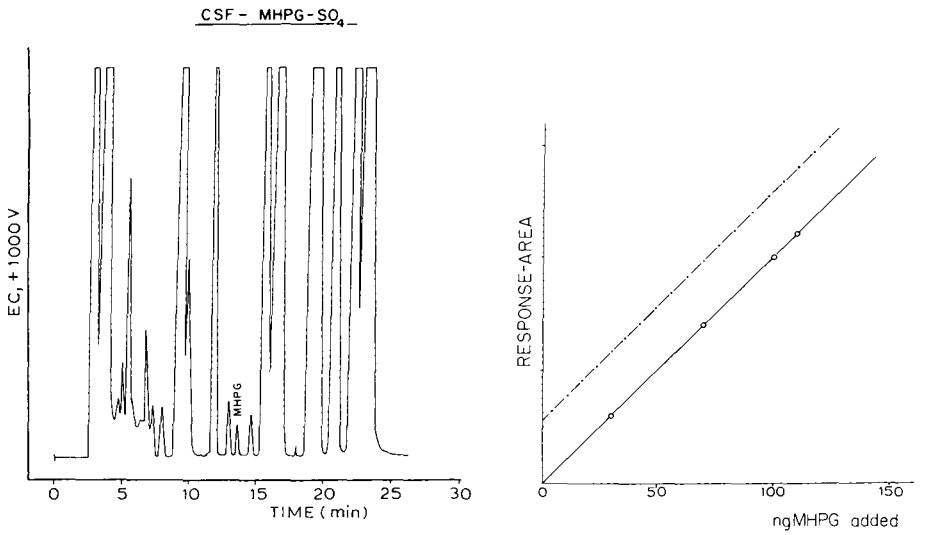

Fig. 7. Chromatogram of the ethyl acetate extract of a CSF sample incubated with sulfatase. Volume injected: 100 μl (corresponding to 2.1 ml of CSF); recorder attenuation: × 256. Chromatographic conditions same as in Fig. 2.
Fig. 8. The standard addition curve.

TABLE I

QUANTITATIVE ANALYSES OF FREE MHPG AND MHPG · SO_4 IN CEREBROSPINAL FLUID

Amounts expressed in ng per ml cerebrospinal fluid.

	N	Range	Mean
Free MHPG	20	0.720—19.510	5.126 ± 4.652 (S.D.)
MHPG · SO_4	12	0.081—0.850	0.2365 ± 0.2269 (S.D.)

dogenous levels of MHPG. The assay is simple, selective and quantitative and does not require derivatization of MHPG. Sample preparation is minimal and only ethyl acetate extraction is needed. Since this method circumvents many problems associated with other currently available analyses for MHPG, we believe it will give the clinicians a tool for routine assessment of the levels of this important catabolite.

ACKNOWLEDGEMENTS

The authors wish to thank Waters Assoc., Kratos, and Brinkmann Instruments for their technical assistance, Dr. L. Sarkozi of the Chemistry Department of Mount Sinai School of Medicine for the CSF samples, and Ms. Sandra Cerqueira for sample preparation. This study was supported in part by a grant from Ayerst Laboratories.

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Journal of Chromatography, 223 (1981) 315—320
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 807

DETERMINATION OF INDOLES AND CATECHOLS IN RAT BRAIN AND PINEAL USING LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC AND AMPEROMETRIC DETECTION

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(Received October 14th, 1980)

SUMMARY

Tryptophan, serotonin, 5-hydroxyindoleacetic acid, and homovanillic acid were determined in rat brain by the direct injection of a centrifuged tissue homogenate into a liquid chromatographic—fluorometric/amperometric system. The above indoles, along with melatonin, were also determined in single rat pineal glands. The utility of the system in determining several additional catechols and indoles in brain was examined.

INTRODUCTION

The measurement of levels of neurotransmitters, their precursors, and metabolites, present in brain is of obvious importance in neurochemistry. Recently, high-performance liquid chromatography (HPLC) coupled with fluorometric [1—5] or amperometric [6—23] detectors has allowed many of the determinations to be made with greater simplicity and sensitivity. We have used a combined liquid chromatographic—fluorometric/amperometric system to determine several neurochemically important indoles and catechols in brain and pineal with a minimum of sample preparation.

MATERIALS AND METHODS

The liquid chromatograph and detection system employed was essentially that described previously [24]. It consisted of an Altex 110A pump (Altex Scientific, Berkeley, CA, U.S.A.), a Waters U6K injector, and a μBondapak C_{18} reversed-phase column (300 mm × 3.9 mm I.D., 10 μm average particle

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size) (Waters Assoc., Milford, MA, U.S.A.). A modified Aminco Fluoromonitor (American Instrument Co., Silver Spring, MD, U.S.A.) was used with a 254-nm low-pressure mercury lamp source and a 360-nm peak transmittance emission filter. The amperometric detector was composed of a Bioanalytical Systems electrochemical controller (LC-2A), a glassy carbon working electrode, a Ag/AgCl reference electrode and a Teflon thin-layer detector cell and reference electrode compartment (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A 51-μm spacer gasket was used, and the potential of the working electrode was set at +0.70 V versus the reference electrode. Background currents of 1—2 nA were observed. The detectors were connected in series, with the amperometric detector downstream. Standards were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions (10 mg/100 ml) were made up in distilled water with 0.1% ascorbate added. Diluted standards (0.1—5.0 ng/μl) were made up daily in distilled water. Solvent systems were prepared by mixing the proper proportion (v/v) of glass-distilled methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) with 0.01 *M* sodium acetate previously adjusted to the desired pH with glacial acetic acid.

Weighed whole rat brains (1—2 g) were placed in polycarbonate centrifuge tubes containing 4.0 ml of 0.1 *M* $HClO_4$ (400 μl of 1 *M* $NaHSO_3$ added per liter). The brain was sonicated at 0—4°C at a medium setting for two 15-sec periods using a Branson Polytron Sonicator (Branson Sonic Power Co., Danbury, CT, U.S.A.). After adding 0.5 ml of 4.0 *M* $HClO_4$ and vortex mixing, the samples were spun at 10,000 *g* for 10 min and a portion of the supernatant stored in a 1.5-ml polyethylene tube. When sonicating brain punches and areas weighing from 2 mg to ca. 1 g, a similar v/w ratio of 0.1 *M* $HClO_4$ was used down to a minimum of 200 μl.

Rat pineals were placed in 1.5-ml polyethylene tubes and sonicated in 200 μl of 0.1 *M* $HClO_4$ and centrifuged at 10,000 *g* without the addition of 4.0 *M* $HClO_4$.

TABLE I

CHROMATOGRAPHIC AND DETECTABILITY DATA

Compound	Chromatographic conditions*	Retention time (min)	Detection limit (pg)**	
			Fluorometric	Amperometric
Serotonin (5-HT)	1	3.2	7	10
	2	2.8		
Tryptophan (TRP)	1	4.7	20	400
	2	4.1		
5-Hydroxyindoleacetic acid (5-HIAA)	1	6.9	35	10
	2	5.1		
Homovanillic acid (HVA)	1	8.5	2000	25
	2	6.3		
5-Hydroxytryptophol (5-HTOL)	2	6.1	35	15
Melatonin (MEL)	3	5.4	25	50

*Chromatographic conditions: 1: 88% pH 4.25 0.01 *M* sodium acetate—12% methanol; 2: 85% pH 4.50 0.01 *M* sodium acetate—15% methanol; 3: 65% pH 4.25 0.01 *M* sodium acetate—35% methanol.

**Injected amount giving a signal twice the peak-to-peak baseline fluctuation.

The compounds were determined in the brain and pineal homogenates by directly injecting 10–100 μl of the supernatants into the chromatograph. The chromatographic conditions are given in Table I. The compounds were quantitated by peak height measurments; single point standards were used as a linear response was observed over the working range.

RESULTS AND DISCUSSION

The chromatographic conditions, retention times, and detection limits for the compounds determined are given in Table I. A chromatogram of serotonin (5-HT), tryptophan (TRP), 5-hydroxyindoleacetic acid (5-HIAA), and homovanillic acid (HVA) standards is shown in Fig. 1. Standards were determined

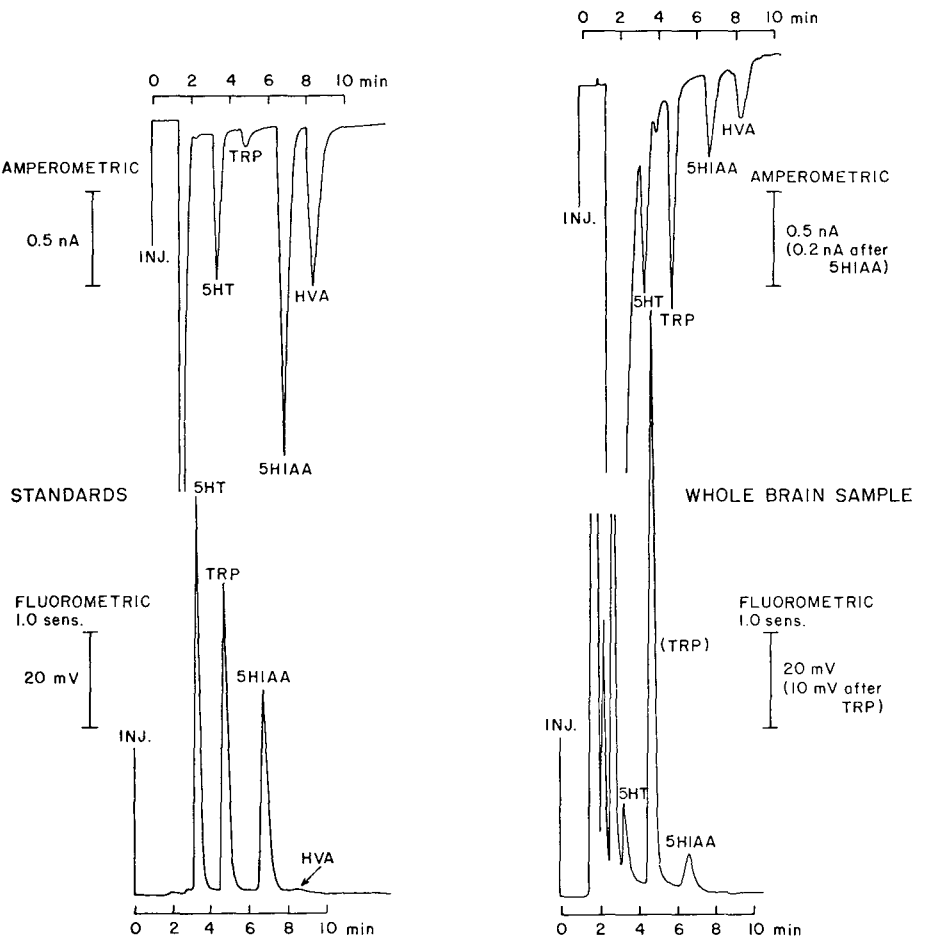


Fig. 1. Chromatogram of standards (5-HT 5 ng, TRP 10 ng, 5-HIAA 10 ng, HVA 10 ng) with fluorometric and amperometric detection. Chromatographic condition 1 (see Table I).

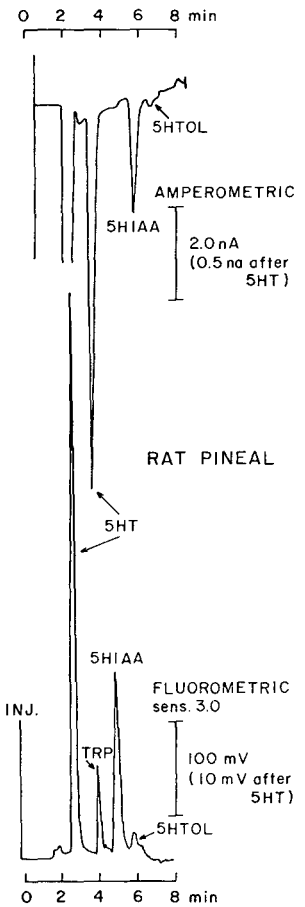
Fig. 2. Determination of 5-HT (130 ng/g), TRP (2500 ng/g), 5-HIAA (110 ng/g), and HVA (53 ng/g) in whole rat brain (1.8 g). Injection volume 20 μl. Chromatographic condition 1 (see Table I).

with typical coefficients of variation of 3—7%. The chromatogram in Fig. 2 depicts the determination of the compounds in whole rat brain. All of the compounds can be determined in brain both fluorometrically and amperometrically (except HVA fluorometrically). The amperometric and fluorometric values agreed well; usually a mean of the two methods was taken. Standard addition studies were performed by adding approximately 1—10 times normal levels of the compounds. The compounds were recovered linearly in yields of 60—92%, depending upon the particular compound, the brain area, and the w/v ratio used for homogenation. The recoveries for whole brain were: 5-HT 82%, TRP 71%, 5-HIAA 72%, and HVA 91%.

Most previous HPLC methods for determining catechols and/or indoles in brain require an extractive and/or chromatographic clean-up before analysis. With our method, the determinations are made easily and sensitively after simple homogenization. Special care must be taken to establish the identity of peaks observed when an unpurified homogenate is directly injected. The peaks observed here were identified on the basis of their relative fluorometric and amperometric response, as well as by chromatographing brain samples in solvent systems differing in pH (3.5, 4.5, and 5.5), and in percent and type of organic modifier (0—15% methanol and acetonitrile). A recent report [5] of the HPLC—fluorometric determination of several catechols and indoles in an unpurified brain homogenate contains several apparently misidentified peaks. Specifically, the peaks assigned to norepinephrine, dopamine, tyramine, 5-hydroxytryptophan, and indolelactic acid all failed to behave similarly to standards in our tests [25]. The peaks observed for tyramine, norepinephrine and dopamine were also several orders of magnitude too large. The observation of large levels of tyramine (>1000 ng/g brain) was of special interest as tyramine is generally considered to be a trace amine with brain concentrations of ca. 1 ng/g [26]. When the brain homogenate was chromatographed using a solvent system similar to that reported, a fluorometric peak did co-chromatograph with the tyramine standard; however, the peak was not electro-active, in contrast to the tyramine standard which was easily detected amperometrically. This reconfirms tyramine's status as a trace amine in rat brain and illustrates the power of the combined fluorometric/amperometric detection system.

Determinations of several indoles in rat pineal are shown in Figs. 3 and 4. In Fig. 3, TRP, 5-HT, 5-HIAA, and a peak tentatively identified as 5-hydroxytryptophol (5-HTOL), were determined by injecting 20 μl of a centrifuged 200-μl 0.1 *M* $HClO_4$ pineal homogenate. In Fig. 4, the important pineal hormone, melatonin, was determined by injecting 100 μl of the same homogenate. A solvent system with a higher percentage of methanol was used to elute this relatively lipophilic compound. Thus, in two runs on the chromatograph, five indoles can be measured in a single pineal. These compounds include melatonin, a hormone with a well established anti-gonadotrophic effect, and 5-HTOL, a compound about which very little is known, but which may have hormonal actions similar to melatonin [27]. The pineal shown was obtained from a young (ca. 30 g) Sprague-Dawley rat sacrificed at 12:00 noon when melatonin is at its lowest level [28, 29]. The compounds determined in pineal have all been recovered in >90% yield from spiked samples.

In conclusion, we have found that the combination of fluorometric and



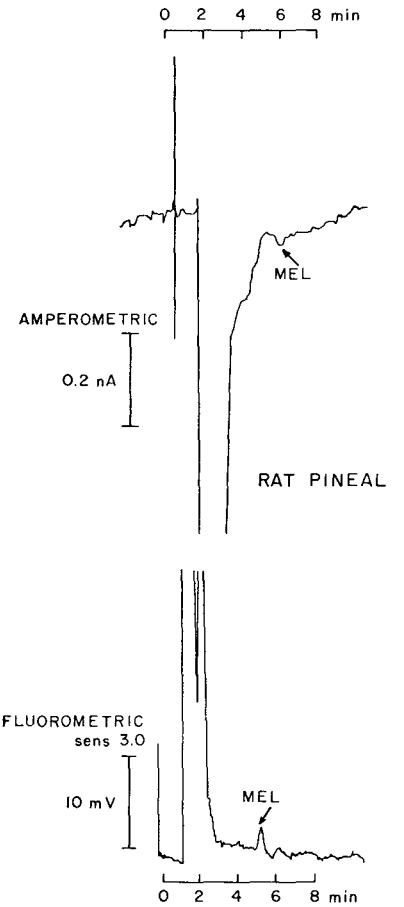


Fig. 3. Determination of 5-HT (100 ng/pineal), TRP (3.1 ng/pineal), 5-HIAA (8.6 ng/pineal), and 5-HTOL (0.74 ng/pineal) in a single rat pineal. Injection volume 20 μl of a 200-μl homogenate. Chromatographic condition 2 (see Table I).

Fig. 4. Determination of melatonin (MEL) in a single rat pineal (MEL, 0.2 ng/pineal). Injection 100 μl of a 200-μl homogenate from a rat pineal obtained at the time of the lowest diurnal MEL level.

amperometric detectors with HPLC has allowed determination of indoles and catechols in rat brain and pineal to be made easily and with a great deal of specificity and sensitivity. The dual detection system affords an extra measure of certainty concerning the identity of the peaks observed. For routine use, we have found fluorometric detection to be practically problem-free, as opposed to amperometric detection, where an occasional problem due to bubble formation, electrode passivation, and non-specific instrumental noise is to be expected.

ACKNOWLEDGEMENTS

This research was supported by MHCRC grant MH 30929, NICHD grant HD-03008, the Solomon R. and Rebecca D. Baker Foundation, and the Gateposts Foundation.

NOTE ADDED IN PROOF

A recent method [30] uses combined fluorometric and amperometric detection to determine several of the compounds in brain extracts.

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Journal of Chromatography, 223 (1981) 321—329
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 818

DETERMINATION OF CARBOHYDRATES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND OPTICAL ACTIVITY DETECTION

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(First received October 22nd, 1980; revised manuscript received December 15th, 1980)

SUMMARY

Improvements in a detector for liquid chromatography based on optical activity of the components have led to a detectability of 100 ng. This allows the simultaneous determination of six naturally occurring carbohydrates in 100-μl samples of human urine, which is injected directly except for a simple deionization step. The reproducibility and reliability of this method should allow better insight into the relation between urinary sugars and physiological conditions.

INTRODUCTION

The identification and the determination of the hundreds of compounds that are present in body fluids have been beneficial to establishing pathological conditions [1], understanding the molecular basis of diseases, monitoring the therapeutic effects of drugs [2], and maintaining proper mental health [3]. Except for the class of highly specific systems, such as enzyme—substrate or antigen—antibody reactions, the body fluids are in general too complex to be analyzed without some sort of a separation scheme. Very high resolution has been achieved in liquid chromatography for such samples [4, 5], but a combination of slow eluent gradient and small particle sizes for the packing material requires an analysis time of the order of many hours. For physiological profiling and for routine clinical usage, these analysis times are prohibitive. It is much more desirable to restrict the studies to individual classes of compounds, so that a simpler, more specific separation procedure can be performed. Or, in cases where sample manipulation needs to be reduced to a minimum, selective detection schemes for liquid chromatography can be employed to minimize interferences.

The carbohydrates that are present in body fluids are an interesting class of compounds, and are related to metabolic processes in general. Much attention has been given to the routine clinical screening for glucose [6], because of the disease diabetes mellitus. The accepted method using hexokinase [7] is relatively free from interferences, but requires 500 μl protein-free filtrate. This can become impractical for fetal or pediatric applications. The other simple sugars are often neglected, primarily because of the lack of reliable quantitative methods at the low concentration levels typical of body fluids. Paper chromatography provides qualitative information in a reasonable time [8, 9], but even semi-quantitative results are difficult to obtain at these concentrations. Automated high-resolution analyzers with sensitivities in the μg range have been used to study carbohydrates in body fluids [10, 11], but again hours are required per analysis. More recently, cation-exchange resins have been successfully used for separating dextrose and fructose from the higher saccharides in food products [12], but the limitation on sensitivity makes the scheme unsuitable for studying body fluids. The use of a refractive index detector in these cases is dictated by the lack of convenient UV absorption bands for the carbohydrates, and is a particularly weak link since the columns must be operated at above-ambient temperatures. Post-column colorimetric methods have been used [13], but should be avoided if more reliable and faster methods can be found. Flame ionization detectors have been used [14], but again are not sensitive enough.

Several uses of carbohydrates other than glucose for physiological profiling are known. Excess fructose in urine can be a sign of an inherited metabolic defect [15]. Lactose is present in urine in late pregnancy and during lactation, but excess can indicate a rare metabolic disease [16]. The inherited disease galactosemia causes the presence of galactose in urine, but galactose can also be an indication of severe hepatitis or biliary atresia in neonatal infants [16]. The last condition can lead to liver damage, mental retardation, and cataracts. The presence of xylulose is related to yet another familial disorder [17]. The D-xylose absorption test can be used to diagnose either enterogenous steatorrhea [18] or kidney malfunction. Since the carbohydrates are directly involved in the metabolic cycles of the body, one would expect dietary and metabolic deficiencies to affect carbohydrate profiles in serum and urine. It is therefore important to have reliable methods for analysis, so that correlations can be studied.

A detector for high-performance liquid chromatography (HPLC) that is particularly suitable for carbohydrate analysis is one based on the optical activity of these compounds [19]. This detector eliminates most of the restrictions on the choice of eluents and gradients so that the chromatography can be optimized independently. Since the work published earlier [19], we have made several improvements on the system. These, and the separation of urinary sugars, will be described in this paper.

EXPERIMENTAL

Chromatography

Separations were performed on a heavy metal cation-exchange column that is commercially available (Bio-Rad Labs., Richmond, CA, U.S.A., HPX-85 Heavy Metal). This column has been optimized for the monosaccharides. The operating conditions were as recommended by the manufacturer, i.e., water at a flow-rate of 0.64 ml/min was used as the eluent and the column was maintained at 85°C with a home-built water jacket. All injections were through a 100-μl loop at a conventional injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7010). To reduce any pressure fluctuations caused by the pump (Milton Roy, Riviera Beach, FL, U.S.A., Model 196-0066-001) at the detector, we used a commercial pulse-dampener (Alltech, Deerfield, IL, U.S.A., Model 9404) in conjunction with a pressure gauge (Alltech, Arlington Heights, IL, U.S.A., Model 9228). Pressure fluctuations were further reduced as a result of having a UV absorbance detector (Spectra Physics Chromatronix, Santa Clara, CA, U.S.A., Model 210) in series and before the optical activity detector. Since the flow-cell was essentially at room temperature, the eluent must be cooled to some extent from 85°C so that turbulence would not exist in the cell. It was found that having the UV detector in series was sufficient for cooling. Alternately, a 50-cm length of standard chromatographic stainless-steel tubing was also satisfactory. Cooling or temperature control, however, was not as critical as in a refractive index detector. Test solutions were all reagent grade material (Fisher Scientific, Fair Lawn, NJ, U.S.A.) dissolved in deionized water. To protect the chromatographic column, urine samples were passed through a mixed-bed (Mallinckrodt, Paris, KY, U.S.A., Amberlite MB-3) ion-exchange column, but otherwise untreated. Identical protection can be achieved using a commercial guard column, so that pretreatment is eliminated from the procedure.

Optical activity detector

The basic arrangement for an optical activity detector for HPLC has been reported earlier [19]. In this work, the laser was operated at 488 nm to better match the spectral response of the photomultiplier tube and to take advantage of the slightly larger specific rotations of these compounds, although these differences are minimal. Since it was found previously [19] that a major source of noise is shot noise from the incompletely extinguished laser beam in the absence of a sample, we introduced intensity stabilization in the laser. This was accomplished by passing the laser light through a Pockels cell (Lasermetrics, Teaneck, NJ, U.S.A., Model 1058-FV) and then a Glan prism (Karl Lambrecht, Chicago, IL, U.S.A., Model MGLS-DW-8) aligned slightly off-axis from the polarization direction of the laser. A photodiode was used to monitor the intensity after this arrangement. After proper amplification, the signal was compared with an adjustable reference level, so that an error signal could be generated. A high voltage operation amplifier (Burleigh Instruments, E. Rochester, NY, U.S.A., Model PZ-70) then provided feedback

to the Pockels cell to control the intensity. The intensity stabilization improved the signal-to-noise ratio and avoided drifting in the baseline in the chromatograms.

The internal volume of the flow-cell has been reduced to 80 μl using a smaller drill-bit without any adverse effects, as predicted in the earlier work [19]. It was found that the cell windows were useful for months unless they were contaminated by the chromatographic effluent, as evidenced by visible deposits. The position of the cell in the optical path was such that the laser beam cleared the cell walls, since scattering caused depolarization. The cell windows were installed for a given position of the cell, but only occasional, minor adjustment by the cell positioner was needed over the period of a week. The entire assembly was mounted on a 2-in. thick optical breadboard (Newport, Fountain Valley, CA, U.S.A., Model LS-48) on a conventional laboratory bench, since we found that sophisticated vibration isolation was not needed. In our effort to reduce stray light, we found that reflections off the window surfaces could be a serious problem, so the cell windows were placed slightly off-normal to the laser beam.

We have improved the Faraday rotators from the previous design [19]. The modulating Faraday cell was placed after the flow-cell. This is an improvement since birefringence in the cell windows depends on the polarization direction, and must be decoupled from the applied modulation for the best signal-to-noise ratio. To increase the efficiency of the modulation driver, we used two matched Faraday rotators with windings in opposite directions. The pair was thus driven during alternate half-cycles of the square wave. This also guaranteed that electronic zero was always maintained in the lock-in amplifier and that the point of maximum extinction with the modulators off was the best setting for the analyzing polarizer. The modulating field was effectively four times that used previously, in view of eqn. 2 in ref. 19.

For the feasibility studies using the He—Ne laser (Spectra Physics, Mountain View, CA, U.S.A., Model 134), a 2X beam expander based on a Galilian telescope was used before the focusing lens so that the proper beam-waist can be achieved at the cell. In that case, an Amperex 56TVP phototube (North American Philips, Hicksville, NY, U.S.A.) was used to provide a better spectral response.

RESULTS AND DISCUSSION

The modifications in the detection system have led to a limit of detection of 100 ng of fructose injected (signal-to-noise ratio = 3) when two standard 10-μm C_{18} columns were used in series to increase the chromatographic efficiency. The limit in detectability seems to be a combination of (1) residual depolarized light through the crossed polarizers, (2) residual pumping noise in the eluent which causes the windows to distort minutely, (3) the remaining ±1% noise in the laser, and (4) dust particles causing flickering in the optical path. It appears that further improvements can be made, perhaps to a detectability of 20 ng.

In the course of identifying sources of noise, we used a 2-mW He—Ne laser as the light source in place of the argon ion laser. There is much more short-

TABLE I

CONCENTRATIONS OF SUGARS IN URINE

Sugar	Concentration (μg/ml)*		
	This work	Ref. 10	Normal range
Sucrose	22	12	0—50
Lactose	10	49	0—100
Glucose	16	24	10—120
Xylose	10	10	0—30
Arabinose	7	26	0—30
Fructose	7	2.7	0—50

*All concentrations are ± 10%.

term fluctuation in the He—Ne laser, so that the intensity cannot be stabilized as well. The lower light level also makes it more difficult to identify sources of stray light and to eliminate them. Even with these problems, we were able to obtain a detectability of 1 μg (signal-to-noise ratio = 3) for fructose under the same conditions. This demonstrates that photon statistics was not the limiting factor in detectability in the case of the argon ion laser. For the He—Ne laser, however, photon statistics was a major contribution. We estimate that a laser with a power of 10—50 mW can be used instead of the expensive argon ion laser, without sacrificing performance. We also found that when the lower power laser was used, the system took less time to warm up. This is because heating causes changes in birefringence in the polarizing crystals as well as the cell windows, and lower laser powers are desirable, up to the photon statistics limit.

Test solutions of individual sugars were run to determine the retention times under our experimental conditions. In general the order of elution and chromatographic efficiency were as specified by the manufacturer. No effort to further optimize was made. The results of the analysis of human urine are shown in Fig. 1. We have identified six urinary carbohydrates, based on the specificity of this column, retention times as correlated to injection of test solutions of sugars, the lack of UV absorption at the corresponding locations, the sign and magnitude of the individual specific rotation, and the estimated concentration levels in normal human urine [10]. The last comparison is shown in Table I, with the method for calculation given below. It is clear that determination of these carbohydrates as outlined here presents no problems. The peaks that appear prior to sucrose represent the unretained components and the higher saccharides, in that order. The major peak at 27 min does not seem to correlate with any of the carbohydrates tested, although one would expect species similar to mannitol and sorbitol to elute about then. It is interesting to note that the three major peaks in the UV detector correspond to a shoulder, a minor peak, and nothing at all in the optical activity detector. This emphasizes the substantial differences in the two detectors for studies in biological fluids. The UV peaks also indicate that even if a refractive index detector can approach this level of sensitivity,

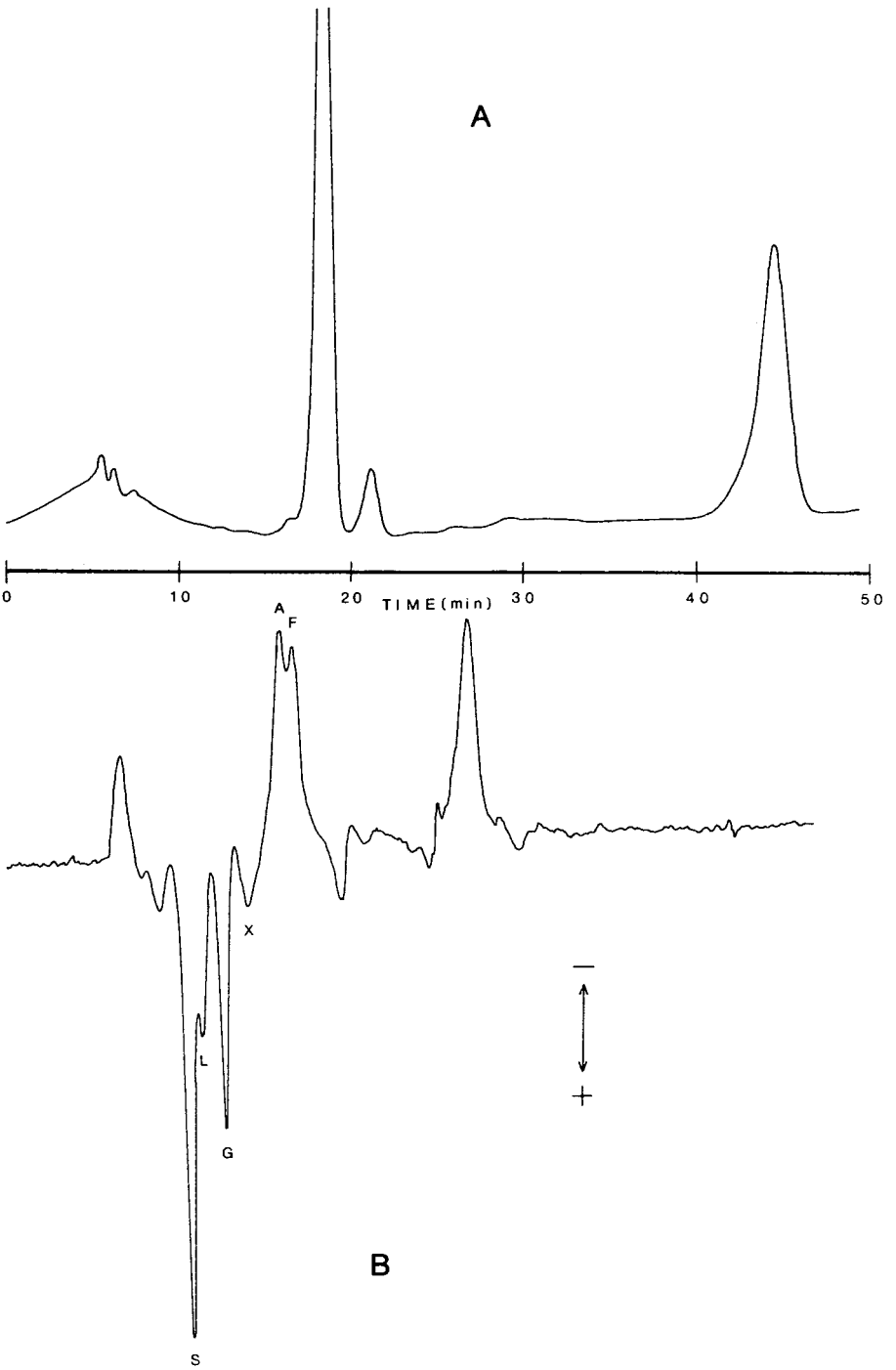

Fig. 1. Separation of components in human urine by HPX-87 heavy metal column. (A) UV detector, (B) optical activity detector. Peaks: S, sucrose; L, lactose; G, glucose; X, xylose; A, arabinose; F, fructose.

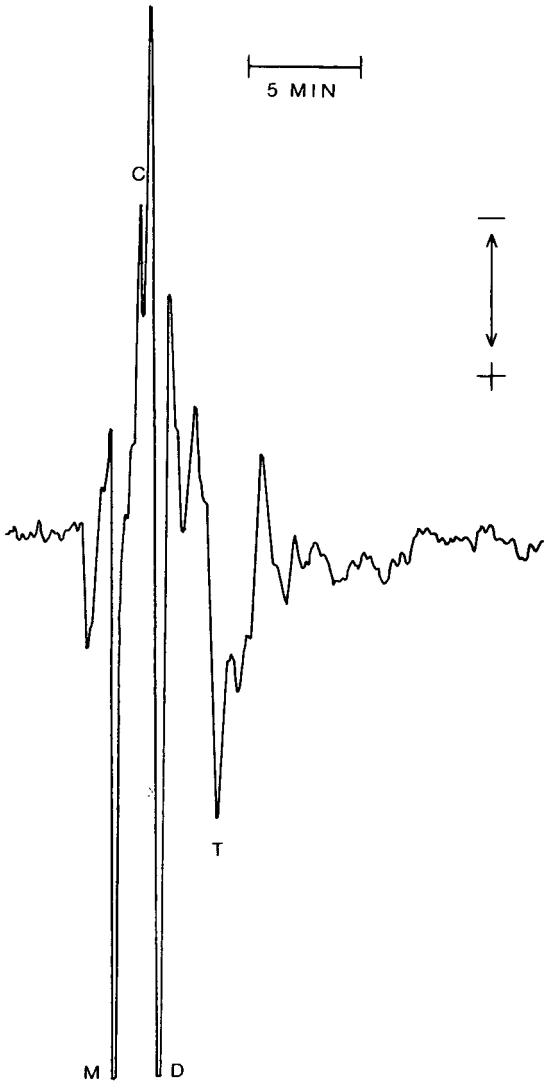

Fig. 2. Separation of components in human urine by 10-μm C_{18} reversed-phase column and optical activity detector. Peaks: C, cystine; M, monosaccharides; D, disaccharides; T, trisaccharides.

additional interferences will be present and limit its usefulness. The chromatographic efficiency can probably be further increased by lowering the flow-rate or by using a second column in series, but the simplicity of the chromatogram makes such a compromise with the analysis time not necessary. We have also studied the same deionized urine sample using two C_{18} reversed-phase columns in series, and the chromatogram shown in Fig. 2 is a substantial improvement over our earlier work [19]. We note that most of the sugars identified in Fig. 1B showed up as the second major positive peak, which was off-scale. This was accompanied by turbulence in the flow-cell caused by the large refractive index change, a condition that never oc-

curred in Fig. 1B. Even though the sugars and cystine are the only components positively identified, one can see that potentially useful information is present, particularly since very few of these peaks also show up in the UV detector.

Calculations of the concentrations are straightforward. Although naturally standards must be used for checking, one can use the absolute standard given by a d.c. Faraday cell of known properties at a known current. In this work we used a current of 0.25 A to produce a field of 250 G. With air, this corresponds to a net rotation of 0.28 millidegrees. Using the specific rotations $[\alpha]$ for the Na D line, which are not too different for this wavelength, one has:

$$c = \frac{\alpha}{[\alpha]} \tag{1}$$

where c is the concentration in g/ml at the detector, and α is determined by the ratio of the peak height and the effective height of the d.c. Faraday rotation, multiplied by 0.28 millidegrees, since our cell is 1 dm in length. The $[\alpha]_D$ values were used taking into account mutarotation. The peaks in Fig. 1 are typically in an eluent volume much larger than that of the flow-cell, so that a triangular approximation can be used for the area. Conversion to total quantity injected is then simple, knowing that the flow-rate is 0.64 ml/min. Since the chromatography was highly reproducible, one could deconvolute the overlapping peaks using a Gaussian shape if desired. However, the fluctuations in human urine did not warrant such an attempt at high accuracy in this work, and the simple triangular approximation was used. The results of this particular urine sample are presented in Table I, together with some other reference values [10]. We find that the determined values are reasonable. For routine applications, one would probably rely on peak height measurements established by standard solutions, rather than use the absolute standard method described here.

In summary, we have refined our method of detection of optically active components in HPLC. Sensitivity is sufficient for the study of urinary carbohydrates, particularly the monosaccharides. We report a straightforward procedure for the determination of six sugars simultaneously in human urine by direct injection, a scheme which should allow more clinical studies correlating these with various physiological conditions.

ACKNOWLEDGEMENTS

We thank Dr. S.D. Woodruff for helping in the electronic designs of the driving circuit and the intensity stabilization circuit. This research was supported by the Director for Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences, WPAS-KC-03-02-03, through the Ames Laboratory, which is operated for the U.S. Department of Energy by Iowa State University under Contract No. W-7405-Eng-82.

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Journal of Chromatography, 223 (1981) 331—339
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 803

SIMULTANEOUS DETERMINATION OF PHENCYCLIDINE AND MONOHYDROXYLATED METABOLITES IN URINE OF MAN BY GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY WITH METHANE CHEMICAL IONIZATION

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(First received July 22nd, 1980; revised manuscript received November 24th, 1980)

SUMMARY

Phencyclidine and monohydroxy metabolites were measured in human urine using gas chromatography—mass fragmentography with methane chemical ionization. Samples were extracted either untreated or following acid hydrolysis, derivatized with heptafluorobutyric anhydride, separated on a 3% SE-30 column and analyzed by mass fragmentography. The assay was sensitive to ca. 0.01 μg/ml for phencyclidine and ca. 0.05 μg/ml for the metabolites. Urine samples from five human subjects enrolled in a methadone maintenance program who had ingested phencyclidine were analyzed. The phencyclidine concentration ranged from 0.3 to 23.7 μg/ml. The concentrations of metabolites ranged from 0 to 1.8 μg/ml. A new monohydroxy metabolite was detected in the samples, but its structure was not fully elucidated. The specificity of the assay was examined.

INTRODUCTION

Phencyclidine (PCP) was developed in the 1950s for use as an anesthetic agent in man. Its therapeutic usefulness was curtailed, however, when extremely disturbing side-effects were noted in postsurgical patients. These effects included drunkenness, blurring of vision, delusions and a general impairment of mental function [1].

PCP appeared later in the drug subculture of the late sixties as the "Peace Pill". The ready availability of starting materials and the ease of synthesis of PCP and derivatives, together with the lure of substantial financial gains, prompted its widespread illicit synthesis and distribution. As a consequence, PCP has become a major drug of abuse in the United States. Recent reports indicate a rising number of overdose cases [2—5] and PCP-related deaths [6—9].

Detection of drug usage is essential in the treatment of overdosage since PCP can produce a psychosis resembling that of schizophrenia [1,10,11]. Of interest also is the role of pharmacologically active metabolites in the overall spectrum of effects of PCP. The monohydroxylated metabolites [4-phenyl-4-piperidinocyclohexanol (PPC) and 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (PCHP), see Fig. 1] have generally been assumed to be inactive [6,9, 12,13], based on the work by McCarthy and Potter [14] who reported the absence of PCP-like activity for PPC and PCHP in the monkey. Recently, however, studies in this laboratory [15,16] and elsewhere [17] have indicated that both PPC and PCHP exhibit significant biological activity.

The assays that have been developed for measurement of PCP include use of gas chromatography (GC) with flame-ionization detection [7,8], GC with a nitrogen-sensitive detector [5,6], gas chromatography—mass spectrometry (GC—MS) [18], mass fragmentography (MF) [4,8,19—21] and radioimmunoassay [22]. At present, however, there is no assay which also measures the hydroxy metabolites. Consequently, we sought to develop an assay for PCP, PPC and PCHP which would be applicable for the simultaneous measurement of these compounds in human urine. This report describes an MF assay specific for PCP, PPC and PCHP and its application to the analysis of human urine from subjects who had smoked or ingested an unknown quantity of illicit PCP.

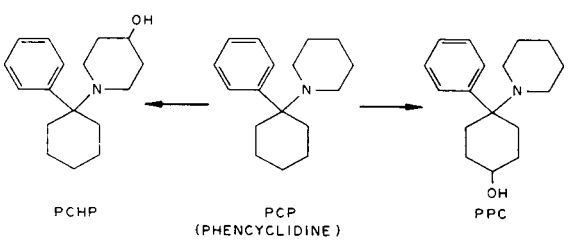

Fig. 1. Metabolism of phencyclidine (PCP) to 4-phenyl-4-piperidinocyclohexanol (PPC) and 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (PCHP).

EXPERIMENTAL

Standards and reagents

PCP, PCC and 1-(1-phenylcyclohexyl)morpholine (PCM, internal standard) were obtained from the Research Technology Branch, Division of Research, National Institute on Drug Abuse, Rockville, MD, U.S.A. Their structural identity and purity were confirmed by thin-layer chromatography, GC and GC—MS.

Heptafluorobutyric anhydride (HFBA) was purchased from Pierce, Rockford, IL, U.S.A. All other chemicals were of reagent grade quality.

Instrumentation

A Finnigan Model 4021 automated gas chromatograph—mass spectrometer—data system operating in the chemical ionization (CI) mode was used. Methane was used as reagent and carrier gas at a flow-rate of 16 ml/min. The gas chromatograph consisted of a glass column (1.83 m × 2 mm I.D.) packed with 3%

SE-30 on Gas-Chrom Q (100—120 mesh) and was coupled to the mass spectrometer by a glass-lined stainless-steel tube and a venting valve. After sample injection, the venting valve was opened for 90 sec, allowing solvent and volatile substances to escape without entering the ion source. The temperatures of the injector, column, interface oven, and ion source were maintained at 165, 190, 250 and 250°C, respectively. The electron energy was set at 70 eV and the multiplier voltage at 1.35 kV. The ions selected for monitoring were *m/e* 242.1, 243.1, 245.1 and 455.1. Under these conditions selective responses for the compounds of interest were obtained at the following relative retention times: PCP, 0.79; PCM, 1.0; PPC, 1.56; PCHP, 1.79. Fig. 2A shows a typical mass fragmentogram of PCP, PCM, PPC and PCHP.

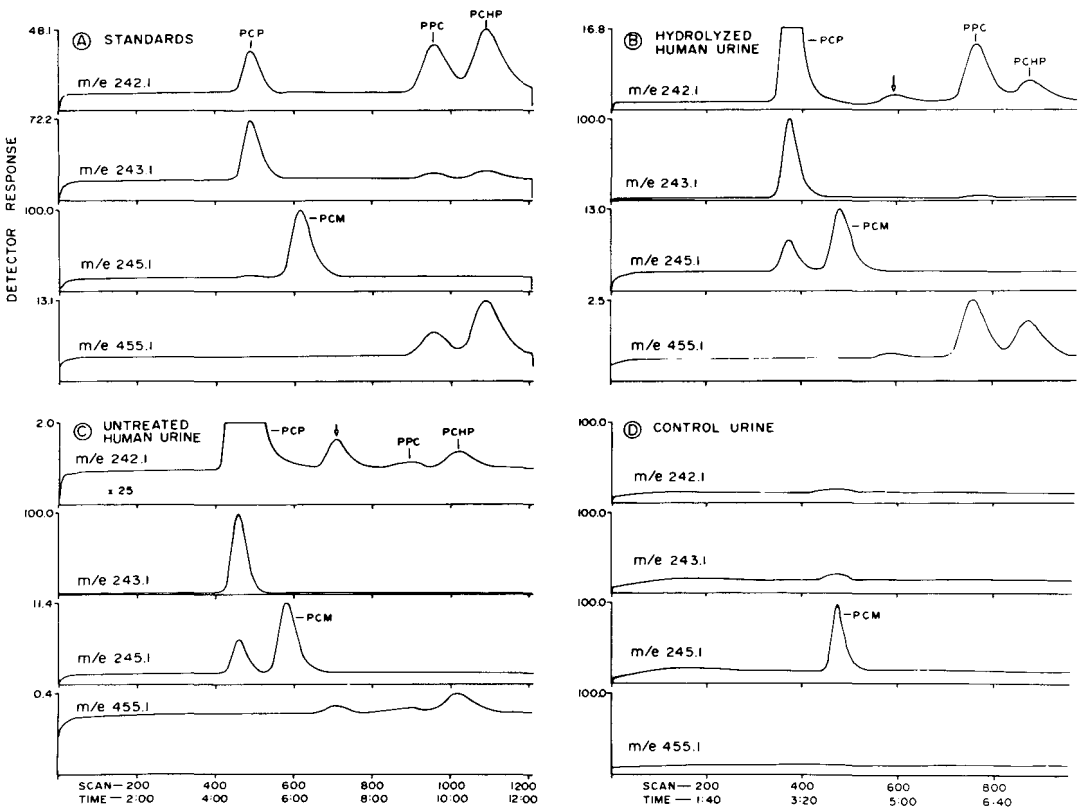

Fig. 2. Mass fragmentograms of phencyclidine (PCP) urine extracts to which 10 μg of 1-(1-phenylcyclohexyl)morpholine (PCM) were added. (A) Normal urine with PCP, 4-phenyl-4-piperidinocyclohexanol (PPC) and 1-(1-phenylcyclohexyl-4-hydroxypiperidine (PCHP) added. (B) Acid-hydrolyzed human urine from subject who ingested an unknown amount of PCP. (C) Same sample as (B) without acid hydrolysis. (D) Control urine. Arrow in (B) and (C) indicates presence of new hydroxy metabolite of PCP.

Human urine samples

Urine specimens from subjects who had taken PCP were obtained from the Veterans Administration Regional Drug Screening Laboratory, Veterans Ad-

ministration Medical Center, Sepulveda, CA, U.S.A. The subjects were enrolled in a methadone maintenance program at the time of sample collection.

Sample preparation and extraction

An aliquot (5—10 ml) of each urine specimen was mixed with internal standard (10 μg of PCM) and extracted either untreated or following acid hydrolysis. These extracts contained the free drug and metabolite fraction or total drug which included both free and conjugated drug species liberated by acid hydrolysis. The samples to be hydrolyzed were treated with a 10% volume of conc. hydrochloric acid and autoclaved at 115°C at 15 p.s.i. for 30 min.

Prior to extraction the pH of all samples was adjusted to ca. pH 10, and 3 ml of 3 *N* potassium carbonate were added to give a final pH of 10.5. Sodium chloride (0.5 g) and hexane (12 ml) were added and the contents were shaken and centrifuged. An aliquot (10 ml) of the organic phase was transferred to a clean tube containing 5 ml of 2 *N* hydrochloric acid. The tubes were shaken, centrifuged and the organic phase was discarded. The pH was adjusted as before to a final value of 10.5. Sodium chloride (0.5 g) and hexane (12 ml) were added and the contents were shaken and centrifuged. An aliquot (10 ml) of the organic phase was transferred to a clean, conical tube and evaporated to dryness under nitrogen at 40°C. Benzene (0.1 ml) and HFBA (20 μl) were added and the tube was sealed and heated at 85—90°C for 60 min. Ammonium hydroxide solution (1 ml of 5% v/v) was added and the tube was shaken and centrifuged. Benzene (50 μl) was added and the aqueous layer was discarded. An aliquot (1—3 μl) of the organic layer was removed for analysis.

Calibration curves and quantitative analysis

Standard curves were constructed from the analysis of standard solutions. The solutions were prepared by adding known amounts of drug and metabolites (0—8 μg) to control urine containing PCM (10 μg) added as internal standard. The samples were hydrolyzed, extracted, derivatized and analyzed by MF in the same manner as that described for the drug urines. Linear relationships for peak intensity ratios of drug or metabolite to internal standard versus concentration were observed throughout the concentration range. Correlation coefficients (r) were typically $\geqslant 0.98$. The lower limits of the assay were ca. 0.01 μg/ml for PCP and 0.05 μg/ml for PPC and PCHP. There was sufficient volume for duplicate analyses of some of the drug urines and the mean ± standard error of the analyses is reported.

Recovery studies

The addition of PCP, PPC and PCHP to urine samples at a concentration of 10 μg/ml resulted in overall recoveries ± S.E. of 65.8 ± 4.5%, 70.2 ± 3.7% and 73.4 ± 2.6%, respectively, for the analysis of free drug and 64.4 ± 1.7%, 69.4 ± 2.3% and 69.8 ± 2.9%, respectively, for the acid-hydrolyzed samples.

Assay specificity

In an attempt to identify substances that might interfere in the assay for PCP and metabolites, a number of compounds commonly encountered in toxicological screens were processed under the same conditions as those de-

TABLE I

RELATIVE GC RETENTION DATA ON 3% SE-30 (190°C) OF COMPOUNDS COMMONLY ENCOUNTERED IN TOXICOLOGICAL SCREENS

Retention times are reported relative to the internal standard, PCM. Relative retention times were reproducible under different operating conditions, i.e. changes of venting time, column temperature. Values in parentheses represent uncorrected retention times in minutes.

Compound	Relative retention time*
Methadone	2.0 (6.4)
Methadone metabolites	1.0 (3.2), 1.3 (4.2)
Morphine	—
Codeine	—
Propoxyphene	0.8 (2.6), 0.9 (2.8), 2.3 (7.4)
Cocaine	2.3 (7.3)
Methaqualone	1.9 (6.0)
Amphetamine	0.1 (0.3)
Diazepam	1.9 (6.1)
Chlordiazepoxide	1.3 (4.1)
Glutethimide	0.7 (2.3)
Phenobarbital	—
Pentobarbital	—
Amobarbital	—
Secobarbital	—
Chlorpromazine	—
Ethchlorvynol	—
Acetylsalicylic acid	—
Doxepin	—
Dextromethorphan	1.8 (5.9)
Meprobamate	—
Ketamine	0.7 (2.2)
1-[1-(2-Thienyl)cyclohexyl]piperidine (TCP)	0.2 (0.6), 0.8 (2.6)
N-Ethyl-1-phenylcyclohexylamine (PCE)	0.2 (0.7)
1-(1-Phenylcyclohexyl)pyrrolidine (PCPY)	0.6 (2.0)
PCP	0.8 (2.7)
PPC	1.5 (4.8)
PCHP	1.7 (5.3)
PCM (internal standard)	1.0 (3.2)

*— indicates that the compound did not elute from the column under these conditions.

scribed for PCP. Mass spectral data were obtained for those substances which eluted from the gas chromatograph with similar retention times to the compounds of interest (see Table I). Limited mass scans were analyzed for possible interferences.

Identification of phencyclidine and metabolites

Total ion current chromatograms were obtained routinely for urine extracts from each subject. PCP and the hydroxy metabolites, PPC and PCHP, were identified by comparison of their spectra (Fig. 3) with those of authentic standards (Table II). A new hydroxy metabolite was detected in extracts from a number of subjects (Fig. 2, relative retention time = 1.2); however, there was not enough sample to obtain total ion scans with sufficient intensity for structural assignment.

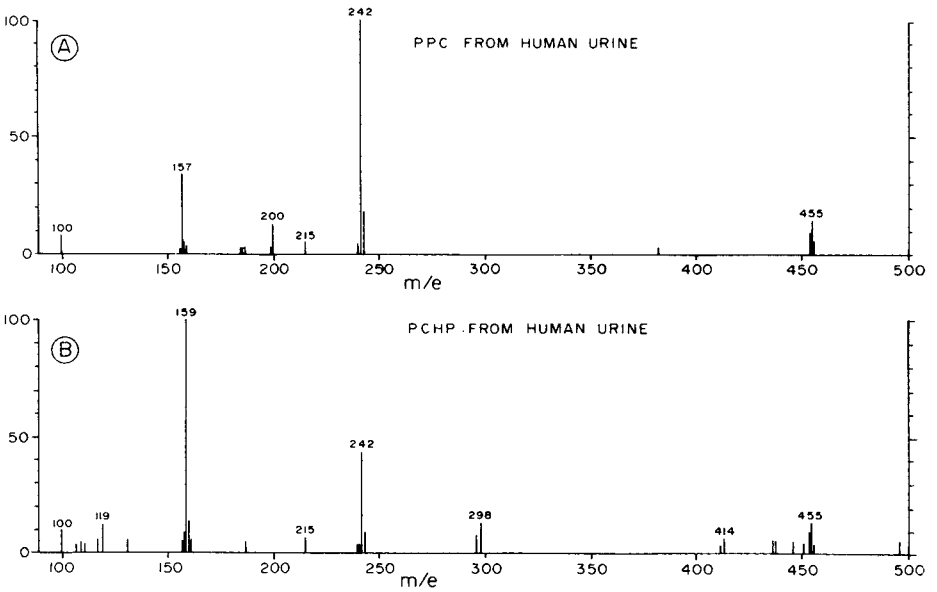

Fig. 3. Methane chemical ionization spectra of metabolites of phencyclidine from human urine identified as: (A) 4-phenyl-4-piperidinocyclohexanol (PPC); (B) 1-(1-phenylcyclohexyl)-4-hydroxypiperidine (PCHP).

TABLE II

METHANE CI SPECTRA OF PHENCYCLIDINE, METABOLITES (HEPTAFLUOROBUTYRYL DERIVATIVES) AND THE INTERNAL STANDARD, PCM [1-(1-PHENYLCYCLOHEXYL)MORPHOLINE]

Compound	Molecular weight	Methane CI spectra (m/e)*			
		$[M+H]^+$	M^+	$[M-H]^+$	Prominent ions**
PCP	243	244 (28)	243 (100)	242 (48)	166 (8), 159 (68), 119 (6)
PPC	455	456 (4)	455 (19)	454 (30)	243 (30), 242 (100), 200 (5), 157 (42)
PCHP	455	456 (7)	455 (22)	454 (9)	298 (19), 296 (10), 243 (16), 242 (86), 215 (7), 187 (11), 160 (13), 159 (100), 158 (5), 157 (9), 119 (9)
PCM	245	246 (6)	245 (31)	244 (13)	160 (11), 159 (100), 116 (11)

*Percentage abundance in parentheses.
**Only ions $\geqslant$ 5% abundance are reported.

RESULTS AND DISCUSSION

A mass fragmentographic (MF) assay was developed for the determination of PCP and the hydroxy metabolites, PPC and PCHP, in human urine. The GC—MS system was operated in the CI mode using methane as the reagent gas. Urine samples were extracted with hexane, derivatized with HFBA, and analyzed by MF. Quantitation was performed by the internal standard method

using PCM, an analog of PCP, for standardization. Standard curves were generated on a daily basis and were linear throughout the ranges tested.

Human urine samples were analyzed either untreated or following acid hydrolysis. The acid-hydrolysis procedure was used to cleave conjugated metabolites and to allow the estimation of their amount in urine. The possibility of loss of PCP or metabolite from this treatment was considered; however, studies showed that there were no differences in recoveries by either method.

The extraction procedure involved extraction with hexane, back-extraction into acid solution followed by re-extraction at high pH with hexane. Recoveries by this method were in the range of 64—73%. Although it was found that recoveries could be improved by extraction with chloroform, the use of hexane provided cleaner extracts with reduced background.

Prior to sample analysis the extracts were derivatized with HFBA in benzene at elevated temperatures. This procedure produced mono-acyl derivatives of the hydroxy metabolites, PPC and PCHP. Although PPC, PCHP and PCP can be separated by GC on SE-30 without derivatization [23], an improvement in peak shape and resolution of the metabolites was achieved by derivatization. The derivatives also exhibited good stability. Derivatized extracts that were 1—5 days old and stored at 0—5°C showed minimal decreases in intensity of response when reanalyzed. PCP and PCM were unaffected by the derivatization process.

For assaying by MF, the ion selected for monitoring for PCP, PPC and PCHP was *m/e* 242.1. This represents the $[M - H]^+$ ion for PCP and the $[M - OOCCF_2CF_3]^+$ ions for PPC and PCHP. The molecular ion (M^+, *m/e* 245.1) was monitored for the internal standard. The ions at *m/e* 243.1 and *m/e* 455.1 (M^+ ions for PPC and PCHP) were monitored for confirmation (see Fig. 2). Standard methane CI spectra are presented in Table II for PCM, PCP and the HFBA derivatives of PPC and PCHP.

Using the procedures described, the concentrations of PCP and hydroxy metabolites were measured in urine from five human subjects who had self-administered PCP (Table III). The amount of PCP was found to be highly variable from subject to subject, ranging from 0.3 to 23.6 μg/ml. Only traces of PPC and PCHP were detected in untreated urine, whereas considerably more was usually present following acid hydrolysis. Apparently PPC and PCHP are excreted for the most part in conjugated form. The amount of PPC ranged from 0.03 to 1.79 μg/ml with a mean of 0.9 μg/ml for the acid-hydrolyzed samples. The amount of PCHP following acid hydrolysis was generally less than that of PPC and ranged from 0 to 0.48 μg/ml with a mean of 0.2 μg/ml. Total ion chromatograms were used for final verification of structure of the metabolites.

A new metabolite of PCP was detected in each of the urines of the five subjects. This metabolite had a relative retention time of 1.2 (see Fig. 3B and C) and an apparent molecular weight equal to that of PPC or PCHP. The metabolite formed a mono-acyl derivative which suggests that the metabolite is a new hydroxylated metabolite of PCP. The molecular weight assignment was based on the presence of the ion at *m/e* 455.1. Unfortunately, there were insufficient amounts of the new metabolite for further spectral work and the assignment of position of hydroxylation awaits further study.

TABLE III

CONCENTRATIONS OF PCP AND MONOHYDROXYLATED METABOLITES IN URINE OF HUMAN SUBJECTS

Values are expressed in μg/ml.

Subject No.	PCP		PPC		PCHP	
	UT*	AH**	UT	AH	UT	AH
8	6.30±0.05	5.94±0.74	Trace	1.79±0.34	0.02±0.0	0.48±0.06
19	0.33	0.37±0.03	0	0.03	0	0.07±0.01
29	4.10	4.13	0	0.10	0	0
39	—	1.70	—	0.81	—	0.10
46	23.65	24.68	0	1.53	0.05	0.28
Mean ± S.E.	8.60±5.2	7.4±4.4	0	0.9±0.4	0.02±0.01	0.2±0.1

*UT = untreated samples.
**AH = acid-hydrolyzed samples.

It is likely that the urine samples contained other drugs in addition to PCP since they were obtained from subjects who were enrolled in a methadone maintenance program at the time of sample collection. Both methadone and metabolites were identified in the total ion chromatogram of several of the urine extracts; however, the specificity of the MF assay was sufficient to measure PCP, PPC and PCHP in the presence of these substances without interferences. In addition, a number of other drugs which are commonly encountered in toxicological screens were added to the assay to determine if they presented interferences (see Table I). Although there was an occasional overlap of retention time, none of these drugs interfered in the MF assay. The procedure is sufficiently sensitive and specific for the determination of PCP and hydroxy metabolites in human urine and can provide the basis for further investigation of the pharmacological roles of PCP metabolites.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Joyce McIntyre, Supervisory Technologist of the Sepulveda V. A. Toxicology Laboratory, V. A. Medical Center, Sepulveda, CA, for her aid in obtaining the biological specimens. We also thank Mr. W.G. Marquardt and Ms. I. Sibcy for their assistance in the preparation of this manuscript.

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Journal of Chromatography, 223 (1981) 341—349
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 826

SPECIFIC DETERMINATION OF PLASMA NICARDIPINE HYDROCHLORIDE LEVELS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received October 24th, 1980)

SUMMARY

A highly specific method for the determination of the plasma level of the potent vasodilator 2-(N-benzyl-N-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine carboxylate hydrochloride (nicardipine hydrochloride) in rats, dogs and humans is described. N-d_3-Methyl derivative was added as an internal standard, then the plasma was extracted with diethyl ether and subjected to thin-layer chromatography (TLC) to remove the pyridine analogue, one of the drug's metabolites. The area corresponding to the unchanged drug was identified with simultaneously run N-d_7-benzyl derivative under UV light. The unchanged drug with a 1,4-dihydropyridine structure was oxidized with nitrous acid to its pyridine analogue, which was stable for gas chromatography, and subjected to mass spectrometry at *m/e* 134 (nicardipine) and *m/e* 137 (N-d_3-methyl derivative). The sensitivity limit was 5 ng ml^{-1}. The ratio of the unchanged drug to the value obtained by the method without TLC separation was 100% for rats and 80% for dogs and humans at almost all times investigated after dosing. These results demonstrate that in these species, the amount of pyridine analogue in plasma was very small compared with that of the parent drug.

INTRODUCTION

Nicardipine hydrochloride, 2-(N-benzyl-N-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine carboxylate hydrochloride, is a new potent vasodilator [1,2], nanogram amounts of which in plasma have been measured by gas—liquid chromatography with electron-capture detection (ECD-GLC) [3] and gas chromatography—mass spectrometry (GC—MS) [4]. In those assays, prior to chromatography, the drug was oxidized with nitrous acid to its pyridine analogue, which is stable for gas chromatography. These

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methods are sufficiently sensitive to determine the plasma concentration of the drug after the oral administration of therapeutic doses to humans. However, when the pyridine analogue is present in the plasma as a metabolite, the values obtained by these methods represents the sum of the unchanged drug and the metabolite. We describe here a highly specific method for determining the unchanged drug by GC—MS after removing the pyridine analogue by thin-layer chromatography (TLC). The plasma concentrations in healthy volunteers were also measured by two previous methods [3, 4], which are the same in principle except for the detector, and the specificities were compared.

EXPERIMENTAL

Chemicals

Nicardipine hydrochloride and two deuterium-labelled compounds (N-d_3-methyl and N-d_7-benzyl derivatives) (Fig. 1) were described in a previous paper [4]. Tablets containing 20 or 30 mg were prepared in our laboratories. Other chemicals and reagents were obtained commercially and were of analytical-reagent grade.

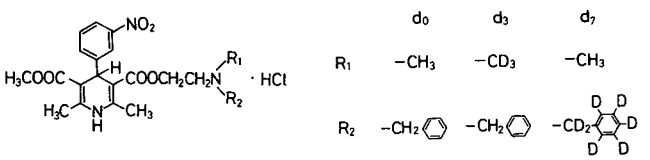

Fig. 1. Structures of nicardipine hydrochloride and its deuterium-labelled compounds.

Gas chromatography—mass spectrometry

A JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) and a Hewlett-Packard 5710A gas chromatograph were used. Separation was effected on a glass column (1.8 m × 2 mm I.D.) packed with 3% OV-1 80—100-mesh Chromosorb W AW DMCS (Nihon Chromato Works, Tokyo, Japan). The column temperature was maintained isothermally at 270°C and the flash heater and separator temperatures were held at 280°C. The flow-rate of the carrier gas (helium) was 30 ml min^{-1}. The ionization potential and trap current were 20 eV and 80 μA, respectively. The entrance and collector slits of the mass spectrometer were adjusted to 0.4 mm. The multiplier voltage supply was set at 2.0—2.3 kV.

Procedure

The ECD-GLC method was as described previously [3]. The GC—MS method has also been reported previously [4]. In the present study, however, the N-d_3-methyl derivative was used as an internal standard (I.S.).

GC—MS combined with TLC (TLC—GC—MS). A plasma sample (1 ml) was introduced into a 10-ml centrifuge tube and 0.5 ml of the internal standard solution (N-d_3-methyl derivative, 200 ng) and 0.5 ml of sodium hydroxide solution (2 *M*) were added, mixed and extracted with two 2-ml volumes of diethyl ether. The organic layer was evaporated to dryness in a water-bath at 45°C. The pyridine analogue of the N-d_7-benzyl derivative in benzene (2 μg,

0.05 ml) was added to the residue as a TLC marker. For TLC (silica gel F_{254}, Merck, Darmstadt, G.F.R.; 0.25 mm thickness) chloroform—acetone—diethylamine (5:4:1) was used as the solvent. The R_F values of nicardipine and the pyridine analogue were 0.57 and 0.64, respectively.

Areas corresponding to unchanged nicardipine and its pyridine analogue were identified with simultaneously run N-d_7-benzyl derivatives and the pyridine analogue of N-d_7-benzyl derivative added to the plasma extract, using UV light (254 nm).

The area corresponding to the unchanged drug was scraped from the plate, introduced into a 10-ml centrifuge tube and 4 ml of hydrochloric acid (0.05 *M*) were added. To the aqueous layer was added 0.3 ml of sodium nitrite solution (0.15 *M*) and the mixture was kept in a water-bath at 45°C for 1 h. This procedure resulted in oxidation of the 1,4-dihydropyridine rings of the labelled and non-labelled drug to pyridine rings. After cooling, the mixture was made alkaline with 0.5 ml of sodium hydroxide solution (2 *M*) and extracted with two 2-ml volumes of benzene. The organic layer was evaporated to dryness under reduced pressure and 50 μl of benzene were added to the residue. A 3-μl aliquot was injected into the gas chromatograph—mass spectrometer. Fragment ions at *m/e* 134 and 137 were used to monitor the pyridine analogues of nicardipine and the internal standard, respectively. The amount of nicardipine hydrochloride in each plasma sample was calculated by measuring the peak-height ratios of the pyridine analogues of the drug and internal standard and comparison with a calibration graph, prepared by subjecting control plasma, to which known amounts of nicardipine hydrochloride (5—200 ng) had been added, to the above procedure and plotting the peak-height ratios of the pyridine analogues of corresponding nicardipine hydrochloride and internal standard against the drug concentration.

Recoveries

Control plasma samples (1 ml) containing 100 ng of nicardipine hydrochloride were carried through the above procedure in the absence of internal standard. The pyridine analogue of the internal standard (200 ng), dissolved in benzene, was added to the benzene extract, the benzene solution was evaporated to dryness under reduced pressure and 50 μl of benzene were added to the residue. A 3-μl aliquot was injected into the gas chromatograph—mass spectrometer. Recoveries were calculated by comparing the peak-height ratios with those obtained when the pyridine analogues of the drug and the internal standard, dissolved in benzene, were processed without the extraction procedure.

Specificity

In the present assay procedure, the pyridine metabolite was removed by TLC prior to GC—MS. To check the specificity of this method, we added 100 ng of nicardipine hydrochloride and 100 ng of its synthesized pyridine derivative to 1-ml plasma samples. These samples were then subjected to the TLC—GC—MS method and the recovery of unchanged drug was calculated.

Animal studies

Male Sprague—Dawley rats (100—130 g) and male beagle dogs (9—11 kg), maintained with free access to food and water, were fasted overnight prior to the oral administration of the drug in aqueous solution. After dosing, the animals were kept in individual metabolism cages. Blood samples, obtained a heparinized syringe from the inferior vena cava of the rats and the antecubital vein of the dogs, were centrifuged at 980 *g* for 15 min. Plasma samples were stored at −20° C until taken for assay.

Human studies

After overnight fasting, four healthy adult males (56—75 kg, age 30—36 years) each received 20 mg of nicardipine hydrochloride in tablet form and another four male subjects (54—64 kg, age 31—37 years) each received a 30-mg tablet. Blood samples were obtained from the cubital vein with heparinized syringes and centrifuged at 980 *g* for 15 min. The plasma samples were stored at −20° C until taken for assay.

Plasma samples from patients

Hypertensive patients recieved 20 mg of nicardipine hydrochloride t.i.d. for several weeks and their physicians submitted the plasma samples for determination of the drug concentration. Residual samples, after analysis by the ECD-GLC method reported previously [3], were used in the present study.

RESULTS AND DISCUSSION

We evaluated nicardipine hydrochloride as a potential therapeutic agent in the treatment of cerebrovascular, hypertensive and angina diseases. This drug, selected from among many 1,4-dihydropyridine derivatives, is water-soluble and is completely absorbed from the gastro-intestinal tract after oral administration [5]. However, in animals and humans, the plasma concentration of the unchanged drug after oral administration was very low owing to its first-pass metabolism [6]. Higuchi and co-workers [3, 4] have reported sensitive methods for the determination of nanogram amounts of nicardipine hydrochloride in plasma and these methods were applied in our earlier studies [6—8]. When nanogram amounts of the drug were injected into the gas chromatograph, the drug was partly oxidized to its pyridine analogue, resulting in two peaks [3]. The difficulties in the assay were overcome by oxidizing 1,4-dihydropyridine to pyridine with nitrous acid before chromatography and the pyridine analogue was detected with an electron-capture detector [3] or by mass spectrometry [4]. Although these methods are sensitive enough to determine drug levels as low as 2—3 ng ml^{-1}, when the pyridine analogue is present in the plasma as a metabolite the values obtained represent the sum of the unchanged drug and the metabolite, whose vasodilative effect is 300 times weaker than that of the parent drug [9]. An experimental study on rats and dogs, in which we used a ^{14}C-labelled compound, revealed that, compared with the unchanged drug, the amount of this metabolite was negligible in the plasma [3]. However, to obtain data on humans, a more specific method is required for determination of the unchanged drug in plasma.

In the present assay, TLC was used to remove the pyridine analogue before the oxidation of dihydropyridine to pyridine analogue with nitrous acid. The amounts of nicardipine and the N-d_3-methyl derivative, added as an internal standard, in plasma were in the nanogram range, so that the area corresponding to the compounds after TLC could not be detected with UV light (254 nm). The area of the unchanged drug was identified with the aid of both simultaneously run N-d_7-benzyl derivative and the pyridine analogue of N-d_7-benzyl derivative, which was added to the plasma extract. R_F values of nicardipine and its pyridine analogue from the plasma extract were slightly different from those of simultaneously run N-d_7-benzyl derivatives because the plasma extracts contained large amounts of endogenous substances. Therefore, the pyridine analogue of the N-d_7-benzyl derivative was added to the plasma extracts as a TLC marker to differentiate the area of the unchanged drug from that of its pyridine analogue. Even if d_7-benzyl derivatives, the amounts of which applied to TLC were large compared with those of nicardipine and the internal standard, slightly contaminated the sample during the procedure, this did not interfere with the assay because the compound does not have fragment ions at *m/e* 134 and 137, which were used to monitor nicardipine and the internal standard in the GC—MS method.

To check the specificity of the assay, we determined the recovery of unchanged drug from plasma containing both unchanged drug and the pyridine analogue. The value obtained of 101.1 ± 2.2% (n = 6) indicates that the method is highly specific.

Chromatograms of human plasma samples, obtained by the TLC—GC—MS method, are shown in Fig. 2. The drug-free control plasma (Fig. 2a) gave no interfering peaks at *m/e* 134 and 137, selected for monitoring pyridines of nicardipine and the N-d_3-methyl derivative, respectively.

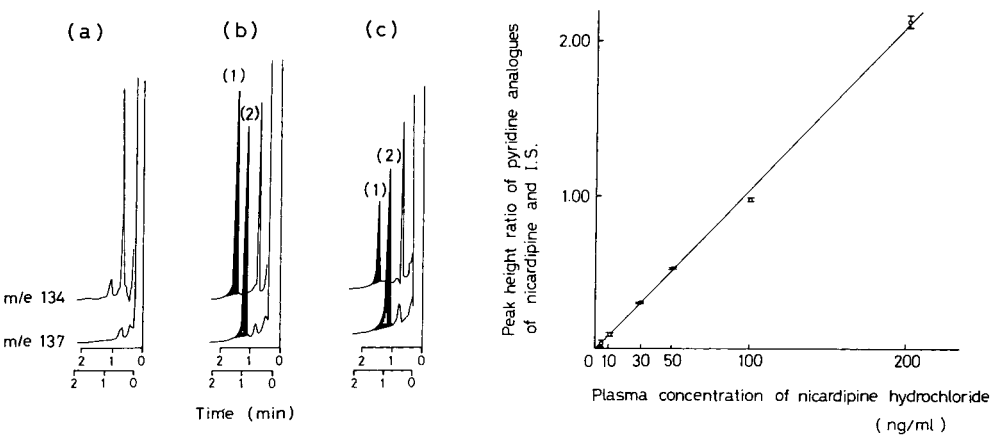

Fig. 2. Chromatograms of human plasma samples obtained by TLC—GC—MS. (a) Control plasma; (b) calibration standard containing nicardipine (1) and the N-d_3-methyl derivative (2) (200 ng ml^{-1} of each); (c) plasma sample obtained at 9.00 a.m. from a hypertensive patient who had received 20 mg of nicardipine hydrochloride t.i.d. for several weeks.

Fig. 3. Calibration graph for determining nicardipine hydrochloride by the TLC—GC—MS method. Each point represents the mean ± standard error from three experiments.

TABLE I

RATIO OF THE PLASMA CONCENTRATION OF THE DRUG OBTAINED BY TLC—GC—MS TO THAT OBTAINED BY GC—MS AFTER THE ORAL ADMINISTRATION OF NICARDIPINE HYDROCHLORIDE TO RATS, DOGS AND HEALTHY HUMANS (%)

Species	Number	Dose	0.5 h	1 h	2 h	4 h	6 h	8 h
Rat	3	5 mg/kg	100 ± 3	87 ± 9	97 ± 10	100 ± 4	—*	—*
Dog	4	5 mg/kg	87 ± 4	95 ± 1	88 ± 6	77 ± 6	84 ± 3	88 ± 1
Man	4	20 mg	95 (n=2)	74 ± 1	87 ± 6	99 ± 3	—*	—*
Man	4	30 mg	82 ± 6 (n=3)	84 ± 6	82 ± 6	109 ± 8 (n=3)	110 ± 7 (n=3)	—*

*The value was below the limit of detection.

The calibration graph obtained from the analysis of animal plasma samples containing various amounts of nicardipine hydrochoride gave a straight line over the concentration range of 5—200 ng ml^{-1} (Fig. 3). The recovery from plasma was 92.5 ± 5.3% (n = 6).

This method was sensitive enough to determine plasma levels of the drug as low as 5 ng ml^{-1} when 1-ml plasma samples were used; it was also found to be applicable to the measurement of plasma concentrations occurring after the administration of therapeutic doses to humans.

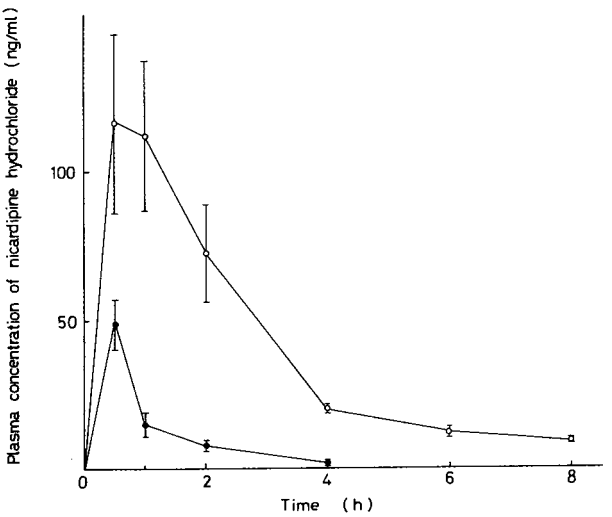

Fig. 4. Plasma concentration of the unchanged drug obtained by TLC—GC—MS after the oral administration of nicardipine hydrochloride (5 mg/kg) to rats (•——•) and dogs (○——○). Each point represents the mean ± standard error from three rat and four dog experiments.

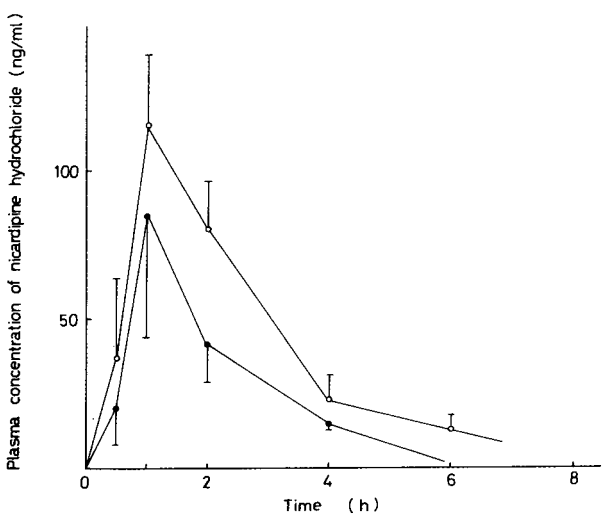

Fig. 5. Plasma concentration of the unchanged drug obtained by TLC—GC—MS after the oral administration of nicardipine hydrochloride [20 mg (•——•) or 30 mg (○——○)] to healthy volunteers. Each point represent the mean ± standard error from four subjects.

The plasma concentrations of the drug after oral administration of nicardipine hydrochloride to rats, dogs and humans were determined by two methods, GC—MS and TLC—GC—MS. The values obtained by the latter method represent the unchanged drug concentration (Figs. 4 and 5), whereas those obtained by the former method represent the sum of the unchanged drug and its pyridine metabolite. There are marked differences in the plasma concentrations among the species examined. This difference is thought to be due to differences in the hepatic metabolizing enzyme activity of the drug [6]. The ratio of the unchanged drug to the value obtained by GC—MS is shown in Table I. This value was about 100% for rats and 80% for dogs and healthy volunteers at almost all times examined after dosing. The value in hypertensive patients was also about 80% (Table II). These results demonstrate that in the species

TABLE II

PLASMA CONCENTRATION OF THE UNCHANGED DRUG OBTAINED BY TLC—GC—MS AND THE RATIO OF THE VALUE TO THAT OBTAINED BY GC—MS IN HYPERTENSIVE PATIENTS

The patients received 20 mg of nicardipine hydrochloride t.i.d. (at 8.00, 13.00 and 18.00) for several weeks.

Time of day	Number of patients	Unchanged drug concentration in plasma (ng/ml)	Ratio of the value obtained by TLC—GC—MS to that obtained by GC—MS (%)
9.00	9	64 ± 22	75 ± 5
13.00	8	14 ± 4	82 ± 6
14.00	11	46 ± 20	85 ± 4
18.00	10	16 ± 4	79 ± 6
19.00	10	40 ± 8	85 ± 3

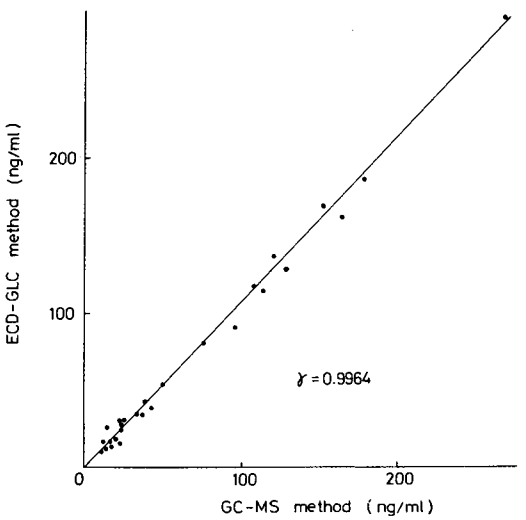

Fig. 6. Correlation between the values obtained by the GC—MS and the ECD—GLC methods.

examined, the amount of plasma pyridine metabolite was very small compared with that of the parent drug.

In the present study the plasma concentrations in healthy volunteers were also measured by ECD-GLC and the values obtained were compared with those obtained by GC—MS. The correlation coefficient (0.9964) indicates that these methods, which are the same in principle except for the detector, have the same specificity (Fig. 6).

ACKNOWLEDGEMENT

The authors express their sincere thanks to Dr. T. Iimura, under whose medical supervision the human study was performed.

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Journal of Chromatography, 223 (1981) 351—356
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 816

QUANTITATIVE DETERMINATION OF NIFLUMIC ACID AND ITS β-MORPHOLINOETHYL ESTER IN HUMAN PLASMA BY GAS—LIQUID CHROMATOGRAPHY

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(First received September 30th, 1980; revised manuscript received December 1st, 1980)

SUMMARY

Niflumic acid and its β-morpholinoethyl ester are extracted from plasma with diethyl ether. After methylation with diazomethane the solution is evaporated to dryness and the residue dissolved in methanol before injection in the chromatographic column. Using a nitrogen-sensitive detector the method permits the determination of 100 ng of each compound in 1 ml of plasma. The coefficient of variation is 5.3% and 4.8% for the acid and the ester, respectively, at the 2-μg level.

INTRODUCTION

Niflumic acid (Nifluril®) is a potent anti-inflammatory drug, and pharmacokinetic studies using [^{14}C] niflumic acid [1] have shown rapid absorption followed by extensive metabolization, essentially hydroxylation and glucuroconjugation. Several methods [2—4] have been published, using spectrophotometry, gas—liquid chromatography (GLC) and high-performance liquid chromatography, but none of them was effective for the analysis of the β-morpholinoethyl ester of niflumic acid, which may be used as a pro-drug of niflumic acid. In order to determine the pharmacokinetics and bioavailability of these two substances, a GLC method has been developed that allows the simultaneous chromatography of the acid and the ester after a single-step extraction.

EXPERIMENTAL

Reagents

Niflumic acid (UP 83) and its β-morpholinoethyl ester (UP 164) (Fig. 1)

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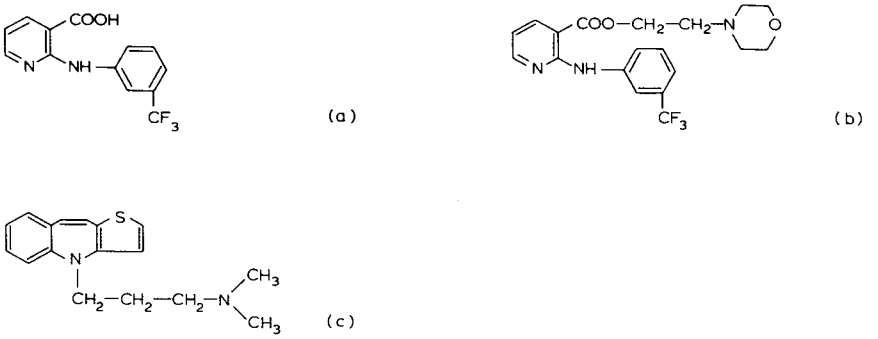

Fig. 1. Chemical structure of (a) niflumic acid, (b) its β-morpholinoethyl ester and (c) the internal standard.

were obtained from UPSA Labs. (Rueil-Malmaison, France). Solutions of these drugs were prepared in methanol.

All reagents were of analytical grade. Methanol, potassium chloride, acetic acid and sodium acetate were obtained from Merck (Darmstadt, G.F.R.). N-Methyl-N-nitroso-*p*-toluenesulphonamide, diethyl ether, ethanol (Merck) and potassium hydroxide (Prolabo, Paris, France) were used as described by Tuong and Tuong [5] for the preparation of diazomethane, which was kept at −20°C until used.

OV-17 and Gas-Chrom Q were obtained from Applied Science Labs. (State College, PA, U.S.A.).

Equipment

A Gilford Model 300 gas chromatograph equipped with a nitrogen-sensitive detector and a Servoriter recorder was used. The glass column (2 m × 2 mm I.D.) was packed with 3% (w/w) OV-17 on Gas-Chrom Q (100—120 mesh) and conditioned overnight at 300°C with nitrogen before use. The injector and detector temperatures were 260 and 305°C, respectively. The column temperature was held at 200°C for 5 min, then raised at 25°C/min for 3.6 min up to 290°C and finally held at that temperature for 5 min. The nitrogen, hydrogen and air flow-rates were 350, 25 and 100 ml/min, respectively.

Preparation of standard solutions

Standard solutions of 1 mg/ml niflumic acid, UP 164, and 5-(3-dimethylaminopropyl)benzazepinethiophene (internal standard, Fig. 1) were prepared in methanol for each series of analyses. The standard solutions of niflumic acid and its ester were then dissolved in drug-free plasma to give final concentrations of 0.1—20 μg/ml. The internal standard solutions were diluted to a final concentration of 0.5 mg/l.

Assay procedure

Plasma (1 ml), 20 μl of internal standard solution and 1 ml of acetate buffer are pipetted into a 20-ml glass-stoppered centrifuge tube. After gentle shaking, 5 mg of potassium chloride are added and mixed carefully. Diethyl ether (6 ml) is added and the tube is shaken for 20 min at 4°C. After centrifugation (7 min

at 3500 *g*), 5 ml of organic phase are transferred into a 5-ml glass tube and carefully evaporated to dryness. A 100-μl volume of an ethanolic solution of diazomethane is added and the tube is kept for 10 min in an ice-bath. The solution is evaporated to dryness and the residue dissolved in methanol (50 μl) with vigorous shaking (Vortex mixer). A 4—5-μl volume of the methanol solution is then injected into the column.

RESULTS AND DISCUSSION

A typical chromatogram of a plasma extract containing niflumic acid, its ester and the internal standard is shown in Fig. 2. The retention times are 4.8,

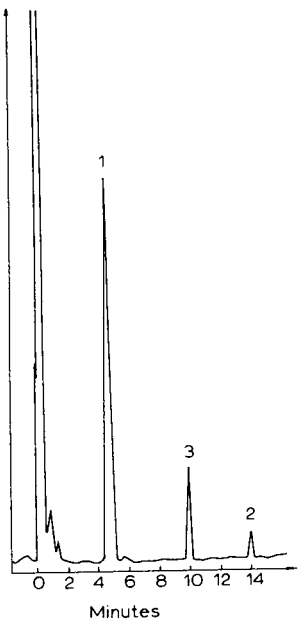

Fig. 2. Typical chromatogram of plasma extract containing 0.72 mg/l of niflumic acid (1), 0.07 mg/l of its ester (2) and 0.01 mg/l of internal standard (3).

14 and 10 min, respectively. Calibration graphs were obtained by a least-squares method of fitting between the peak-height ratio of the sample substance and the internal standard versus amount of substance added. The equations of these regression lines were

$$y_a = 1.20x_a - 0.012 \quad (1)$$

and

$$y_e = 0.40x_e - 0.04 \quad (2)$$

with correlation coefficients of 99.85 and 99.16% for the acid (eqn. 1) and the ester (eqn. 2), respectively. This indicates reasonable linearity for both products between the detector response and amount added to plasma in the range of concentrations tested, i.e., 0.1—20 mg/l.

TABLE I

APPARENT RECOVERIES OF NIFLUMIC ACID AND ITS ESTER

Sample	Niflumic acid		Ester	
	Internal std.		Internal std.	
	Without extraction	After extraction	Without extraction	After extraction
1	0.750	0.335	0.800	0.557
2	0.745	0.377	0.792	0.580
3	0.746	0.325	0.795	0.525
4	0.752	0.343	0.795	0.548
5	0.749	0.370	0.794	0.602
6	0.750	0.380	0.798	0.575
7	0.754	0.375	0.796	0.585
8	0.750	0.330	0.795	0.542
9	0.748	0.345	0.797	0.580
10	0.749	0.354	0.794	0.592
$\bar{m}$	0.749	0.353	0.796	0.569
S.D.	0.003	0.021	0.002	0.035
C.V. (%)	0.4	5.9	0.3	6.2

Apparent recoveries of the acid and ester are indicated in Table I. Comparison of the peak-height ratio after and without extraction for samples containing 2 mg/l of each drug gives apparent recoveries of 47.1% for niflumic acid and 71.5% for its ester.

The detector sensitivities for niflumic acid and its ester are 1 and 1.5 ng, respectively. According to apparent recovery, the overall sensitivity is 100 ng/ml for each substance when 5 μl of a methanolic solution of the sample are injected.

Replicate analyses of plasma samples to which known amounts of acid or ester had been added demonstrated that the method has acceptable accuracy and precision (Table II).

TABLE II

PRECISION AND ACCURACY OF ANALYSIS OF NIFLUMIC ACID AND ITS ESTER

The mean ± standard deviation in mg/l for 10 determinations is given, followed by the coefficient of variation.

Concentration added (mg/l)	Concentration found using internal standard	
	Niflumic acid	Ester
0.1	0.119 ± 0.029 (24.4%)	0.111 ± 0.028 (25%)
0.2	0.190 ± 0.024 (13.0%)	0.204 ± 0.024 (11%)
0.5	0.492 ± 0.03 (7%)	0.488 ± 0.055 (11%)
1.0	1.01 ± 0.12 (12%)	1.00 ± 0.10 (10%)
2.0	1.958 ± 0.1165 (5.9%)	1.986 ± 0.095 (4.8%)

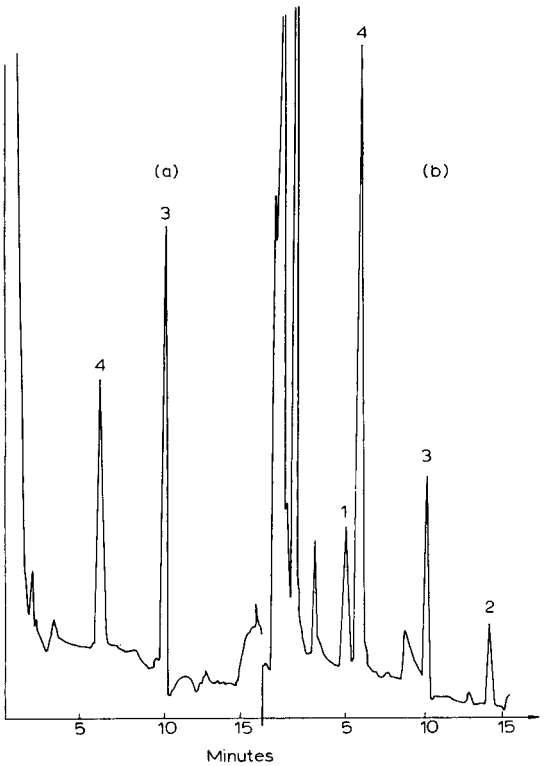

Fig. 3. Gas chromatograms from volunteers samples: (a) sample obtained prior to the oral administration of the ester; (b) sample obtained 2 h later. Peaks: 1 = niflumic acid; 2 = ester; 3 = internal standard; 4 = impurity from plasma.

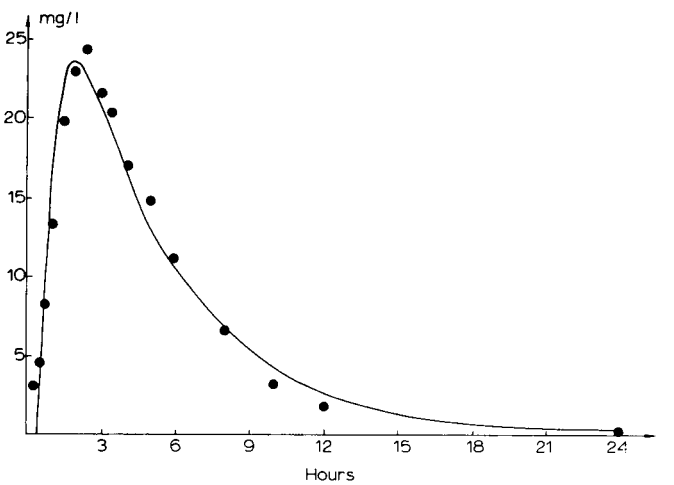

Fig. 4. Plasma profile of niflumic acid in a volunteer after a 350-mg oral dose of the ester.

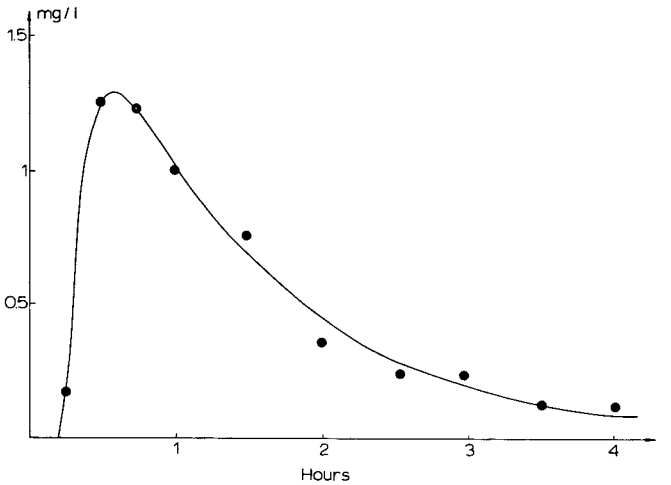

Fig. 5. Plasma profile of β-morpholinoethyl ester in a volunteer after a 350-mg oral dose of the ester.

The method was applied to the determination of niflumic acid and its ester in plasma using twelve healthy volunteers in order to estimate the bioavailability of commercial tablets. None of them showed any contaminant in the zero-time plasma sample corresponding to the retention times of the tested drugs (Fig. 3).

Plasma profiles of niflumic acid and its ester over 24 h for one of the subjects after a single oral dose of 350 mg of β-morpholinoethyl ester are shown in Figs. 4 and 5, respectively. The maximum niflumic acid plasma level occurred after 3 h and that of the ester after about 1 h, with apparent half-lives of 2.87 and 0.89 h, respectively. These figures show that the described technique is sufficiently sensitive for the determination of plasma levels of niflumic acid and its ester in humans, allowing pharmacokinetic or drug monitoring studies after therapeutic doses.

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Journal of Chromatography, 223 (1981) 357—363
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 820

GAS CHROMATOGRAPHIC DETERMINATION OF THE HYPOGLYCAEMIC AGENT GLICLAZIDE IN PLASMA

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(First received September 1st, 1980; revised manuscript received December 17th, 1980)

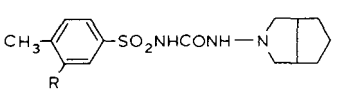
SUMMARY

A gas chromatographic method has been developed that permits the accurate and specific determination of the hypoglycaemic agent gliclazide in plasma. Gliclazide is extracted with chloroform and, after clean-up, derivatized with diazomethane followed by heptafluorobutyric anhydride to form N-methyl-N′-heptafluorobutyrylgliclazide, which is assayed on a gas chromatograph equipped with a flame ionization detector, an electron-capture detector or a nitrogen—phosphorus sensitive detector.

Accurate determinations are possible with flame ionization detection over a concentration range of 1—15 μg/ml of gliclazide in plasma with a relative standard deviation of 5.2%. The minimum detectable concentration with electron-capture detection is 0.02 μg per sample. Plasma levels of gliclazide in dogs following single oral administration (40 mg per dog) have also been determined.

INTRODUCTION

Gliclazide {1-(4-methylbenzenesulphonyl)-3-(3-azabicyclo[3.3.0]octyl)urea} (Fig. 1) [1, 2] is a new oral hypoglycaemic drug of the sulphonylurea type. In order to study the pharmacokinetics of gliclazide, a sensitive and specific assay method for the unchanged drug is necessary.



R = H Gliclazide
R = Cl Internal standard

Fig. 1. Structural formulae of gliclazide and internal standard.

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Sulphonylurea drugs have been most frequently determined by gas chromatography [3—8] after methylation. These methods utilize the thermal fragmentation of the N-methyl derivative to N-methylsulphonamide in the injection port, and therefore the unchanged drug cannot be determined in the presence of some metabolites or other sulphonylurea drugs such as tolbutamide and tolazamide. These disadvantages were eliminated after the thermally stable N-methyl-N′-perfluoroacyl derivative was introduced [9, 10], and the specific determination of the unchanged drug is possible.

In this paper we describe a gas chromatographic method for the determination of unchanged gliclazide in plasma based on methylation with diazomethane followed by acylation with heptafluorobutyric anhydride (Fig. 2).

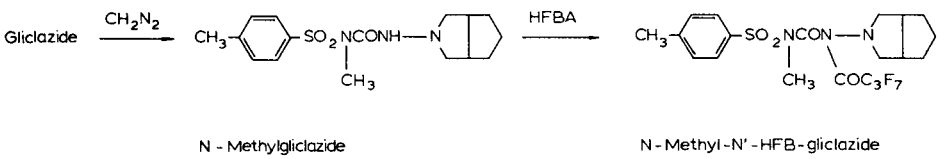

Fig. 2. Derivatization of gliclazide with diazomethane and heptafluorobutyric anhydride.

EXPERIMENTAL

Chemicals and reagents

Gliclazide was a gift from Lab. Servier (Suresnes, France). 3-Chlorogliclazide (m.p. 161—162°C), used as an internal standard, was synthesized in our laboratory [11] (Fig. 1). Heptafluorobutyric anhydride (HFBA) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). An ethereal diazomethane solution was prepared as described previously [12]. All other chemicals were of analytical-reagent grade.

Gas chromatography

Flame ionization and electron-capture detection. A JEOL Model JGC-20K gas chromatograph equipped with a flame ionization detector (FID) and a 10-mCi ^{63}Ni electron-capture detector (ECD) was used. A silanized glass column (100 cm × 2 mm I.D.) was packed with 3% XE-60 on Chromosorb W AW DMCS (80–100 mesh). Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min. The column temperature was 220°C and the injector and detector temperatures were 280°C.

Nitrogen—phosphorus sensitive detection. A Hewlett-Packard Model 5840A gas chromatograph equipped with a nitrogen—phosphorus sensitive detector (N—P FID) was used. A silanized glass column (120 cm × 2 mm I.D.) was packed with 2% OV-101 on Chromosorb W AW DMCS (100—120 mesh). Helium was used as the carrier gas at a flow-rate of 30 ml/min. The column, injector and detector temperatures were 220, 280 and 300°C, respectively.

Preparation of N-methylgliclazide

Diazomethane solution was added to a solution of gliclazide (2 g) in methanol and the mixture was allowed to stand at room temperature for 1 h,

then evaporated to dryness in vacuo. The residue dissolved in chloroform was applied to a silica gel column (20 × 2 cm I.D.), eluted with chloroform and evaporated to dryness in vacuo. The residue was recrystallized from diethyl ether–light petroleum to give 1.1 g of N-methylgliclazide (53% yield, colourless needles, m.p. 133—134.5°C). Calculated for $C_{16}H_{23}N_3O_3S$: C, 56.95; H, 6.87; N, 12.45; S, 9.50%. Found: C, 56.92; H, 7.04; N, 12.19; S, 9.36%.

Preparation of N-methyl-N'-heptafluorobutyryl gliclazide

HFBA (2 ml) and pyridine (0.5 ml) were added to a solution of N-methylgliclazide (1 g) in ethyl acetate and the mixture was heated at 65°C for 30 min. The reaction mixture was shaken with 0.5 *M* sodium carbonate solution followed by water. The organic layer was dried over anhydrous sodium sulphate and evaporated in vacuo, leaving a colourless oil. The oil was distilled to give 1.17 g of N-methyl-N'-heptafluorobutyrylgliclazide (74% yield, b.p. 110—113°C at 30 mmHg). Calculated for $C_{20}H_{22}F_7N_3O_4S$: C, 45.24; H, 4.16; F, 24.93; N, 7.88; S, 6.01%. Found: C, 45.24; H 4.40; F, 24.67; N, 7.67; S, 6.18%.

Assay procedure for gliclazide in plasma

The assay procedure with the FID was as follows. To 1 ml of plasma sample were added 2 ml of 0.25 *M* acetate buffer (pH 3.95) and 8 ml of chloroform containing 15 μg of internal standard in a glass-stoppered 15-ml centrifuge tube, and the tube was shaken for 15 min. The organic layer (6 ml) was shaken with 4 ml of 1 *N* sodium hydroxide solution for 10 min. The aqueous layer (3 ml) was transferred into a glass-stoppered 15-ml centrifuge tube containing 1 ml of 3 *N* hydrochloric acid and 5 ml of 0.25 *M* acetate buffer (pH 3.95), and then shaken with 5 ml of chloroform for 10 min. The organic layer (4 ml) was transferred into another centrifuge tube and evaporated to dryness under a gentle stream of nitrogen.

The residue was dissolved in 0.1 ml of methanol, 0.5 ml of diazomethane solution was added and the solution was allowed to stand at room temperature for 10 min. The reaction mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in a mixture of 45 μl of ethyl acetate, 5 μl of pyridine and 50 μl of HFBA, and the solution was heated at 65°C for 10 min. The reaction mixture was shaken with 4 ml of *n*-hexane for 30 sec, 4 ml of 5% ammonia solution were added and the mixture was again shaken for 5 min. After centrifugation, the organic layer (3 ml) was evaporated to dryness. The residue was dissolved in 30 μl of ethyl acetate and a 5-μl aliquot of the solution was injected into the gas chromatograph.

When the ECD or N—P FID was used, the assay procedure was as described above except that the amount of the internal standard was reduced to 2 μg in each instance.

Calibration graph

Samples (1 ml) of the control plasma containing 1—15 μg of gliclazide were treated as described under *Assay procedure for gliclazide in plasma*. Peak-area ratios of gliclazide to the internal standard were measured with the FID and plotted against the amount of gliclazide present.

Animal experiment

Gliclazide was administered orally at a dose of 40 mg to male beagle dogs (9—12 kg) fasted for 16 h and about 5 ml of blood were drawn by venipuncture 1, 2, 4, 6, 8, 10, 24 and 48 h after dosing. Blood samples were centrifuged and plasma samples were kept frozen until taken for analysis.

RESULTS AND DISCUSSION

Derivatization of gliclazide

Sulphonylurea drugs have usually been methylated with diazomethane [3, 4] or dimethyl sulphate [5—7] for determination by gas chromatography. The reaction of gliclazide with these reagents was evaluated.

Treatment of gliclazide with excess of diazomethane gave two products, N-methylgliclazide and N,N′-dimethylgliclazide (Fig. 3). The former was a major product and the yield was 74.8 ± 1.2% (n = 6). N,N′-Dimethylgliclazide (m.p. 144—145°C) was considered to be an inner salt of the quaternary amine [12], as revealed by nuclear magnetic resonance spectrometry and elemental analysis, and did not volatilize under the usual gas chromatographic conditions. Dimethyl sulphate also gave two products, but the quaternary amine was the major product and the yield of N-methylgliclazide was less than 5%. Similarly, extractive alkylation [8] with methyl iodide afforded largely the quaternary amine derivative. Therefore, diazomethane was used for the methylation of gliclazide.

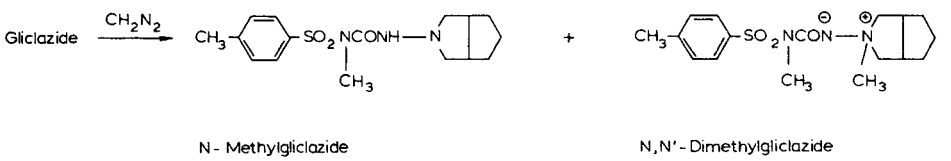

Fig. 3. Reaction of gliclazide with diazomethane.

The reaction of N-methylgliclazide with HFBA proceeded to completion at 65°C within 5 min, and no decomposition products were found after 120 min. The reaction was not affected by the temperature used in the range 50—80°C.

The mass spectrum of N-methyl-N′-HFB-gliclazide obtained by gas chromatography—mass spectrometry showed prominent ions at m/z 476 ($[M-57]^+$, 2%), 321 ($[M-212]^+$, 100%), 155 ($[M-378]^+$, 64%) and 91 ($[M-442]^+$, 19%), suggesting that no thermal fragmentation had occurred.

The purification step after derivatization, viz., addition of n-hexane followed by washing with 5% ammonia solution, was necessary owing to the large solvent peaks of pyridine and HFBA. However, treatment with these reagents in the reverse order resulted in decomposition of the HFB derivative.

N-Methyl-N′-trifluoroacetyl(TFA)gliclazide (m.p. 93—93.5°C) was also synthesized. Both TFA and HFB derivatives had excellent gas chromatographic properties and gave sharp and symmetrical peaks, but were less stable to the usual washing with alkaline solution to remove acyl reagents. The TFA derivative was hydrolysed to N-methyl-p-toluenesulphonamide, whereas the

HFB derivative was stable when dissolved in *n*-hexane followed by washing with 5% ammonia solution. Therefore, the HFB derivative was used in this work.

Specificity

Gliclazide is known to be metabolized in animals and man with major metabolic conversions of the tolyl and azabicyclooctyl groups [13]. The pyrolysis gas chromatographic method commonly used for the determination of sulphonylurea drugs would be simpler and more rapid than our method. However, two hydroxyazabicyclooctyl metabolites have already been isolated from urine of rats receiving 10 mg/kg of gliclazide and therefore these metabolites, if present in plasma, would also be determined as the unchanged drug. In contrast, in the present method these metabolites gave individual peaks (t_R 7.4 and 9.5 min) that were well separated from gliclazide (t_R 6.0 min).

Sensitivity

The minimum detectable concentrations of gliclazide in plasma using the FID, ECD and N—P FID methods are shown in Table I. The sensitivity increased in the order FID < N—P FID < ECD, and the ECD and N—P FID methods were ten times or more sensitive than the FID method.

As the mean plasma level of gliclazide in humans receiving a single 80-mg dose of the drug was about 0.8 μg/ml 24 h after dosing [13], the FID method required a sample volume of 1 ml for study of the pharmacokinetics of gliclazide. In contrast, the sample volume could be decreased to 0.1 ml when using the ECD and N—P FID methods.

TABLE I

SENSITIVITY TOWARDS N-METHYL-N'-HFB-GLICLAZIDE USING THE FID, ECD AND N—P FID

Detector	Minimum detectable concentration (mole/sec)*	Sample volume (ml)
FID	$9.4 \cdot 10^{-14}$	1
ECD	$3.1 \cdot 10^{-16}$	0.1
N—P FID	$1.0 \cdot 10^{-15}$	0.1

*The amount that gives a signal three times the background noise level.

Calibration graph

The calibration graph obtained with 1—15 μg of gliclazide in 1 ml of plasma using the FID was rectilinear and passed through the origin. The precision of the method was 5.2% (relative standard deviation), and the recovery of gliclazide was 85.1 ± 2.7% (n = 8) of the theoretical value.

Fig. 4 shows typical chromatograms for plasma samples obtained using the FID, ECD and N—P FID.

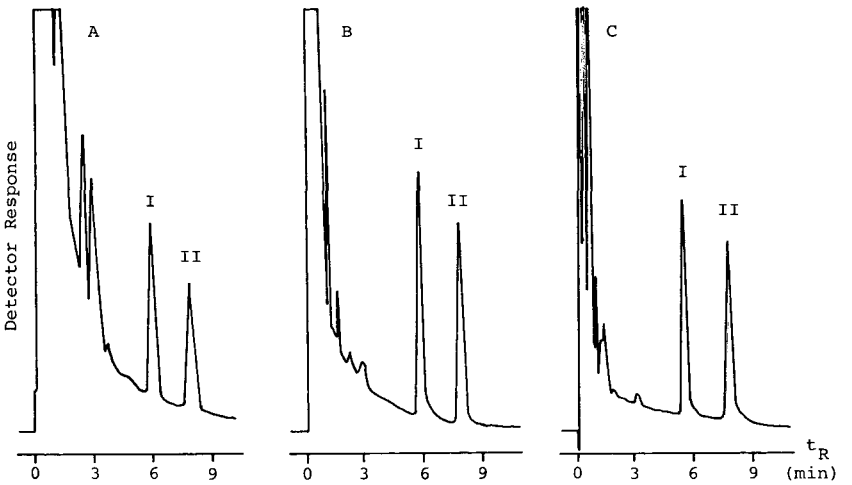

Fig. 4. Typical chromatograms of gliclazide in plasma obtained using (A) FID, (B) ECD and (C) N—P FID. A: Gliclazide (I, 10 μg) and internal standard (II, 15 μg) were added to 1 ml of plasma. B and C: I (1 μg) and II (2 μg) were added to 0.1 ml of plasma.

Determination of gliclazide in dog plasma

Plasma levels of gliclazide following single oral administrations of 40 mg (ca. 4 mg/kg) are shown in Fig. 5. The drug levels were maximal 4 h after dosing with a mean peak level of 7.9 μg/ml, followed by a first-order decrease with a half-life of 10 h. These results are consistent with previous findings [13].

The method described here should be sufficiently sensitive and specific for the determination of unchanged gliclazide in plasma and would therefore

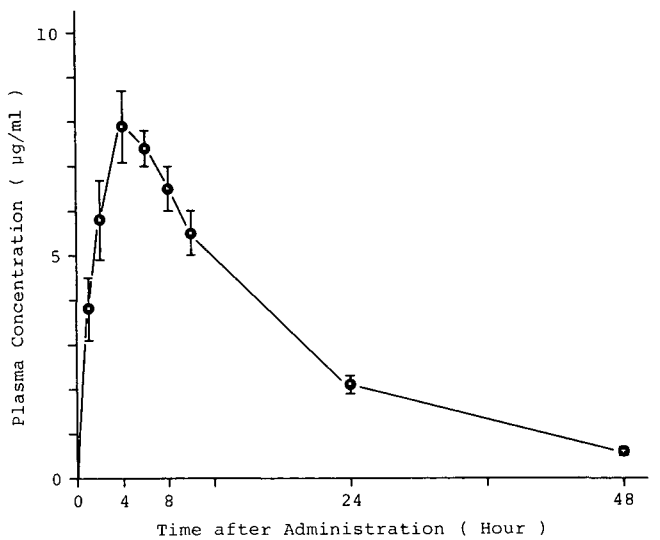

Fig. 5. Plasma levels of gliclazide in dogs following single oral administration of 40 mg of gliclazide. The points are mean values ± standard errors from six dogs.

permit pharmacokinetic studies of gliclazide in both man and experimental animals, as the peak plasma levels of gliclazide were 2—5 μg/ml in human subjects receiving a clinical dosage of 80 mg [13].

ACKNOWLEDGEMENTS

The authors are grateful to Dr. H. Nishimura, Director of the laboratory, for his support, and Mr. A. Kagemoto for the synthesis of the internal standard.

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Journal of Chromatography, 223 (1981) 365—370
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 825

SIMULTANEOUS DETERMINATION OF CYCLANDELATE AND ITS METABOLITE IN HUMAN PLASMA BY CAPILLARY COLUMN GAS—LIQUID CHROMATOGRAPHY

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(Received October 28th, 1980)

SUMMARY

A method was developed for the simultaneous determination of cyclandelate and mandelic acid concentrations in plasma, involving extraction from plasma followed by trimethylsilylation and chromatography of the derivatives on a glass capillary column with hydrogen flame-ionization detection. Calibration graphs were linear down to at least 20 μg/ml for each substance. The precision was excellent with a pooled relative standard deviation of 6.3% and 6.4% for cyclandelate and mandelic acid serum samples, respectively. Concentrations below 500 ng/ml of each substance could be detected in human plasma. The method was developed for use in bioavailability and metabolism studies.

INTRODUCTION

Since cyclandelate (I) was first synthesized, it has become a much used drug for the treatment of vascular disease [1—5], but little information is available concerning its pharmacokinetic behaviour in humans [6—10]. The use of polarography [6] for the determination of cyclandelate in urine is not sensitive enough for the study of its pharmacokinetic properties in plasma. A spectrophotometric method [11] has been used to measure cyclandelate in biological samples, but it is non-specific for the determination of metabolites.

A chromatographic method was described previously for the determination of cyclandelate in human plasma [12], but the determination of its metabolite (mandelic acid) is difficult because of interfering peaks. This paper presents a modified method for the simultaneous determination of cyclandelate (I) and mandelic acid (II) using a capillary column and ethyl mandelate (III) as an internal standard.

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Reagents

Ethyl mandelate, anhydrous sodium sulphate, hydrochloric acid and pyridine were of analytical-reagent grade and were used without further purification. Diethyl ether was purified by distillation in an all-glass apparatus. A commercially prepared mixture of N-bis(trimethylsilyl)fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1) was used for methylating the derivatives.

Standard solutions of ethyl mandelate (Roth, Karlsruhe, G.F.R.), mandelic acid (Fluka, Buchs, Switzerland) and cyclandelate (Synthedis, Bobigny, France) were prepared in ethyl acetate.

Instrumentation

The analysis was performed on a gas chromatograph (Girdel) equipped with a flame-ionization detector and a splitless mode injector (Ross) connected to a recorder fitted with an automatic programmable integrator (Sigma 1, Perkin-Elmer). The gas chromatograph input attenuation was $1 \cdot 10^{-11}$ A/mV and the integrator attenuation was $\times 4$. The 25 m $\times$ 0.3 mm I.D. glass capillary column was deactivated and wall-coated with SE-30 (Girdel, Paris, France). The carrier gas was nitrogen at a flow-rate of 2 ml/min.

The injector and detector temperatures were 250°C. The column oven was operated at 125°C, with a programme involving a 13-min initial hold, a 3°C/min increase to 180°C and a final hold at 180°C for 1 min.

Extraction and preparation of samples for chromatography

The extraction procedure reported earlier [12] was slightly modified. To 1 ml of plasma in a glass-stoppered 15-ml centrifuge tube were added 20 μl of the internal standard, ethyl mandelate (20 μg/ml in ethyl acetate). The plasma was extracted with 10 ml of diethyl ether by mixing for 1 min on a vortex mixer. This organic portion contained cyclandelate. After separation from the aqueous phase by centrifugation (5 min at 4300 *g*) the organic phase was removed into another 30-ml tube containing anhydrous sodium sulphate.

Mandelic acid was obtained by another 10-ml extraction with diethyl ether, after the plasma had been acidified with 30% hydrochloric acid and shaken as described above. The organic phases were pooled, filtered and evaporated to 1—2 ml in a rotary flash evaporator with a 20—30°C water-bath. The residual material was transferred to a silylation vial and the solvent was removed by evaporation with a stream of nitrogen at room temperature. The dried residue in each vial was dissolved in 50 μl of pyridine, 25 μl of the trimethylsilylating reagent were added and the solution was allowed to stand for 5 min at 60°C. Approximately 1 μl of this solution was injected into the gas—liquid chromatographic (GLC) column.

Method of quantitation

The peak heights of I and II were measured by computer. Peak-height ratios were obtained by dividing the peak heights of I and II by the peak height of III.

Calibration graphs for known concentrations of I and II in plasma were

prepared by plotting peak-height ratios versus I and II concentrations. Values for unknown concentrations of I and II in plasma samples, obtained in the same manner, were calculated by the Sigma 1 computer from the slope of the calibration graph. The slopes were obtained by a least-squares linear regression analysis for the cyclandelate and mandelic acid standards.

RESULTS AND DISCUSSION

Analysis of plasma

The GLC analysis of control (blank) human plasma carried through the extraction procedure, is presented in Fig. 1A. Fig. 1B shows a gas chromatogram of cyclandelate (I), mandelic acid (II) and the internal standard (III), obtained by adding cyclandelate, mandelic acid and ethyl mandelate (internal standard) to control plasma and carrying the mixture through the extraction procedure.

The spiked samples showed excellent resolution. The retention times were 31.3, 8.2 and 7.1 min for I, II and III, respectively.

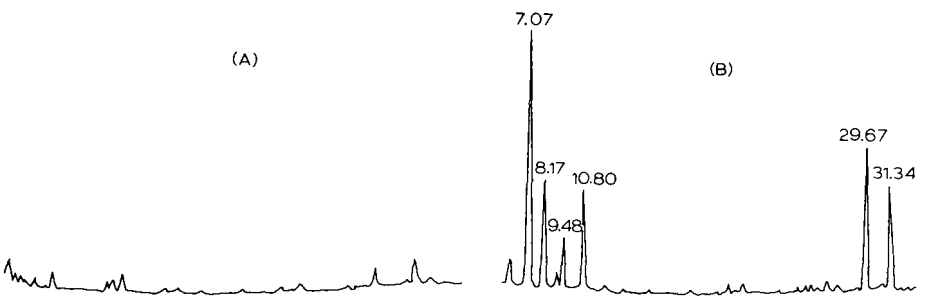

Fig. 1. Gas chromatograms of 1-ml human plasma extracts. (A) Blank plasma; (B) plasma spiked with 20 μg of ethyl mandelate (retention time 7.07 min), 10 μg of mandelic acid (8.17 min) and cyclandelate (31.34 min).

Recovery and linearity

Cyclandelate and mandelic acid recoveries were determined by adding various known amounts of product to human plasma and analysing each sample in quadruplicate according to the described procedure (Table I). Compared to a similar series of unextracted reference standards, the mean recoveries varied from 95% to 106% (mean 101%) for mandelic acid and from 95% to 102% (mean 98%) for cyclandelate. They were independent of concentration within the range used for plasma.

The calibration graphs obtained from plasma spiked with cyclandelate and mandelic acid in the range 1—20 μg/ml were linear for both products, as reflected by the values of the correlation coefficients (r = 0.997 for cyclandelate and r = 0.996 for mandelic acid) (Fig. 2).

Precision

The precision was determined by carrying out eight consecutive calibration runs on plasma samples containing both cyclandelate and mandelic acid (Table II). The coefficients of variation (C.V.) ranged from 4 to 8% and 3 to 7% for

TABLE I

RECOVERY OF MANDELIC ACID AND CYCLANDELATE ADDED TO HUMAN PLASMA

Compound	Amount added (μg/ml)	*n*	Amount recovered (μg/ml)	Recovery (mean ± S.D.) (%)
Mandelic acid	1	8	1.06 ± 0.08	106 ± 8
	5	8	5.02 ± 0.19	100.4 ± 3.8
	10	8	9.57 ± 0.69	95.7 ± 6.9
	20	8	20.17 ± 0.91	100.9 ± 4.6
Cyclandelate	1	7	0.95 ± 0.12	95 ± 12
	5	7	4.76 ± 0.19	95.2 ± 3.8
	10	7	10.27 ± 0.72	102.7 ± 7.2
	20	7	19.84 ± 0.66	99.2 ± 3.3

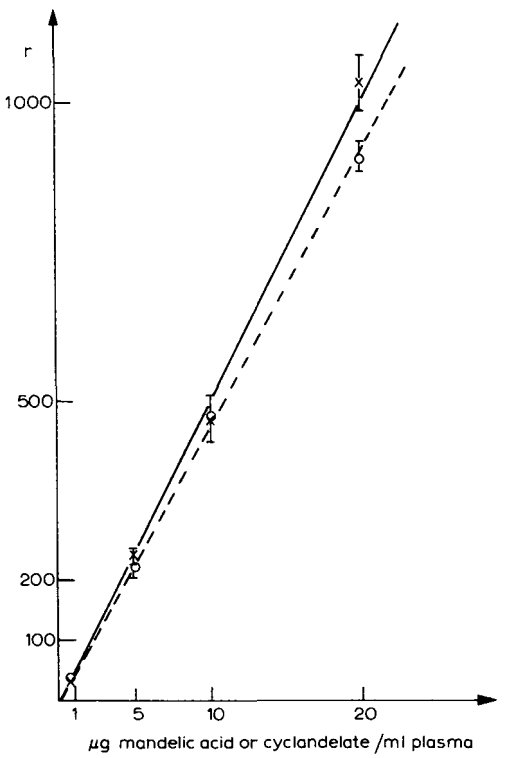

Fig. 2. Calibration graphs of amounts of cyclandelate (— — —) and mandelic acid (——) added to 1 ml of human control plasma versus measured peak-area ratio (r) of mandelic acid or cyclandelate to internal standard (ethyl mandelate). The concentration of the ethyl mandelate was 20 μl/ml.

TABLE II

PRECISION OBSERVED ON ANALYSIS OF CONSECUTIVE CALIBRATION GRAPHS FOR PLASMA CONTAINING CYCLANDELATE AND MANDELIC ACID AT THE CONCENTRATIONS INDICATED

Amount added to plasma (μg/ml)	Peak-height ratio			
	Mandelic acid:ethyl mandelate		Cyclandelate:ethyl mandelate	
	Mean ± S.D. (n = 8)	C.V.(%)	Mean ± S.D. (n = 7)	C.V.(%)
1	0.048 ± 0.004	8.3	0.052 ± 0.006	11.5
5	0.239 ± 0.010	4.2	0.224 ± 0.009	4
10	0.471 ± 0.038	8.1	0.474 ± 0.033	7
20	1.031 ± 0.047	4.6	0.907 ± 0.030	3.3

mandelic acid and cyclandelate, respectively, for 5—20 μg/ml levels. Only the 1 μg/ml level for cyclandelate had a high coefficient of variation (11.5%). These results indicate the good reproducibility of the technique.

Detection limits

The detection limit, as determined at the 95% confidence level, was 500 ng/ml for both cyclandelate and mandelic acid.

Application to pharmacokinetic studies

The suitability of the proposed method for the determination of cyclandelate and its metabolite in plasma was tested with six healthy volunteers who received 400 mg of cyclandelate as a single oral dose. Blood samples (10 ml) were drawn by venipuncture at 0, 0.5, 1, 1.5 and 2 h. Plasma was separated, and the samples were frozen until taken for the determination of the cyclandelate and mandelic acid concentrations by the described method. An interesting observation concerning metabolism of cyclandelate was made; these results will be published elsewhere [13].

CONCLUSION

The method described is linear, precise, accurate and sensitive. It has been used for over 6 months in our pharmacokinetic laboratory, where it has been applied to the generation of pharmacokinetic profiles on humans and animals who have been administered cyclandelate orally.

The reason for preparing a derivative of cyclandelate and mandelic acid is that the unchanged drugs are highly polar compounds that show marked tailing during chromatography. However, there are two important disadvantages with I and II derivatives: they are time consuming to prepare and are readily hydrolysed by moisture, even in the presence of excess of reagents.

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Journal of Chromatography, 223 (1981) 371—378
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 808

DETERMINATION OF 1-β-D-ARABINOFURANOSYLCYTOSINE AND 1-β-D-ARABINOFURANOSYLURACIL IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received September 16th, 1980; revised manuscript received December 2nd, 1980)

SUMMARY

A method is described for the determination of 1-β-D-arabinofuranosylcytosine (Ara-C) and its metabolite 1-β-D-arabinofuranosyluracil (Ara-U) in human plasma. After deproteinization of the plasma sample, separation is performed by reversed-phase liquid chromatography.

For Ara-C concentrations exceeding 0.05 mg/l and for Ara-U concentrations exceeding 1 mg/l, injection volumes of 100 μl are applied. For lower concentrations an injection volume of 500 μl is used. Ara-C is detected at 280 nm with a lowest detection limit of 0.002 mg/l in plasma. Ara-U is detected at 264 nm with a lowest detection limit varying from 0.01 to 0.1 mg/l in plasma. This variation is caused by an unknown substance with the same elution properties as Ara-U and which appears to be present in plasma in variable concentrations. The coefficient of variation of the whole procedure is about 6% for Ara-C concentrations above 0.005 mg/l and for Ara-U concentrations above 0.1 mg/l. For lower concentrations the coefficient of variation is about 14%.

INTRODUCTION

The pyrimidine analogue 1-β-D-arabinofuranosylcytosine (Ara-C) is one of the most effective drugs in the treatment of acute non-lymphocytic leukemia [1,2]. After administration, Ara-C is inactivated rapidly by deamination to 1-β-D-arabinofuranosyluracil (Ara-U). Both compounds are eliminated simultaneously by renal excretion [3,4]. Plasma concentration curves of Ara-C as a function of time vary considerably after the same intravenous dose in different patients. Since treatment results have been related to the plasma concentrations achieved [5—7], a suitable method for the determination of the Ara-C concentration in plasma is required in order to optimize therapy. For detailed pharmacokinetic studies, measurement of both Ara-C and Ara-U is required.

Various methods have been used for the determination of Ara-C. Among them are an enzymatic assay [8], bioassays [7,9,10], radioimmunoassays [11,12], gas chromatography [13], and determination of radioactivity after administration of labeled Ara-C [3,4]. Most of these methods are rather insensitive or not suitable for routine application. Recently, several methods for the determination of Ara-C by high-performance liquid chromatography (HPLC) have been reported [14—16]. At concentrations far below the detection limits of these procedures, Ara-C is still biologically active [17—19]. Therefore these procedures are not adequate for proper pharmacokinetic studies.

This paper describes an HPLC procedure for the determination of very low concentrations of Ara-C and Ara-U in plasma.

EXPERIMENTAL

Chemicals

Cytidine (Cyd), 5-methylcytidine (5-CH_3-Cyd), deoxycytidine (dCyd), uridine (Urd), and deoxyuridine (dUrd) were purchased from Sigma, St. Louis, MO, U.S.A. Ara-C was purchased from Upjohn, Kalamazoo, MI, U.S.A., and Ara-U from Calbiochem, San Diego, CA, U.S.A. Tetrahydrouridine (THU) was generously supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Bethesda, MD, U.S.A. All other chemicals were purchased from E. Merck, Darmstadt, G.F.R. All chemicals were of analytical grade.

Blood sample preparation

Blood samples of 5 ml were collected in heparinized tubes, containing 50 μg of THU to prevent deamination of Ara-C [20]. After centrifugation (10 min, 2000 *g*, 4°C) plasma was collected and then ultrafiltrated through conical ultrafilters (Centriflo F 25, Amicon) by centrifugation (30 min, 800 *g*, 4°C) in order to remove most of the protein. To remove small molecular weight proteins still present in the ultrafiltrate, 1 ml of ultrafiltrate was mixed with 50 μl of ice-cold 8 *M* perchloric acid and the mixture was placed immediately in an icebath. After 10 min, precipitated protein was removed by centrifugation (10 min, 2000 *g*, 4°C). To 1 ml of the supernatant were added 100 μl of 4 *M* dipotassium hydrogen phosphate. The precipitated potassium chloride was removed by centrifugation. The term blank plasma is used for plasma samples taken before Ara-C administration.

HPLC equipment

The HPLC equipment exists of a Pye Unicam LC 3-XP pump, an injection valve (Rheodyne Model 7120) equipped with a 500-μl loop, and two reversed-phase columns (Nucleosil 10 C-18, 250 × 4.6 mm I.D., particle size 10 μm) linked together to improve separation (see HPLC procedure). Two UV detectors are used: a UV III monitor LDC 1203 with a 10-μl flow cell and a fixed wavelength of 280 nm (Ara-C), and a Pye Unicam LC-UV with an 8-μl flow cell and a variable wavelength set at 264 nm (Ara-U).

HPLC procedure

A 500-μl sample of deproteinized plasma is injected into the two linked reversed-phase columns. Elution is carried out at a constant flow with 0.2 *M* potassium dihydrogen phosphate, adjusted to pH 2.0 with phosphoric acid. Helium gas is led through the eluent to prevent development of air bubbles at the low pressure side. The detection of Ara-C appears to be optimal at 280 nm, whereas for the detection of Ara-U 264 nm is more appropriate (Fig. 1). Peak heights are used for the quantitation of the assay. Ratios of the absorption at 280 nm to that at 264 nm are used to detect impurities underlying the peaks of Ara-C and Ara-U.

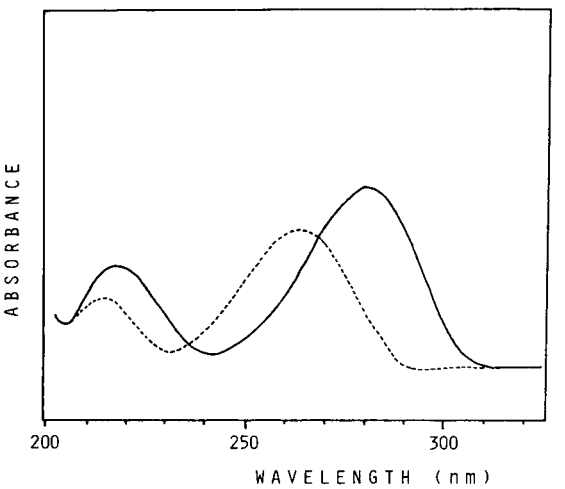

Fig. 1. UV spectrum of Ara-C (——) and of Ara-U (— — —) at pH 2.0

For concentrations of Ara-C above 0.05 mg/l and of Ara-U above 1 mg/l an injection volume of 100 μl was used, and separation on a single column proved to be sufficient. The maximal run-time for the complete separation of a 500-μl sample with the two-column system is 45 min, whereas separation of a 100-μl sample with the one-column system can be carried out in 22 min.

RESULTS

To prevent dilution of the plasma samples, most protein is removed by ultrafiltration. Thus, further precipitation can be carried out with a small volume of perchloric acid. This procedure combines maximal deproteinization with minimal dilution (1 ml of the injection sample equals 0.866 ml of plasma).

Deproteinization of the plasma samples led to a recovery of Ara-C and Ara-U of 92 ± 8% (n=20). This is in agreement with the observation of Van Prooijen et al. [21] that 13 ± 2% of Ara-C is bound to plasma protein.

Chromatographic separation

The chromatogram of a standard mixture of Ara-C, Cyd, dCyd, 5-CH_3-Cyd,

Ara-U, Urd, dUrd and uric acid shows that Ara-C and Ara-U can be separated from their analogues (Fig. 2). Fig. 3 depicts the chromatograms of a blank plasma sample, spiked before deproteinization with Ara-C (final concentration 0.006 mg/l) and with Ara-U (final concentration 0.24 mg/l) for which the detection was at 280 nm (Fig. 3a) and at 264 nm (Fig. 3b), respectively.

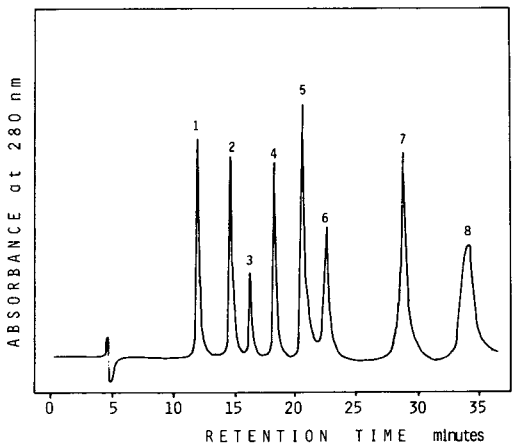

Fig. 2. Separation of a standard mixture of Ara-C and Ara-U and some of their analogues by HPLC. Injection volume 500 µl, separation on two-column system (see Methods), detection at 280 nm. 1=Cyd, 2=Ara-C, 3=dCyd, 4=5-CH_3-Cyd, 5=Urd, 6=uric acid, 7=Ara-U, 8=dUrd.

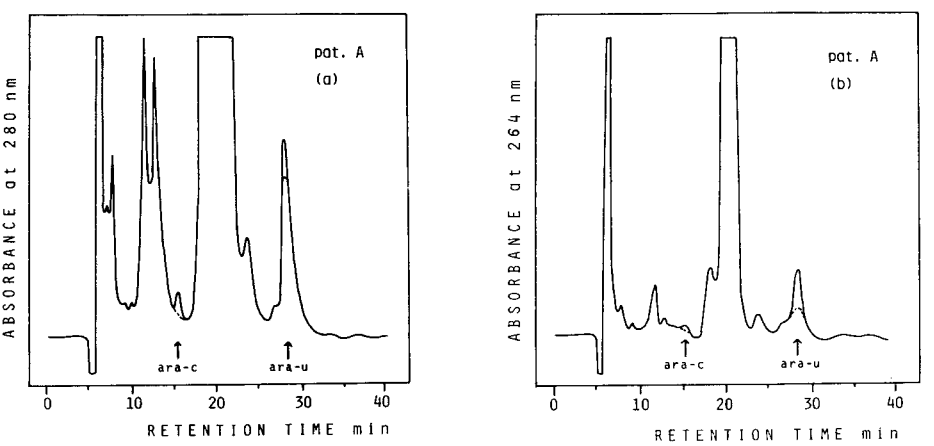

Fig. 3. High-performance liquid chromatogram of blank plasma of patient A spiked with Ara-C (0.006 mg/l) and Ara-U (0.24 mg/l). Injection volume 500 µl, separation on two-column system, detection at 280 nm, 0.002 a.u.f.s. (a) and at 264 nm, 0.005 a.u.f.s. (b). Dotted lines indicate chromatogram without Ara-C and Ara-U.

Blank plasma of patients with acute leukemia reveals great differences. In Fig. 4 the chromatograms of blank plasma of two patients are shown. All plasma samples appeared to be free of compounds interfering with the detection of Ara-C, but several plasma samples contain a substance with the same elution properties as Ara-U. The concentration of this compound in plasma

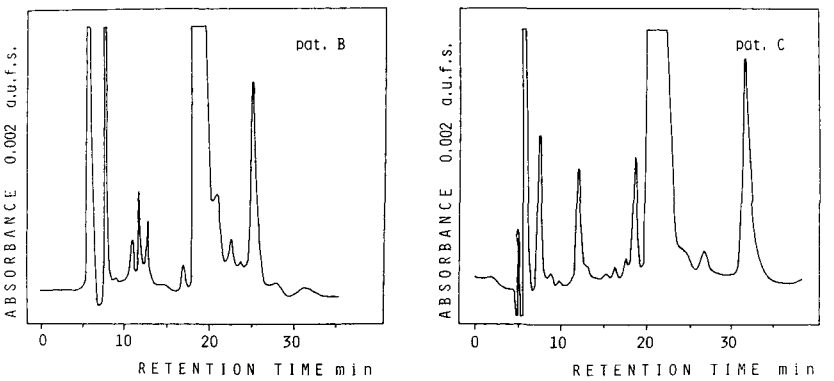

Fig. 4. Chromatograms of blank plasma of patients B and C. Conditions as in Fig. 3. Detection at 280 nm, 0.002 a.u.f.s.

of individual patients varies considerably (see Figs. 3a and 4) and accounts for the variable detection limit of Ara-U in plasma. Since the ratio of the absorption at 280 nm and at 264 nm is different for Ara-U and this compound (see Fig. 3), the influence of this compound on the determination of Ara-U can be eliminated mathematically.

Calibration curves and accuracy

Calibration curves of Ara-C and Ara-U are linear and pass through the origin. Detection limits in plasma are 0.002 mg/l for Ara-C and about 0.1 mg/l for Ara-U. The detection limit of Ara-U is estimated to be 0.01 mg/l when the interfering compound is absent. The relative coefficient of variation for the determination of plasma concentrations, including the deproteinization step, ranges for Ara-C from 14% at the detection limit to 6% at higher concentrations. Detailed information about accuracy at different concentrations is given in Table I.

TABLE I

COEFFICIENT OF VARIATION IN THE DETERMINATION OF ARA-C AND ARA-U

Concentration (mg/l)	*n*	Coefficient of variation (%)
Ara-C		
0.1	10	6
0.02	6	4
0.005	9	14
0.002	8	12
Ara-U		
0.4	5	7
0.1	7	14
0.05	8	12

Application

Fig. 5 illustrates the concentration curves of Ara-C and Ara-U after an intravenous bolus injection of 100 mg/m^2 in a patient with acute leukemia. Ara-C is eliminated rapidly and Ara-U rapidly reached maximal values. Elimination of Ara-U is a relatively slow process with a half-life time of 360 min. Also the concentrations of Ara-C and Ara-U could be monitored during infusion of a very small amount of Ara-C as is shown in Fig. 6. This patient received 17 mg of Ara-C in a 1-h infusion. This led to a concentration of Ara-C of 0.07 mg/l during infusion. A rapid decline was measured after cessation of the drug administration. Ara-U reached equilibrium within 20 min; elimination after infusion was again much slower than the elimination of Ara-C.

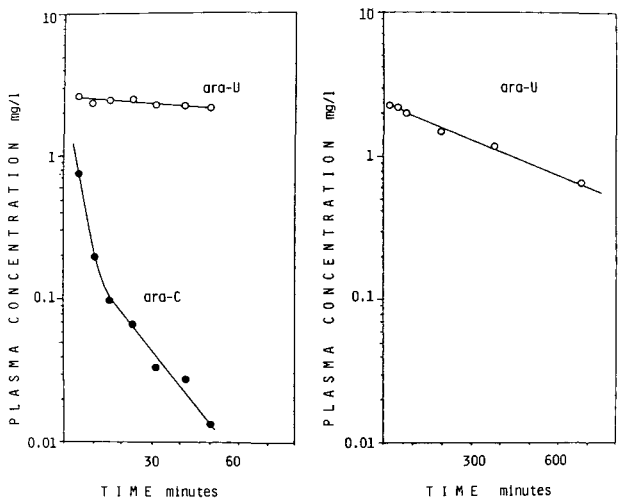

Fig. 5. Plasma concentration—time curves of Ara-C (•) and Ara-U (○) after an intravenous bolus injection of 100 mg/m^2 in a patient with acute leukemia.

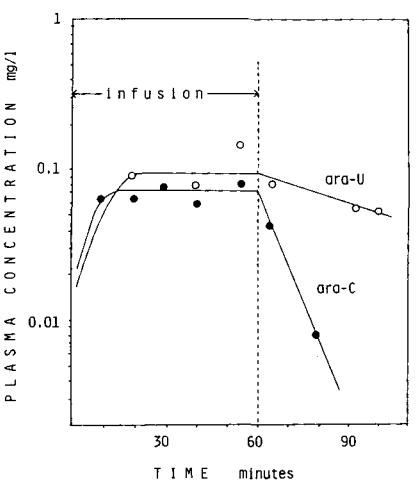

Fig. 6. Plasma concentration—time curves of Ara-C (•) and Ara-U (○) during and after infusion of 17 mg of Ara-C (10 mg/m^2).

DISCUSSION

Several studies in patients with acute non-lymphocytic leukemia treated with Ara-C indicate that treatment results are correlated with differences in pharmacokinetics [5—7]. Adjustment of dosages in individual patients requires a rapid and reliable method for the determination of Ara-C and its metabolite Ara-U.

All previously described methods of measuring Ara-C exhibit more or less disadvantages. Radioimmunoassays may be disturbed by endogenous nucleosides, which are sometimes present in the plasma of patients with leukemia in very high concentrations [22]. This objection may also hold for bioassays. Furthermore, the application of bioassays is limited since Ara-C is often used in combination with other cytostatic drugs. Both radioimmunoassays and bioassays require a calibration curve and since these curves are linear over only a small concentration range, appropriate dilutions have to be made. This makes these procedures time-consuming and laborious. Gas chromatography combined with mass spectroscopy, although very sensitive, is difficult to perform routinely. Radioactive drug administration requires special precautions to prevent environmental contamination. All these objections can be overcome by the use of HPLC. However, the detection limits of the HPLC procedures described until now are higher than those of other methods. The HPLC procedure described in this paper offers a rapid and reliable method for the determination of very low concentrations of both Ara-C and Ara-U in plasma.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. J.M. Lintsen for packing the columns, and to Mrs. G. Vierwinden for skilful assistance. This study was supported by a grant from the Queen Wilhelmina Foundation, The Netherlands Organisation for Cancer Research.

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Journal of Chromatography, 223 (1981) 379—392
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 813

DETERMINATION OF THREE MAIN ANTILEPROSY DRUGS AND THEIR MAIN METABOLITES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received April 29th, 1980; revised manuscript received December 7th, 1980)

SUMMARY

The simultaneous analysis of main antileprosy drugs such as 4,4′-diaminodiphenyl sulfone (DDS), clofazimine, rifampicin and their main metabolites in serum was examined by high-performance liquid chromatography using a μBondapak C_{18} column. When the drugs dissoluted from serum were developed by tetrahydrofuran—0.5% acetic acid (40:60), clofazimine and rifampicins could be analyzed separately. Apart from the mutual separation of water-soluble conjugates of DDS, the individual analysis of DDS, its main liposoluble metabolite and a few related sulfone compounds is possible when the drugs are first developed by acetonitrile—water (20:80). By the use of tetrahydrofuran—water (50:50) containing PIC B-5, the rapid measurement of clofazimine isolated from the other compounds is also possible.

INTRODUCTION

The main antileprosy drugs presently used are 4,4′-diaminodiphenyl sulfone (DDS) [1], 3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylamino)-phenazine (clofazimine) [2] and rifampicin (RFP) [3], of which the former two have pharmacodynamic activities [4]. Moreover, we have found that DDS and clofazimine have depressive and potentiative effects, respectively, on

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cell-mediated immunity of thymectomized guinea pigs when examined by a rabbit red blood cell rosette formation method [5].

Up to now, Gordon and Peters [6] and Burchfield et al. [7] have reported, respectively, fluoriphotometric and gas chromatographic analysis of DDS and its acetylated metabolites. Both of these highly sensitive methods brought a significant advancement in pharmacokinetic and clinical studies on leprosy. A favorable monitoring method for detecting excreted urinary DDS from patients was also reported [8]. On the other hand, Dill and Glazko [9] reported a fluoriphotometric method for analyzing clofazimine using titanous chloride and sulfuric acid. Vlasáková et al. [10] reported the separation of RFP

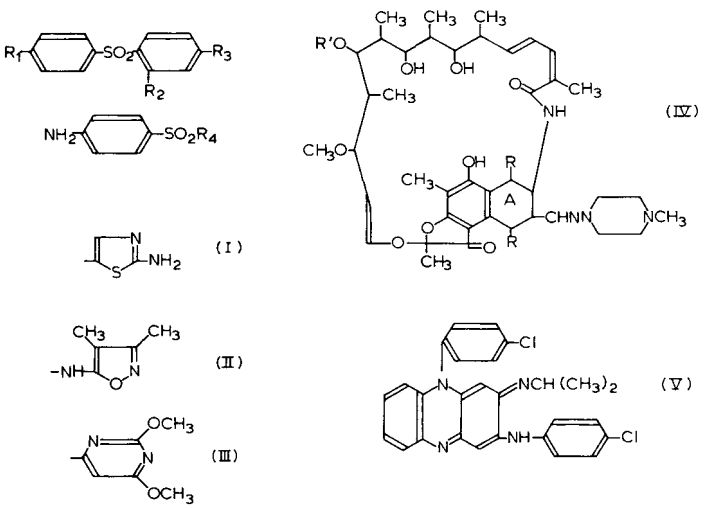

Fig. 1. Structural formulae of antileprosy drugs and related substances.

	R_1	R_2	R_3
DDS	NH_2	H	NH_2
MADDS	NH_2	H	$NHCOCH_3$
DDSG	NH_2	H	$NHC_6H_8O_6Na$
DDSS	NH_2	H	$NHSO_3K$
s-DDS	NH_2	SO_2NH_2	NH_2
Proethyl	NH_2	H	$NHCH_2CH_2OH$

	R_4
Promizole	I
Sulfisoxazole	II
Sulfadimethoxine	III

IV	R	R'	A
RFP	OH	CH_3CO	naphthol
DARFP	OH	OH	naphthol
RFPQ	O	O	naphthoquinone

V: Clofazimine

and its hydrogenated substances by high-performance liquid chromatography (HPLC). However, no complete chromatographic separation of all of these antileprosy drugs and their metabolites has yet been achieved.

In the meantime, since the occurrence of patients resistant to DDS therapy has gradually become a serious problem in the field of leprosy where DDS has long been used as the single and economical drug especially established for antileprosy chemotherapy, attention has been given to a combined therapy using DDS with the other two drugs.

In order to facilitate the analysis of serum specimens of patients under the combined therapy, we have examined the simultaneous analysis of the drugs in a pooled guinea pig serum by HPLC.

EXPERIMENTAL

Analytical instruments

A liquid chromatograph system (Waters Assoc., Milford, MA, U.S.A.) consisted of a high-pressure pump (Model 6000A) and a universal injector (Model U6K) equipped with a Jasco Uvidec 100-II UV spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) was used. A recorder (Model VP-6621-A, Matsushita Communication Ind. Co., Yokohama, Japan) was linked to the detector. The attenuations of the UV spectrophotometric detector were between 0.005 (minimum range) and 0.04 depending on drug concentration. The column used was μBondapak C_{18} (300 mm × 3.9 mm I.D., Waters Assoc.).

For UV spectrophotometry, an Hitachi recording spectrophotometer, Model EPS-3T (Hitachi-Nissei, Tokyo, Japan) was used.

Mobile phases

The compositions of the mobile phases used were: system A, acetonitrile—water (20:80); system B, tetrahydrofuran (THF)—0.5% acetic acid (40:60); and system C, THF—water containing PIC B-5 (50:50). The reagent PIC B-5 (Waters Assoc.) contains 1-pentanesulfonic acid and glacial acetic acid, the concentration of the acetic acid being a trade secret. The concentration of the 1-pentanesulfonic acid in water was 0.005 *M* , thus its final concentration in system C was 0.0025 *M*. The flow-rates for systems A, B and C were 2.0 ml/min (70 kg/cm^2), 1.5 ml/min (112 kg/cm^2) and 1.5 ml/min (116 kg/cm^2), respectively. All the organic solvents in systems A—C were filtered through a Teflon filter (FHLP04700, pore size 0.5 μm, Millipore). The re-distilled water was filtered through a cellulose ester filter (GSWP04700, pore size 0.22 μm, Millipore). After mixing an organic solvent with an aqueous layer inside a graduated glass cylinder, which was well washed with the filtered water, every mobile phase was degassed in a specially cleansed glass bottle using a sonicator (Ultrasonic Cleaner B-220, Branson and Smithkline) for at least 20 min before use.

Switching of mobile phase

Since RFPs and clofazimine could first be separated by development with system B, whereas sulfones were separable with system A, in order to achieve simultaneous analysis of all the drugs, the switching of the mobile phases from

system A to B or from B to A was performed. For switching from A to B, system B was run into the analytical system after analysis of sulfones with system A. However, for switching from B to A, system B was first removed by pumping methanol—water (50:50) through the system at 1.0 ml/min for at least 20 min before starting column conditioning with system A.

Chemicals

All of the following drugs were kindly supplied by the following companies. DDS, 2-amino-5-sulfanilylthiazole (promizole, PZ) and 4-hydroxyethyl DDS (proethyl, PE), Yoshitomi, Fukuoka, Japan; DDS 2-sulfonamide (s-DDS) [11], Tanabe, Saitama, Japan; sulfisoxazole, Yamanouchi, Tokyo, Japan; sulfadimethoxine, Chugai, Tokyo, Japan; clofazimine, Ciba-Geigy, Basle, Switzerland; purified standards of RFP and its analogues, 25-desacetyl rifampicin (DARFP) and rifampicin 1,4-quinone (RFPQ), Daiichi, Tokyo, Japan under licence from Lepetit, Milan, Italy. DDS 4-mono-N-acetylate [12] (MADDS) was synthesized in this laboratory.

The other compounds used in search of a suitable internal standard for the RFPs and clofazimine were kindly supplied by Nihon Waters, Tokyo, Japan.

All of the solvents used for HPLC and UV spectrophotometry were those specially purified for HPLC and obtained from Tokyo Kasei Kogyo, Tokyo, Japan, and Wako Pure Chemical, Osaka, Japan. The solvents and chemicals used for extraction or the other procedures were of guaranteed reagent grade or special reagent grade of the two respective companies.

Standard solutions

All the substances were dissolved in suitable solvents usually at a concentration of 2 μmol/ml. Sulfone compounds other than s-DDS were dissolved in methanol. s-DDS and sulfonamides excluding sulfisoxazole were dissolved once in a small volume of 0.1 *M* NaOH and diluted with methanol to a final alkali concentration below 0.01 *M*. But when s-DDS was used as an internal standard it was dissolved in the solvent system distilled water—chloroform—dimethylformamide (DMF) (1:1:5) which was used for the dissolution of drugs in serum and named SEx. Sulfisoxazole was dissolved in water, clofazimine was dissolved in chloroform. The stock solutions of these drugs were kept in an Ultra-low Revco Freezer and used within 2 weeks after preparation. All RFPs are dissoluble in chloroform or isoamyl alcohol. However, since they are unstable in both solvents when examined spectrophotometrically [13], in cases when they were analysed individually they were dissolved in DMF just before use because DMF strongly stabilizes RFPs [14]. However, for determination of the redissolution ratios from serum of RFPs and for analysis of their serum samples by HPLC, they were once dissolved in chloroform and the chloroform was immediately evaporated to 70—1400 nmol and 42—420 nmol, respectively.

UV spectrophotometry

The stock solutions (2 μmol/ml) were diluted to 10 nmol/ml (clofazimine) or 20 nmol/ml (sulfone compounds, sulfonamides and RFPs) with the developing solvents to be used for HPLC and immediately analyzed. However, the

substances selected as standards were analyzed at the final concentrations of 5–50 nmol/ml.

Selection of standards

The standards were selected based on their retention times and UV absorbances which were analyzed spectrophotometrically. On the basis of these results, we selected s-DDS for analysis of sulfone compounds and phenylthiohydantoin(ε-phenylthiocarbamyl)-lysine (PEPL) for analysis of RFPs and clofazimine (Table I). All of the reagents shown in Table I were of guaranteed reagent grade of Tokyo Kasei Kogyo.

Sera

In order to use guinea pigs as an animal model for examining the influences of antileprosy drugs on cell-mediated immunity, which is supposed to be closely connected with the main cause of relapsed or persistently positive patients [5], we preferentially used a guinea pig serum pooled after collection from hearts of more than 20 healthy animals (Hartley, 600—750 g). Blood

TABLE I

SELECTION OF STANDARDS BASED ON THEIR UV ABSORBANCE (MEASURED BY SPECTROPHOTOMETRY) IN SYSTEM A OR B, AND THEIR RETENTION TIMES WHEN DEVELOPED BY HPLC

Substance	Spectrophotometry		HPLC	
	Peak (nm)*	Molecular extinction × 10^{-4}	t_R (min)	Developing solvent**
Sulfisoxazole	255	2.27	2.5	System A
Promizole (PZ)	269, 334	2.09, 3.41	5.4	System A
s-DDS	262, 297	1.84, 2.45	6.7	System A
DDS	261, 296	1.70, 2.86	7.5	System A
Proethyl (PE)	266, 306	1.80, 3.29	7.8	System A
MADDS	259, 295	2.01, 2.51	9.9	System A
Sulfadimethoxine	272	2.53	12.6	System A
DARFP	242, 256, 338	3.38, 3.30, 2.60	6.9	System B
PTH-phenylalanine***	271	1.01	8.2	System B
RFP	242, 256, 337	4.45, 3.95, 3.13	8.2	System B
RFPQ	242, 278, 337	2.82, 2.61, 1.75	9.4	System B
PTH(ε-PTC)-lysine§ (PEPL)	270	2.93	12.6	System B
Acenaphthene	234, 283, 292, 303, 309, 323	2.22, 0.88, 0.99, 0.62, 0.44, 0.26	18.8	System B
Clofazimine	287	8.82	19.1	System B
Fluoranthene§§	216, 239, 279, 289	4.35, 5.55, 2.35, 4.40	20.1	System B

*UV absorbance was measured by an Hitachi Recording Spectrophotometer, Model EPS-3T.
**System A: acetonitrile—water (20:80), flow-rate 2.0 ml/min, pressure 70 kg/cm^2. System B: tetrahydrofuran—0.5% acetic acid (40:60), flow-rate 1.5 ml/min, pressure 112 kg/cm^2.
***PTH = phenylthiohydantoin.
§ PTC = phenylthiocarbamyl.
§§ Only main peaks recorded.

TABLE II

DISSOLUTION RATIO FROM SERUM SPECIMENS OF EACH DRUG (AVERAGED ON THE BASIS OF THOSE AT VARIOUS CONCENTRATIONS) AND THE LINEARITY BY OPTICAL DENSITY OF VARIOUS CONCENTRATIONS OF DRUG IN THE REDISSOLUTE

One millilitre each of various concentrations of each drug (70, 140, 210, 350, 700 or 1400 nmol/ml) prepared by diluting the stock solution (2 μmol/ml) was separately dried in vacuo. The residues were dissolved by 7 ml of water—chloroform—DMF (1:1:5) (SEx) as the comparative controls for 100% redissolution. In addition, in order to examine the sufficient redissolution from serum specimens, 1 ml of bovine serum, 1 g of $(NH_4)_2SO_4$, 1 ml of chloroform and 5 ml of DMF were stepwisely added to the dried drug in various amounts (70, 140, 210, 350, 700 or 1400 nmol) under stirring. After centrifugation followed by filtration, the optical densities of the supernatants were measured using an Hitachi Recording Spectrophotometer, Model EPS-3T, and compared with those of comparative controls (SEx solutions). The composition of the final supernatants obtained from serum specimens is an aqueous layer of serum—chloroform—DMF (1:1:5), which is named SExS in this paper, in addition to various amounts of drug. Always, three specimens were prepared at each drug concentration. All of the optical densities (comparative controls, n = 18) and of redissolution ratios (serum specimens, n = 18) at the final concentrations of 10—200 nmol/ml were found to be within ± 10% error, and 1—3 values at each concentration were used for determining the redissolution ratio averaged and statistically examined by C.V.

Substance	nm	Y (optical density) = aX (nmol) + b						Averaged dissolution ratio (%)	C.V. (%)
		Control			Redissolute				
		a*	b	n**	a'	b'	n		
DDS	310	0.0325	−0.0486	16	0.0314	0.0114	12	102.9	5.11
MADDS	303	0.0263	0.0073	15	0.0254	0.0028	12	100.3	1.40
PZ	310	0.0309	−0.0090	16	0.0303	−0.0590	13	101.9	2.50
s-DDS	303	0.0237	−0.0318	14	***	—	—	—	—
Clofazimine	289	0.0849	−0.0831	13	0.0865	−0.0472	12	99.8	4.33
Clofazimine	452	0.0592	−0.0026	16	0.0599	−0.0266	14	99.6	5.32
RFP	342	0.0273	0.0226	13	0.0270	0.0315	13	100.6	2.19
DARFP	342	0.0271	−0.0023	14	0.0275	0.0053	10	100.0	1.74
RFPQ	263	0.0264	−0.0262	13	0.0253	−0.0365	10	101.5	4.87
RFPQ	330	0.0125	−0.0370	15	0.0128	−0.0226	12	104.8	4.11
PEPL	272	0.0293	0.0203	16	***	—	—	—	—

*Linear coefficients determined by the least squares method.
**Number of specimens used for determining linear coefficients.
***Since both s-DDS and PEPL used as standard materials were added to the supernatants after redissolution, their redissolution ratios are not given.

samples were taken whenever needed and preserved at −80°C as 5—10-ml aliquots in sterilized disposable centrifuge tubes (Polyspitz-S, Nissui). However, for determination of redissolution ratios of drugs, in order to decrease the frequency of sampling, bovine serum (Japan Biotest Institute) was used.

Determination of redissolution ratios of drugs

Starting from each stock solution, 70, 140, 210, 350, 700 or 1400 nmol of each drug were dried in vacuo below 40°C in a glass test tube (100 mm long, 15 mm I.D.) by the use of a shaking evaporator, Vapour-Mix (Tokyo Rikakikai). One millilitre of bovine serum was added and stirred for 20 sec by a Micro Thermo-Mixer, Model TM-101 (Tokyo Thermonics) to take up the drug in the serum. Then 1 ml of chloroform and 1 g of $(NH_4)_2SO_4$ fine crystals were added and again stirred for 20 sec. To the resultant homogenate, 5 ml of DMF were added and stirred further for 1 min. The tube was centrifuged at 4000 *g* for 10 min at 0°C on a Marusan Model 50M (-L) centrifuge (Sakuma Seisakusho). The supernatant was filtered once through a filter paper (Toyo Roshi No. 7, dial 9 cm, Toyo Roshi). In addition, 8 ml of the bovine serum were extracted with increased amounts of redissolution reagents. The final filtrate, with a composition equal to SEx except that it contains protein-free supernatant of bovine serum instead of water as in SEx, was named SExS. It was used for diluting the portions of redissolutes where the optical densities were above 1.0 due to the high drug concentrations.

As the comparative control, the drug solutions of 10, 20, 30, 50, 100 and 200 nmol/ml of SEx were used as standards for 100% extraction.

In each case three specimens were prepared at each drug concentration. The average redissolution ratio of each drug represented by the coefficient of variation (C.V.) was elicited on the basis of 1—3 ratios at each concentration. The linearity of response of each drug was examined by the least squares method. This deproteinated redissolution could minimize the volume of denaturated protein block-precipitated after centrifugation. Without the addition of chloroform, a slight turbidity was occasionally observed in the supernatant. The results are shown in Table II.

Redissolution of drugs from serum for HPLC analysis

Initially, the following solutions were prepared. (I) Sulfone compounds (each 100 μmol) excluding s-DDS and 100 μmol of clofazimine together dissolved in 50 ml of methanol. (II) RFP and DARFP (each 64 μmol) dissolved in 8 ml of chloroform. (III) RFPQ (64 μmol) dissolved in 8 ml of chloroform. (IV) s-DDS (40 μmol) dissolved in 200 ml of SEx. (V) PEPL (40 μmol) dissolved in 200 ml of SEx. By diluting solution I with methanol, 10-ml solutions of drugs containing 200, 500, 1000 or 2000 nmol/ml were prepared; 0.21-ml aliquots of each were immediately dried in vacuo in 20 glass test tubes. These were preserved at −20°C until use. Solution II was immediately washed by stirring for 30 sec with 0.5 ml of 1% ascorbic acid aqueous solution. It was washed further twice with 0.5 ml each time of distilled water. The chloroform layer was diluted with chloroform to 0.8, 2.0 or 4.0 μmol/ml. An aliquot (0.21 ml) of each of the original solution or dilutions were dried in vacuo below 40°C in 12—15 glass test tubes. The residues contain 168, 420, 840 or 1680

nmol of RFP and DARFP. They were preserved at −80°C until use. Just before use, 1 ml of chloroform was added and an aliquot (0.25 ml) of the resultant solution was added to the residue dried from solution I. The mixture was immediately dried in vacuo below 40°C. Solution III was diluted with chloroform as in the case of solution II but without washing the chloroform layer. It was layered on to the dried mixture of solutions I and II and immediately dried again in vacuo below 40°C. Thus, the final dried residue contains 42, 105, 210 or 420 nmol of PZ, DDS, MADDS, RFPs and clofazimine.

To the dried drug mixture, 0.3 ml of pooled guinea pig serum and 0.3 g of $(NH_4)_2SO_4$ were added. It was made to redissolute by the further addition of 0.3 ml of chloroform and 1.5 ml of DMF. After centrifugation at 4000 *g* for 10 min at 0°C, 1 ml of the supernatant whose drug concentration is to be 20, 50, 100 or 200 nmol/ml was diluted with 1 ml of solution IV or V, in which the concentration of s-DDS or PEPL is equal or twice that of the drugs, respectively. In order to estimate the practical minimum measurable quantity, in some cases the redissolute of the lowest final concentration (10 nmol/ml) was further diluted with SExS.

All of the redissolutes were filtered through a Teflon-type filter (FHLP01300, pore size 0.5 μm, Millipore) and analyzed by HPLC; the injection volume was 3—8 μl, a Hamilton micro-syringe, Microliter No. 820 being used.

RESULTS AND DISCUSSION

At the first step, the discovery of a suitable solvent in which are freely dissoluble all the sulfone drugs, clofazimine and RFPs in serum was requested.

Ellard and Gammon [15] and Peters and co-workers [16, 17] extracted DDS and MADDS from serum with ethyl acetate and from urine with dichloroethane at a strongly basic pH. Barry et al. [18] and other investigators [19, 20] used chloroform or acetic acid—benzene (or toluene) (1:3) for extracting clofazimine from biological materials. RFPs are extractable quantitatively with chloroform, isoamyl alcohol [21] or other solvent mixtures [22]. We also noticed that clofazimine and RFPs could be sufficiently extracted by deproteinative extraction with chloroform and ammonium sulfate even in final concentrations above 200 nmol/ml. However, since extraction at acidic or basic pH is undesirable for the unstable RFPs, we could not use chloroform as the extraction solvent because especially the extraction ratios of sulfones after agitation with chloroform at neutral pH were found to be incomplete above a final concentration of 50 nmol/ml. Although the method used in this report is not extraction but a mere redissolution, this method showed two advantages: (1) the minimization of denaturated protein block-precipitated after centrifugation of redissolute; and (2) the stabilization of RFPs by the effect of DMF.

The redissolute was first developed by system A which is the usual developing solvent system for analyzing sulfonamides on a μBondapak C_{18} column [23]. Various quantities between 5 and 500 pmol of liposoluble sulfone compounds were analyzed. The chromatograms at lower concentrations of drugs and the linearities examined by coefficients of variation are shown in Fig. 2 and Table III, respectively. The linearities by both peak height and peak area could clearly be found in the range of attenuator on the UV spectrophotometric detector linked to the outlet of the column from 0.005 (minimum

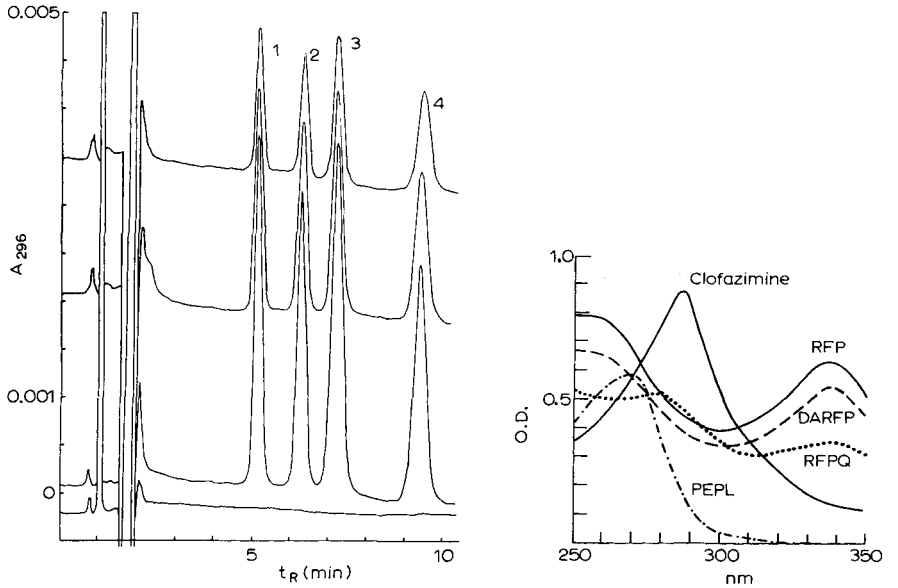

Fig. 2. Chromatograms of several sulfone compounds developed with system A and detected at 296 nm. Developing solvent A = acetonitrile—water (20:80); flow-rate 2.0 ml/min; pressure, 70 kg/cm^2; temperature, 20 ± 2°C. Peaks: 1 = promizole (PZ); 2 = 4,4′-diaminodiphenyl sulfone 2′-sulfonamide (s-DDS); 3 = 4,4′-diaminodiphenyl sulfone (DDS); 4 = DDS 4-mono-N-acetylate (MADDS). The chromatograms from top to bottom correspond, respectively, to 30 pmol, 50 pmol, and 80 pmol of each drug. Range on attenuator: 0.005. Both of the two sharp large peaks detected at t_R 1.1 and 1.8 min are those of the basal medium [SExS, the aqueous layer of guinea pig serum—chloroform—DMF (1:1:5)]. By developing with system A, only the sulfone compounds eluted and all the RFPs, PEPL and clofazimine remained on the μBondapak C_{18} column.

Fig. 3. UV spectra of RFPs, clofazimine and PEPL (as the standard for HPLC analysis). Solvent system B. Concentrations of substances: clofazimine, 10 nmol/ml; the others, 20 nmol/ml. Spectrophotometer: Hitachi Recording Spectrophotometer, Model EPS-3T.

range) to 0.04. Based on a signal-to-noise ratio above 30, all of the practical minimum measurable quantities of DDS, MADDS and PZ were found to be 15 pmol when injection volumes of 3—8 μl of 2 nmol/ml of redissolute were analyzed. The averaged recovery ratios (peaks of redissolute/peaks of SEx solution, %) and C.V. (%) at low molecular concentrations (10 pmol per 5 μl and 16 pmol per 8 μl) were: PZ 102.4%, 3.05%, n = 3; DDS 99.0%, 5.09%, n = 3; MADDS 99.1%, 4.72%, n = 4.

When the redissolute was developed with system B, DDS, MADDS and PZ were eluted at the same time as a single peak. Then, the peaks of RFPs were detected in the order DARFP, RFP and RFPQ. The peaks of PEPL and clofazimine were found later than the elution of RFPs.

However, as the wavelengths of the main RFPs peaks are shorter or longer than those of clofazimine and PEPL, the selection of a suitable detection wavelength was requested.

As shown in Fig. 3, the molecular extinction of PEPL markedly decreased from the peak at 270 nm to a longer wavelength (29,300, 9500, 2520, 890 and 310 at 270, 287, 300, 310 and 320 nm, respectively); 2, 5 or 20 times higher concentrations of PEPL as standard than that of drugs were needed when analyzed by HPLC at 287, 300, and 310—320 nm, respectively. The

TABLE III

STATISTICAL RESULTS OF CHROMATOGRAMS OF SULFONE COMPOUNDS DEVELOPED WITH SYSTEM A AND DETECTED AT 296 nm

Substance	t_R (min)	Peak height* (= mm/nmol) (R: 1.0)	C.V. (%)	Peak height (= aX (pmol) + b)		Peak area** (mm^2/nmol) (R: 1.0)	C.V. (%)	Peak area (= $a'X$ (pmol) + b')		Pract. min.§ (pmol)
				a***	b***			a'	b'	
PZ	5.2	11.6	3.53	0.0107	0.0512	47.7	2.83	0.0448	0.0867	15
s-DDS§§	6.4	10.0	2.00	0.0098	0.0181	47.9	2.00	0.0460	0.1868	15
DDS	7.2	11.0	3.45	0.0105	0.0468	59.9	3.99	0.0577	0.1995	15
MADDS	9.4	8.2	1.83	0.0070	0.0268	59.1	3.37	0.0529	0.0156	15

*Each peak height (mm) was converted into that at 1 nmol and the range (R) on the attenuator at 1.0 after correction by that of s-DDS as the standard. Then all the peak heights were averaged and examined statistically by C.V.

**The peak areas (height × width at half height) were statistically treated in the same way as the peak heights.

***The linearity was examined by the least squares method. The linear coefficients (a and b for peak heights and a' and b' for peak areas) based on heights or areas of five adequate peaks (from 10 to 500 pmol) are recorded.

§ The practical minimum measurable quantity was estimated from chromatograms based on a signal-to-noise ratio above 30.

§§ The values of s-DDS peaks were based on those developed in SEx solutions alone because it is the standard for sulfone compounds.

sensitivity in peak detection of PEPL itself by HPLC analysis examined by minimum measurable quantity was also found to be lower when the solution or dissolute of PEPL which was dissolved in SEx [water—chloroform—DMF (1:1:5)] or in SExS [serum—chloroform—DMF (1:1:5) with added ammonium sulfate], respectively, was analyzed.

However, when all RFPs, clofazimine and PEPL were analyzed at 287 nm, which is the optical peak of clofazimine dissolved in system B, the peaks of RFPs, especially of RFPQ, were larger than those at 300 nm, due to the gradual increase in the UV absorbance of RFPs from 300 nm to 287 nm. For this reason, we concluded that analysis at 287 nm after mixing a two-times higher concentration of PEPL than that of the drugs would be the most rational method.

Various quantities of RFPs (10—500 pmol) and clofazimine (5—500 pmol) were analyzed. The representative chromatogram and the linearities examined at 287 nm are shown in Fig. 4 and Table IV, respectively. In Table IV, the practical minimum measurable quantities were estimated on the basis of a signal-to-noise ratio above 30. The averaged recovery ratio (%) and C.V. (%) at low molecular concentrations (10 pmol per 2 μl, 30 pmol per 6 μl, 50 pmol

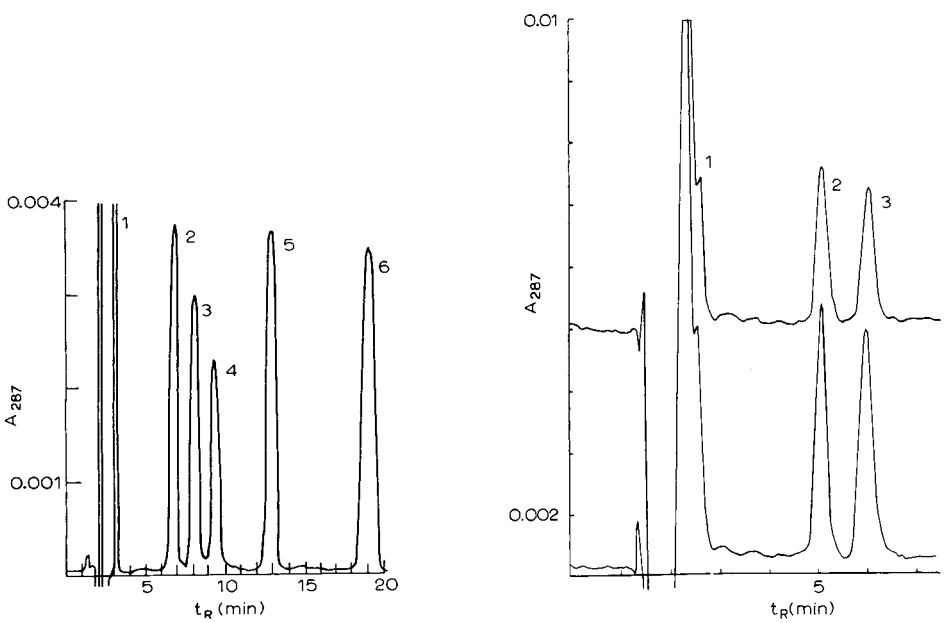

Fig. 4. Chromatograms of RFPs, clofazimine and PEPL (as the standard) developed with system B and detected at the optical peak of clofazimine (287 nm). Developing solvent B, THF—0.5% acetic acid (40:60); flow-rate, 1.5 ml/min; pressure, 112 kg/cm^2; temperature, 20 ± 2°C. 1 = Peaks from mixture of DDS, MADDS and PZ; 2 = 25-desacetyl RFP (DARFP); 3 = RFP; 4 = RFP 1,4-quinone (RFPQ); 5 = PTH(ϵ-PTC)-lysine (PEPL); 6 = clofazimine. Concentrations: PEPL, 200 pmol; all of the others, 100 pmol. Range on attenuator, 0.01.

Fig. 5. Chromatograms of clofazimine isolated from RFPs when developed with system C. Developing solvent C, THF—water (50:50) containing PIC B-5; flow-rate, 1.5 ml/min; pressure, 116 kg/cm^2; temperature, 20 ± 2°C. 1 = Peaks from mixture of solvent, sulfone compounds and RFPs; 2 = peaks of clofazimine, upper peak with 15 pmol, lower peak with 25 pmol; 3 = peaks of PEPL, upper peak with 60 pmol, lower peak with 100 pmol. Range on attenuator: 0.01.

TABLE IV

STATISTICAL RESULTS OF CHROMATOGRAMS OF RFPs AND CLOFAZIMINE DEVELOPED WITH SYSTEM B AND DETECTED AT 287 nm

The meanings of linear coefficients and the practical minimum measurable quantities, or the statistical method, are the same as those explained in the footnotes of Table III.

Substance	t_R (min)	Peak height (=mm/nmol) (R: 1.0)	C.V. (%)	Peak height (=aX (pmol) + b)		Peak area (=mm²/nmol) (R: 1.0)	C.V. (%)	Peak area (=$a'X$ (pmol) + b')		Pract. min. (pmol)
				a	b			a'	b'	
DARFP	6.5	7.1	2.54	0.0070	0.0019	57.1	3.71	0.0553	0.3007	40
RFP	7.8	5.4	5.56	0.0059	−0.1322	42.8	7.71	0.0521	−1.8824	40
RFPQ	9.2	4.3	6.51	0.0045	0.0833	59.6	4.40	0.0574	0.1135	50
PEPL	12.5	3.4	3.24	0.0034	−0.0357	32.9	3.25	0.0350	−0.6501	50
Clofazimine	18.9	6.3	4.60	0.0059	0.0701	104.3	2.36	0.1012	0.5444	15

TABLE V

STATISTICAL RESULTS OF CHROMATOGRAMS OF CLOFAZIMINE ISOLATED FROM RFPs WHEN DEVELOPED WITH SYSTEM C AND DETECTED AT 287 nm

The meanings of linear coefficients and the practical minimum measurable quantities, or the statistical method, are the same as those explained in the footnotes of Table III.

Substance	t_R (min)	Peak height (=mm/nmol) (R: 1.0)	C.V. (%)	Peak height (=aX (pmol) + b)		Peak area (=mm²/nmol) (R: 1.0)	C.V. (%)	Peak area (=$a'X$ (pmol) + b')		Pract. min. (pmol)
				a	b			a'	b'	
Clofazimine	5.1	42.8	11.42	0.0397	0.0745	190.9	2.34	0.1844	0.4005	5
PEPL	6.1	8.7	1.61	0.0083	0.1439	52.1	1.63	0.0503	0.7157	20

per 5 μl) were: DARFP 104.6%, 2.78%, n = 3; RFP 98.1%, 7.90%, n = 3; RFPQ 103.0%, 5.31%, n = 3; clofazimine 97.8%, 8.85%, n = 4.

Some other solvent systems were examined together. However, clofazimine could not be eluted with methanol systems containing 0.5% phosphoric acid, 1% acetic acid or PIC B-5, and the elution rates with solvent systems containing THF and various volumes of 0.5% phosphoric acid were too fast.

If the redissolute was developed with THF—water (50:50) containing 0.0025 M 1-pentanesulfonic acid and acetic acid as PIC B-5, the elution pattern was faster than that in development by system B. As the result, the rapid measurement of clofazimine separated from RFPs became possible. In this case, the peak of PEPL was found later than that of clofazimine. Representative chromatograms and the statistical results are shown in Fig. 5 and Table V, respectively. For analyzing clofazimine by development with system C, a final concentration of PEPL four times higher than that of clofazimine was used. The averaged recovery ratio (%) and C.V. (%) at low molecular concentrations (3 pmol per 3 μl, 5 pmol per 5 μl, 10 pmol per 5 μl, 16 pmol per 8 μl) was: clofazimine 97.8%, 6.68%, n = 4.

Insofar as the analysis could be finished within a day, each of the purified RFP standards showed a fine single peak when dissolved in the DMF or SEx system and preserved in an ice bath. At the same time, even if they were kept under these conditions before analysis, a non-negligible quantity of RFPQ was detected in commercially available RFP capsules, when developed without prior washing with ascorbic acid. Moreover, even though they were purified RFP standards, if they were dissolved in methanol and kept at room temperature for more than 6 h before analysis, the peak of RFPQ due to the oxidation of RFP was clearly detected.

One of the problems still remaining is the mutual separation of water-soluble conjugates of DDS and MADDS such as 4-N-glucosiduronates and 4-N-sulfamates which are respectively called DDSG [24], DDSS [25], MADDSG and MADDSS [26]. No single method of determination for these compounds can be found except for their approximate determination by stepwise hydrolysis with HCl [27]. Although these compounds are mutually separable on silica plates by developing with isopropanol—chloroform—methanol—10 N ammonia (10:10:5:2) [28] or with isopropanol—n-butanol—phosphate buffer (pH 7.4) (3:2:1) [29], further work is necessary to establish a means of quantitating them.

On the other hand, no references can be found in the literature which demonstrate any metabolite of clofazimine except a presumption based on in vitro experiments where the production of a labile leuco-type of clofazimine was detected [9, 30].

So far as we have examined, no disturbance of either the mutual separation or sensitivity in analyzing sulfone compounds due to lowering of the theoretical plate number or instability of the baseline was observed even after analyzing several tens of redissolutes containing RFPs, clofazimine and PEPL, all of which accumulated inside the column when developed by system A. Moreover, all of the compounds were eliminated during conditioning of the column by system B. The order of development from system A to B was exchangeable with that from system B to A.

Since a uniform layer could be preserved up to an increased ratio of serum to

chloroform and DMF (no higher than 3:1:5), the minimum measurable concentrations of every drug may be lowered further. At the same time, minimization of the volumes of both blood and the SEx system will be possible by the use of a micro-analytical technique; for example, the rapid separation of SExS supernatant on a micro-centrifuge following the redissolution inside a smaller test tube will achieve this purpose and will further facilitate the analysis of a number of specimens, if all of the redissolutes from the specimens contain these drugs and their main metabolites in final concentrations not less than the respective practical minimum measurable quantities.

ACKNOWLEDGEMENT

The authors wish to thank Dr. T. Nakayama, the Liquid Chromatography People of Nihon Waters Ltd., for his valuable suggestion and supply of reagents in the search for the standard material for RFPs and clofazimine.

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Journal of Chromatography, 223 (1981) 393—399
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 819

DETERMINATION OF CEPHALOSPORINS IN BIOLOGICAL MATERIAL BY REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY

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(First received October 28th, 1980; revised manuscript received December 17th, 1980)

SUMMARY

Seven cephalosporins (β-lactam antibiotics), viz. cefazolin, cephalotin, cefoxitin, cefotaxime, cefamandole, cefuroxime and cefoperazone (T 1551) were determined in biological material. The compounds were extracted from acid-treated body fluids into chloroform—1-pentanol (3 : 1) and re-extracted into a small volume of an aqueous phase at pH 7, which was injected into the chromatographic column. The chromatographic support was μBondapak C_{18} (10 μm) and the mobile phase was a mixture of 0.01 *M* acetate buffer (pH 4.8) and methanol or acetonitrile. Detection limits are about 50 ng/ml for extractions from 1 ml of serum and have permitted pharmacokinetic studies of the seven cephalosporins.

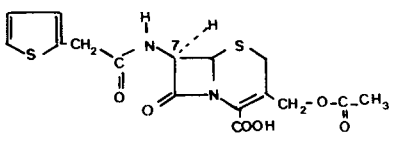
INTRODUCTION

Pharmacokinetic studies of cephalosporins are usually performed by conventional microbiological assay procedures [1—6]. However, high-performance liquid chromatography is specific, rapid and sensitive, and is to be preferred when analysing samples of biological fluids, for separating the drug from interfering substances or for dosing eventual metabolites. A number of liquid chromatographic methods have been reported for the determination of cephalosporins in blood serum and urine [7—10]. Although some of these methods used anion-exchange columns for the separation of the drugs [11], some recent methods [12—14] recommended the reversed-phase technique with octadecyl-bonded packings. The major differences between these methods were in the serum sample preparation procedure. We have previously used serum protein precipitation techniques for sample preparations [15, 16] but with a low sensitivity (500 ng/ml). Although rapid and convenient, this method is not readily adaptable to trace amounts of drugs.

0378-4347/81/0000—0000/$02.50

This paper describes a method for the preparation and determination of seven cephalosporins, cefazolin, cefoxitin, cephalotin, cefuroxime, cefotaxime, cefamandole and cefoperazone (T 1551) and metabolites, using reversed-phase HPLC after extraction.

These semi-synthetic cephalosporins have similar structures (Fig. 1), based on the 7-aminocephalosporanic nucleus. The extraction depends on the carboxylic group at the C_3 position. Cephalosporins are isolated from the biological material in an organic phase at pH 3 and brought into a small volume of aqueous phase at pH 7. The drugs are then separated from co-extractants by reversed-phase chromatography and quantified by UV absorbance measurement at an appropriate wavelength. Detection limits are about 50 ng/ml for extraction from 1 ml of serum.



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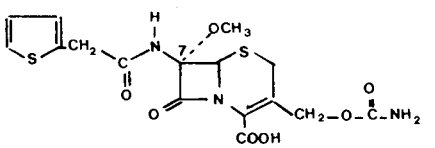

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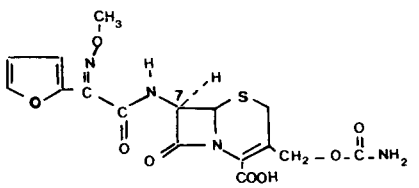

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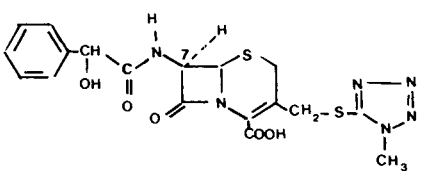

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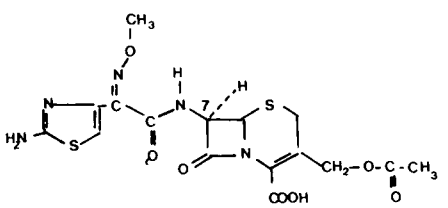

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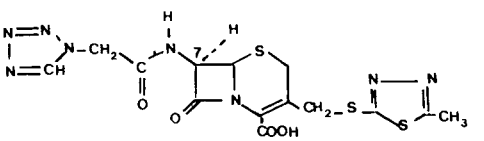

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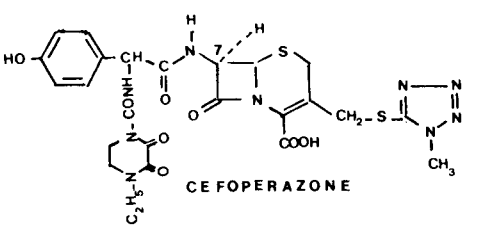

CEFOPERAZONE

Fig. 1. Structures of cephalosporins.

EXPERIMENTAL

Drug standards

Pure drug samples were obtained as follows: cefazolin from Allard (Paris, France), cefoxitin from Merck, Sharp & Dohme (Paris, France), cephalotin and cefamandole from E. Lilly (Saint-Cloud, France), cefotaxime from Roussel (Paris, France), cefuroxime from Glaxo (Paris, France) and cefoperazone (T 1551) from Pfizer (Orsay, France).

Chemicals

Reagent-grade acetic acid, sodium acetate, hydrochloric acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, chloroform, 1-pentanol (R.P. Normapur, Prolabo, Paris, France), acetonitrile and methanol (Uvasol grade, E. Merck, Darmstadt, G.F.R.) were used without further purification.

Plasma and urine samples

Blood samples (5 ml) were collected in 10-ml glass test-tubes (Vacutainer) containing heparin, immediately before and at appropriate times after the start of drug administration. The samples were rapidly centrifuged at 1000 *g* for 15 min. The plasma fraction was carefully separated using a sera-clear and frozen at −20°C until taken for assay. Urine samples were stored at −20°C until taken for assay.

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used, which included a dual 6000A solvent delivery system, a WISP 710A sample processor and an M450 variable-wavelength UV detector. An Omniscribe B5000 recorder (Houston Instruments, Austin, TX, U.S.A.) was used. A reversed-phase μBondapak C_{18} (10 μm; 30 cm × 3.9 mm I.D.) column was employed (Waters Assoc.). Filtration of solvents was carried out using a Pyrex filter holder (Millipore Corp., Bedford, MA, U.S.A.). The curves were plotted by a Tektronix 4052 computer with an interactive graphic package for pharmacokinetic analysis [17].

Procedure for plasma samples

Extraction. The plasma sample (1 ml) was mixed with 0.50 ml of 0.4 *M* hydrochloric acid in a 10-ml centrifuge tube. The mixture was extracted with 7 ml of chloroform—1-pentanol (3 : 1), the extraction time being 10 min. After centrifugation at 1000 *g*, 5 ml of the organic phase were transferred into another centrifuge tube and cephalosporins were back-extracted (10 min) into 350 μl of phosphate buffer (pH 7). After centrifugation at 1000 *g*, part (10—50 μl) of the (upper) aqueous phase was injected into the chromatographic column.

Chromatography. The mobile phase was a mixture of 0.01 *M* acetate buffer (pH 4.8) and methanol or acetonitrile. The flow-rate was 1.5 ml/min at a pressure of about 150 bar. The separations were carried out at room temperature.

Quantitation. The chromatograms were quantitated by measuring the peak heights manually with a ruler. Calculations were performed by using a calibra-

tion graph of peak height versus concentration obtained by analysing known amounts of the compounds added to plasma samples obtained before administration of the drugs.

Procedure for urine samples

After centrifugation, urine samples were diluted with doubly distilled water and injected into the liquid chromatograph without extraction. To avoid interfering peaks, a mobile phase containing a lower percentage of acetonitrile or methanol was generally used. Calculations were performed by using a calibration graph obtained by analysing known amounts of the cephalosporins added to urine samples obtained before administration of the drugs.

RESULTS AND DISCUSSION

Extraction

Results for extraction from serum using various solvents are given in Table I.

TABLE I

PERCENTAGE EXTRACTION OF CEFAZOLIN WITH DIFFERENT SOLVENTS

Solvent	Extraction (%)	Solvent	Extraction (%)
Benzene	6.5	Amyl acetate	12
Chloroform	3	Ethyl acetate	83.5
Cyclohexane	5	Chloroform—1-butanol (3 : 1)	84.5
Methylene chloride	9	Chloroform—1-propanol (3 : 1)	84.5
Diethyl ether	7	Chloroform—1-pentanol (3 : 1)	85
n-Heptane	4		
n-Hexane	5		

Chloroform—1-pentanol (3 : 1) gave the best quantitative extractions. A theoretical elucidation of the extraction mechanism is probably possible based on the pK_a of the cephalosporins given by the carboxylic group at the C_3 position on the cephem nucleus: this pK_a is about 4—5. When the pH is lower than the pK_a, cephalosporins are in non-ionized form and therefore soluble in organic phase, whereas when the pH is about 7 (higher than the pK_a) they are extracted by an aqueous phase. The ratio of the volume of the organic to that of the aqueous phase is 20. This extraction cannot be used for orally absorbed cephalosporins, which contain an amino group that is ionized at pH 3.

Liquid column chromatography

Retention. We first performed the chromatographic analysis of cephalosporins using anion-exchange techniques, but a continuously decreasing retention was obtained.

The present method, using reversed-phase chromatography, can be used for all cephalosporins, even the orally absorbed cephalosporins cefadroxil, cephradine, cefalexin and cefaclor [18]. The retention times of the seven cephalosporins are given in Table II for a mobile phase consisting of 0.01 *M* acetate buffer (pH 4.8)—15% methanol.

TABLE II

ELUTION TIMES OF CEPHALOSPORINS

Column, μBondapak C_{18} (10 μm, 30 cm × 3.9 mm I.D.); eluent, methanol—0.01 *M* acetate buffer (pH 4.8) (15 : 85); flow-rate, 1.5 ml/min.

Cephalosporin	λ_{max}	Elution time (min)
Cefuroxime	254	3.1
Cefoxitin	245	3.3
Cefotaxime	234	3.45
Cefazolin	275	4.20
Cefamandole	270	6.15
Cephalotin	240	8
Deacetylcephalotin	240	2.7
Cefoperazone (T 1551)	240	8.35

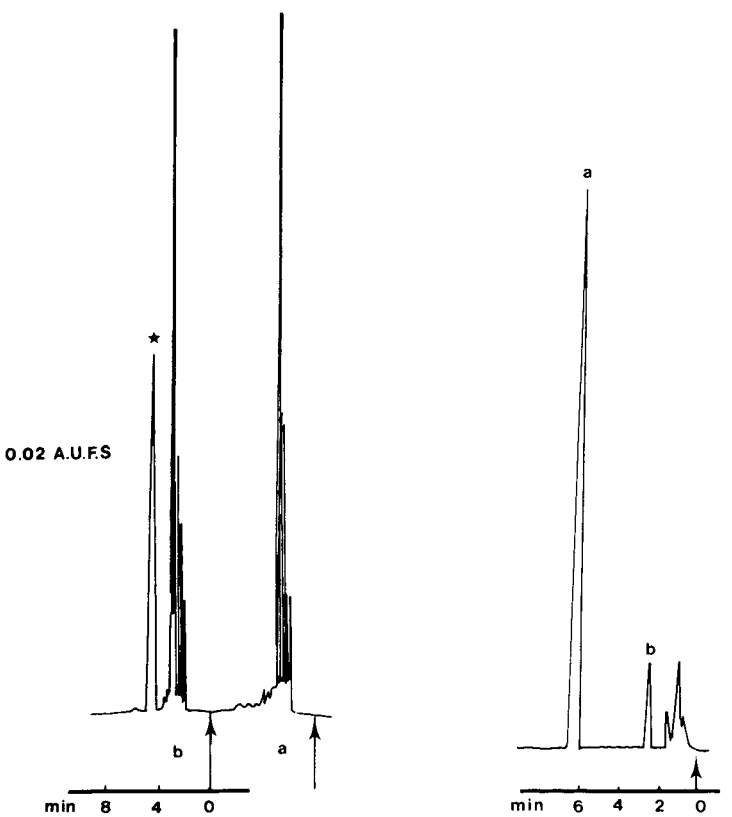

Fig. 2. (a) Chromatogram of extract from blank plasma (1 ml). (b) Chromatogram of extract from 1 ml of plasma containing 2.5 μg of cefoperazone (★) (λ = 240 nm; 0.02 a.u.f.s.; injection volume 40 μl).

Fig. 3. Chromatogram of extract from 1 ml of plasma after administration of cephalotin: a, cephalotin; b, deacetylcephalotin.

Detection and linearity. The detection wavelengths (Table II) were chosen so as to maximize the sensitivity. For instance, as shown in Fig. 2, cefoperazone (T 1551) was monitored at a UV wavelength of 240 nm with a detector sensitivity of 0.02 a.u.f.s. The detection limit under these conditions is about 50 ng/ml. The linearity of the method was determined for all cephalosporins and is illustrated in Table III.

TABLE III

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES

Cephalosporin	Concentration range (μg/ml)	Correlation coefficient	Slope	Intercept
Cefazolin	0.075—1.25	0.9979	40.16	0.213
Cephalotin	0.15—5	0.9999	28.71	−0.055
Cefamandole	0.15—5	1.0000	26.92	0.189
Cefoxitin	0.15—5	0.9999	37.47	1.8
Cefuroxime	0.075—5	0.9999	82.09	−0.088
Cefotaxime	0.15—2.5	0.9999	57.16	0.924
Cefoperazone	0.15—2.5	0.9995	46.07	0.975

Selectivity

Serum protein precipitation techniques have not permitted the separation of cephalosporins from their metabolites. The method proposed here permits the quantitative determination of cephalotin and deacetylcephalotin (Fig. 3), cefotaxime and deacetylcefotaxime after administration of cephalotin or cefotaxime to a patient.

Precision

The precision of the method was studied, for each cephalosporin, by repeated analysis of serum solutions containing the drug. The data in Table IV show that the peak height for eight samples ranged from 106 to 108 mm [mean = 106.5 ± 0.47 mm (S.E.M.) for 2.5 μg/ml of cefoperazone].

TABLE IV

REPEATABILITY OF ANALYSIS OF CEFOPERAZONE

Analysis of 8 blood serum samples containing 2.5 μg/ml of cefoperazone

Sample No.	Peak height (mm)	Sample No.	Peak height (mm)
1	106	5	109
2	106	6	106.5
3	105.5	7	107
4	105	8	108

Plasma samples from patients

Chromatograms of plasma sample from a patient treated with cefoperazone are shown in Fig. 2. The plasma levels after intravenous administration of cefoperazone (1 g, bolus) are presented in Fig. 4.

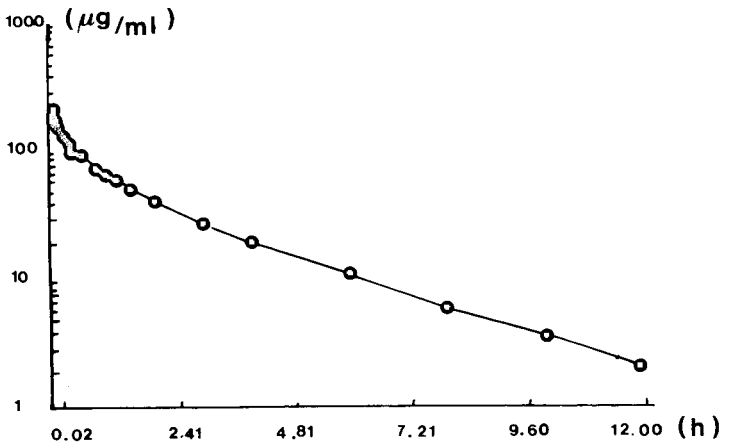

Fig. 4. Decrease of plasma levels of cefoperazone with time after intravenous administration (1 g, bolus).

This method permitted us to determine and compare the pharmacokinetic properties of the seven cephalosporins cefazolin, cephalotin, cefoxitin, cefamandole, cefotaxime, cefuroxime and cefoperazone (T 1551) under the same conditions [19].

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Journal of Chromatography, 223 (1981) 401—408
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 821

DETERMINATION OF TRITIATED DIGOXIN AND METABOLITES IN URINE BY LIQUID CHROMATOGRAPHY

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(First received March 18th, 1980; revised manuscript received December 19th, 1980)

SUMMARY

A liquid chromatographic method for the determination of digoxin, digoxigenin, its mono- and bisdigitoxoside and dihydrodigoxin in urine is described. Doses of 100 μCi of [12α-^{3}H]digoxin and 0.5 mg (640 nmol) of digoxin were administered orally to eight healthy volunteers. The compounds were extracted from urine with methylene chloride containing 3% of heptafluorobutanol. After separation, fractions corresponding to digoxin and the metabolites were measured by liquid scintillation counting. Conjugates of the glycoside metabolites were determined indirectly after pre-treatment of the samples with β-glucuronidase—arylsulphatase. The detection limit was 0.1 nmol/l. Metabolites amounting to 0.5% of digoxin were assayed with a relative standard deviation of 5%.

The advantages of the method are a high recovery in the extraction step, short separation times and the possibility of separate assay of dihydrodigoxin.

INTRODUCTION

Digoxin is hydrolysed under acidic conditions to form digoxigenin and its mono- and bisdigitoxoside [1, 2]. In vivo, the corresponding dihydro compounds also appear [3—5], in addition to conjugates with glucuronic and sulphuric acids [6, 7]. The extent and clinical importance of digoxin metabolism have been discussed by several investigators [4, 5, 8—10], but our knowledge of the biotransformation remains incomplete. The methods available for the assay of digoxin and its metabolites are often tedious and time consuming. Watson et al. [11] used a gas chromatographic method with electron-capture detection. Liquid chromatographic separations using reversed-

or normal-phase systems have been described [12—14] and applied to biological samples [9, 15—17].

In this study we aimed to combine a more efficient solvent extraction process than in previous methods and a high-performance liquid chromatographic system permitting short separation times. Moreover, the separate determination of dihydrodigoxin was desired. This method was applied to urine samples from volunteers given an oral dose of tritiated digoxin, the tritiated substance being used to obtain sufficient sensitivity.

EXPERIMENTAL

Materials

The liquid chromatograph consisted of a Model 71147 pump (LDC, Riviera Beach, FL, U.S.A.), an injection valve (Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl loop, a stainless-steel separation column (150 × 4.5 mm I.D.) and a model 212 UV spectrophotometer (Cecil, Cambridge, Great Britain) with an 8-μl flow cell, operated at 220 nm. The liquid scintillation system was a Nuclear-Chicago Mark III (Searle, Des Plaines, IL, U.S.A.). Digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, digoxin and β-glucuronidase—arylsulphatase were obtained from Boehringer (Mannheim, G.F.R.). [12α-^{3}H]digoxin with a specific activity of 14 Ci/mmol was purchased from New England Nuclear Chem. (Dreieich, G.F.R.). Dihydrodigoxin and [12α-^{3}H]dihydrodigoxin were supplied by the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). 1H,1H-Heptafluorobutanol was obtained from Bristol Organics (Berkeley, Great Britain) and Insta-Gel from Packard Instruments (Downers Grove, IL, U.S.A.). Acetonitrile, *n*-heptane, methylene chloride, 1-pentanol, 2-propanol, 1-butanol and the buffer substances were of analytical-reagent grade and were obtained from E. Merck (Darmstadt, G.F.R.).

Extraction system

The distribution constants (K_D) between phosphate buffer of pH 6.5 (I = 0.1) and various organic phases were determined for digoxin and its hydrolysis products. Before and after extraction and equilibration with the organic phase the compounds were assayed in the aqueous phase by reversed-phase chromatography, UV detection and peak-height measurement. Tritiated dihydrodigoxin was used owing to its low UV absorbance. All extraction experiments were carried out in duplicate.

Chromatographic systems

The separation columns were packed with LiChrosorb SI 60 (normal phase) or LiChrosorb RP-8 (reversed-phase), 5- or 7-μm particles (Merck). In the normal-phase system the eluent was *n*-heptane—1-pentanol—acetonitrile—water (64:26:9:1) and the flow-rate was 1.5 ml/min. The reversed-phase system used a mobile phase of phosphate buffer of pH 6.3 (I = 0.1)—2-propanol (83.5:16.5) and the flow-rate was 1.0 ml/min.

Studies with volunteers

The study on eight healthy volunteers was approved by the Ethical Committee, University Hospital, Linköping. Renal and hepatic functions, as judged from routine laboratory tests, were normal. The volunteers fasted for 8 h before and 2 h after the intake of 100 μCi of [12α-^{3}H]digoxin and 0.5 mg (640 nmol) of unlabelled digoxin given as rapidly dissolving tablets specially prepared by AB Hässle. Urine was collected in polythene bottles during the following intervals after drug intake: 0—4, 4—8, 8—12, 12—24, 24—48 and 48—72 h. Aliquots were kept at −18°C until taken for assay. The gastric pH was analysed using duplicate gastric aspirates taken immediately before drug intake.

Performance of analyses

Frozen urine samples were thawed and homogenized by shaking and 20-ml volumes were extracted for 15 min with 20 ml of methylene chloride containing 3% of heptafluorobutanol. After centrifugation at 1000 *g* for 10 min, 15 ml of the aqueous phase were transferred into a clean tube and re-extracted with 15 ml of new organic phase. Volumes of 10-ml of the organic phase from each extraction were combined and evaporated under nitrogen until about 0.5 ml remained, then 20 μl of 1-pentanol were added to prevent complete dryness and evaporation was continued until 20 μl remained. The residue was dissolved in 250 μl of mobile phase and 100 μl were injected on to the normal-phase chromatographic column. Fractions of the eluate corresponding to digoxin and its hydrolysis metabolites were collected in liquid scintillation vials and 10 ml of Insta-Gel were added before counting.

In order to determine dihydrodigoxin separately from digoxin, the same procedure was performed except that only one extraction was needed and phosphate buffer instead of 1-pentanol was added to prevent complete dryness. Reversed-phase chromatography was used for the separation process.

Conjugated metabolites were determined as the difference between an untreated sample and a sample incubated with β-glucuronidase—arylsulphatase at 37°C for 20—24 h. 25 μl of the enzyme were added per millilitre of sample, the pH of which was adjusted to 5.0 by the addition of buffer solution.

RESULTS AND DISCUSSION

Extraction

Different organic solvents and solvent mixtures were tested for the extraction of digoxin and its hydrolysis metabolites. Results from some of the systems are shown in Table I. Methylene chloride containing 1-butanol or heptafluorobutanol was found to be the most efficient extractant. Heptafluorobutanol is more volatile than the corresponding non-fluorinated alcohol, which facilitates evaporation. Repeated extraction with methylene chloride containing 3% of heptafluorobutanol according to the analytical procedure gave recoveries of 90—99% for the four components.

In order to determine approximately 0.5% of metabolites, it was necessary to concentrate the sample so that the volume injected (100 μl) corresponded to about 4 ml of urine. It was important that the organic phase was not

TABLE I

DISTRIBUTION CONSTANTS (K_D) FOR DIGOXIN AND HYDROLYSIS METABOLITES EXTRACTED FROM PHOSPHATE BUFFER (pH 6.5, I = 0.1)

Substance*	Log K_D		
	Methylene chloride	Methylene chloride—heptafluorobutanol (97:3)	Methylene chloride—1-butanol (90:10)
dg-g	0.23	0.57	0.56
dg-mono	0.00	0.34	0.52
dg-bis	0.43	0.86	1.05
dg	0.90	1.40	1.56

*dg = digoxin; dg-bis = digoxigenin bisdigitoxoside; dg-mono = digoxigenin monodigitoxoside; dg-g = digoxigenin.

completely evaporated as this resulted in hydrolysis of digoxin.

Adsorption of digoxin to polythene and glass vials from aqueous solutions has been observed [18]. In this study, samples stored frozen for 6 months showed no loss of digoxin.

Chromatography

Normal- and reversed-phase chromatography were investigated. It was possible to achieve separations of digoxin and its hydrolysis products with both kinds of system. Well separated peaks were necessary in order to avoid overlapping of the radioactivity in the collection of the eluate fractions. A normal-phase system similar to that suggested by Lindner and Frei [13] was chosen for the analysis (Fig. 1), as short separation times and coinciding retentions for the dihydro compounds and the corresponding unsaturated compounds were obtained. For the separation of dihydrodigoxin from digoxin a reversed-phase system was used in which the dihydro compound was less retained than digoxin (Fig. 2). The k' and α values are given in Table II.

The order of elution of digoxin and its hydrolysis products was the same with both the normal- and the reversed-phase systems. Lindner and Frei [13] suggested that the retention mechanism was partition between polar solvent components enriched on the silica surface and the non-polar mobile phase. A reverse elution order would then be expected for hydrophobized silica with an aqueous mobile phase, but obviously other factors are involved in the retention [12, 16].

Recovery and relative standard deviation

The relative standard deviation was 2% on repeated analysis of urine samples containing 125 pmol (20 nCi) of [^{3}H]digoxin per millilitre. For the determination of hydrolysis products in urine, the relative standard deviation was 5% when measuring metabolites amounting to 0.5% of digoxin. The absolute yield from urine samples was in the range 85% (digoxigenin monodigitoxoside) to 99% (digoxin) in comparison with a standard solution injected directly on to the column.

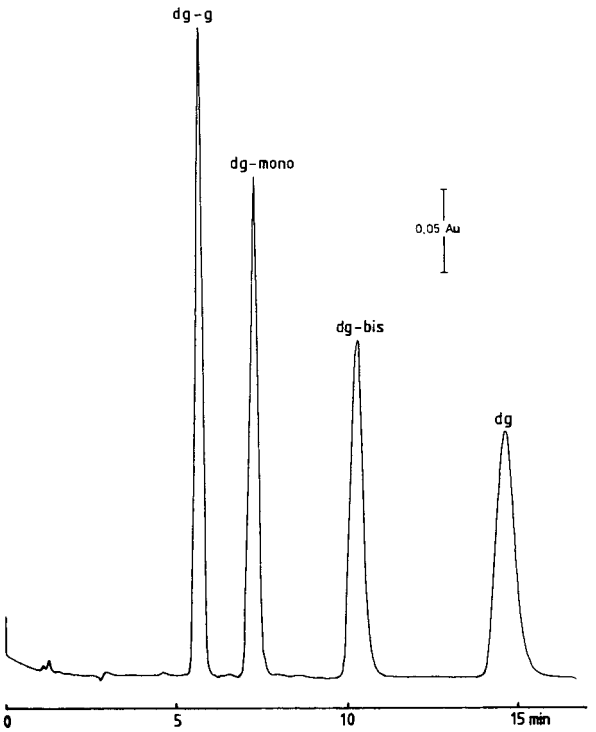

Fig. 1. Chromatogram obtained using a normal-phase system. Column packing: LiChrosorb SI 60 (5 μm). Mobile phase: *n*-heptane—1-pentanol—acetonitrile—water (64:26:9:1). Sample: 10 nmol of each substance in 100 μl of mobile phase. Detection 220 nm, a.u.f.s. 0.5. For abbreviations see Tables I and II.

In comparison, Nelson et al. [16] reported a recovery of 54—78% for digoxin, Loo et al. [15] an average of about 50% for digoxigenin and Gault et al. [17] a mean recovery of about 73%. The greater extraction with our method will increase the precision and the uniformity of recovery between samples from different subjects.

When repeated analysis of urine samples to which 0.5 pmol (2 nCi) or [^{3}H]-dihydrodigoxin per millilitre had been added was performed, the relative standard deviation was 2% and the absolute yield was 99%.

Application

The method was applied to urine samples from eight subjects who had been given an oral dose of tritiated digoxin. The amounts of digoxin and metabolites (after deconjugation) excreted in the urine during 72 h averaged 42.2% (range 29.9—51.9%) of the dose (640 nmol). Digoxin constituted 90.3% (82.0—96.4), dihydrodigoxin 1.4% (0—6.2), digoxigenin bisdigitoxoside 3.6% (1.7—6.0), digoxigenin monodigitoxoside 2.2% (0.8—4.6) and digoxigenin 2.5% (0.5—6.0). These findings are within the ranges reported in other studies [9, 19].

The variation in metabolic pattern between different individuals is illustrated in Fig. 3, where the amounts of metabolites for two of the subjects (C and G)

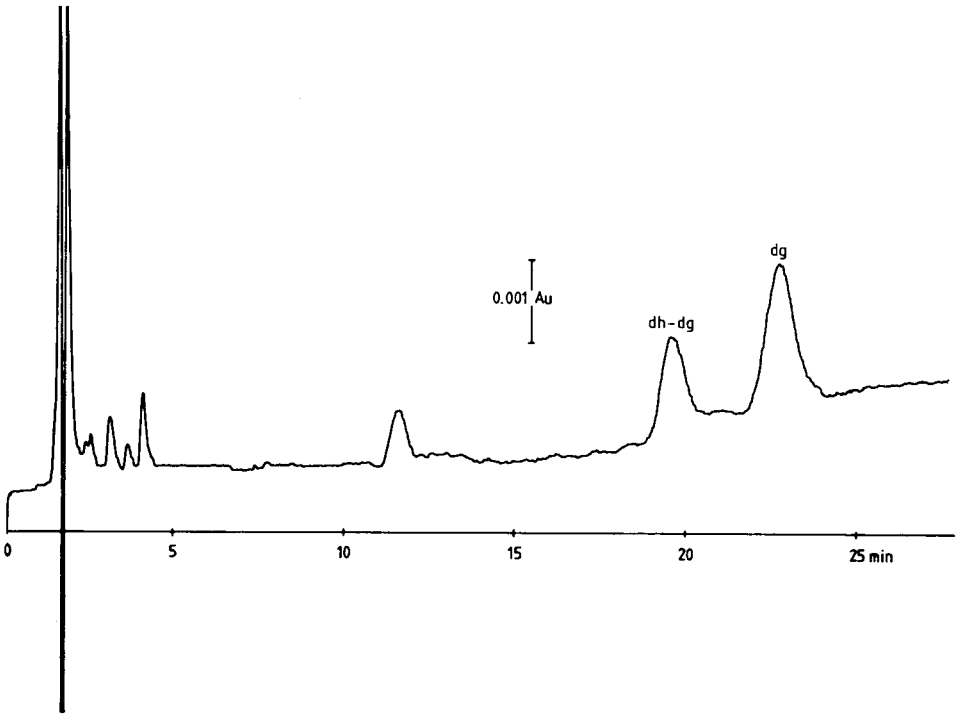

Fig. 2. Chromatogram obtained using a reversed-phase system. Column packing: LiChrosorb RP-8 (5 μm). Mobile phase: phosphate buffer (pH 6.3)—2-propanol (83.5:16.5). Sample: 100 nmol of dihydrodigoxin and 0.2 nmol of digoxin in 100 μl of mobile phase. Detection: 220 nm, a.u.f.s. 0.01. For abbreviations see Tables I and II.

TABLE II

CAPACITY FACTORS (k') AND SEPARATION FACTORS (α) FOR DIGOXIN AND METABOLITES

Substance*	Normal-phase LC		Reversed-phase LC	
	k'	α	k'	α
dg-g	3.15		1.81	
dg-mono	4.15	1.32	2.81	1.55
dg-bis	6.23	1.50	6.66	2.37
dg	9.15	1.47	13.44	2.02
dh-dg	9.62	1.05	11.69	1.15

*Abbreviations as in Table I; dh-dg = dihydrodigoxin.

are given. Both subjects had a gastric pH of 1.5 prior to drug intake and during 72 h 39.5 and 40.8% of the dose was recovered. The metabolites expected to be formed by intragastric hydrolysis, digoxigenin and its mono- and bis-digitoxoside, amounted to 3.0% for subject C and 11.8% for subject G. More than half of the amount of digoxigenin and its monodigitoxoside were found to

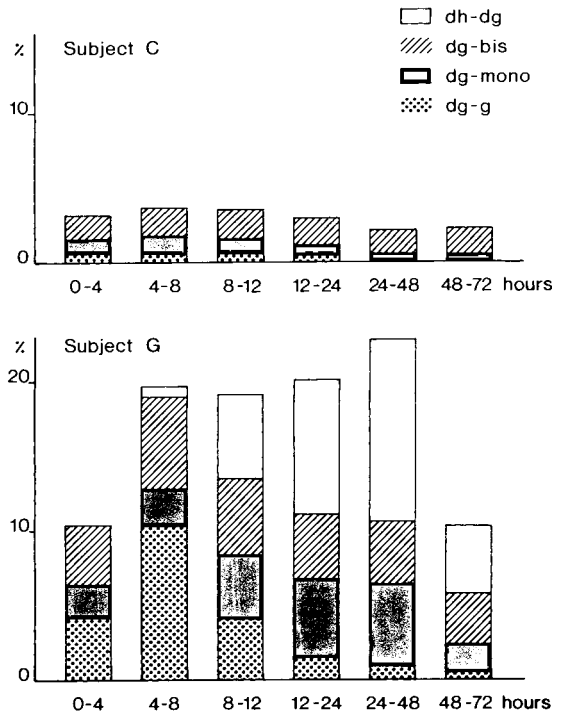

Fig. 3. Excretion of digoxin metabolites after deconjugation in two volunteers expressed as a percentage relative to the total recovery in each collection interval. For abbreviations see Tables I and II.

be present in the conjugated form. In relation to the rate of hydrolysis in vitro [2], the amounts of metabolites are low, which may reflect a rapid gastric emptying rate or a slower hydrolysis in vivo.

In subject G, 6.2% of the recovered dose was in the form of dihydrodigoxin. It has been suggested that this metabolite is formed by bacteria in the lower intestine, but this has not been proved [5]. As can be seen in Fig. 3, dihydrodigoxin is excreted later than the other metabolites, which does not seem to be consistent with the reported half-life of 1.2 h [9].

In conclusion, the proposed method for the determination of tritiated digoxin and its metabolites shows advantages over previous liquid chromatographic methods in terms of improved recovery in the solvent extraction procedure, short separation times and the possibility of the separate assay of the dihydro metabolite of digoxin. The use of ^{3}H-labelled digoxin and selective measurement of digoxin by liquid chromatography give increased accuracy and additional important information compared with conventional radioimmunoassay.

ACKNOWLEDGEMENTS

The support by Drs. Ulf Jonsson and Bengt-Arne Persson during the course of this work and in the preparation of the manuscript is gratefully acknowledged.

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Journal of Chromatography, 223 (1981) 409—416
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 828

ASSAY OF α-DIFLUOROMETHYLORNITHINE IN BODY FLUIDS AND TISSUES BY AUTOMATIC AMINO-ACID ANALYSIS

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(Received December 23rd, 1980)

SUMMARY

A procedure is described for the measurement of DL-α-difluoromethylornithine (DFMO), a selective irreversible inhibitor of ornithine decarboxylase, in biological specimens. The drug is separated from other amino acids with a commercial amino-acid analyser and detected by formation of its alkylthio-isoindole derivative with *o*-phthalaldehyde. DFMO concentrations of 0.1 nmol can be determined in a sample volume of 100 μl. The assay has been used to determine the half-life of DFMO in serum of several species and the relationships between serum and tissue concentrations.

INTRODUCTION

α-Difluoromethylornithine (RMI 71.782, DFMO) has been synthesized in this Centre to inhibit ornithine decarboxylase specifically and hence block production of putrescine and of the polyamines, spermidine and spermine [1]. The concentration of these three amines, which are essential to cell growth, is increased in rapidly proliferating tissues and they thus may be clinically relevant as biochemical markers of cancer [2]. DFMO has interesting properties when used in animal models of rapid cellular proliferation. Danzin et al. [3] have demonstrated that repeated doses of DFMO markedly slow the testosterone-induced weight gain of the prostate in castrated rats, while Prakash and co-workers have shown prolonged survival of L1210 leukemia-bearing mice [4] and retardation of the growth of solid EMT-6 mammary tumors in mice [5].

In order to carry out metabolic, pharmacological and distributional studies an assay for DFMO in biological samples was required. Procedures employing extraction and subsequent concentration of the drug were not applicable since DFMO is extremely water-soluble. Conventional automated amino-acid analysis using colorimetry with ninhydrin was tried initially. This was

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found, however, to be time-consuming and too insensitive for pharmacological studies. A method has therefore been developed where the chromatographic separation is made with a single buffer, so that only a certain section, or "window", of the amino acids measured in normal physiological assays is considered. By employing micro-columns and fluorimetric detection of the *o*-phthalaldehyde derivative, sensitivity has been increased so that 0.1 nmol of DFMO in 100 μl of an injected sample can easily be measured every 40 min.

METHODS

Chemicals

All materials were of A grade and were purchased from E. Merck (Darmstadt, G.F.R.) except the lithium citrate sample dilution buffer, which was obtained from Pierce (Rotterdam, The Netherlands). DFMO was synthesized in our centre [1].

Equipment

A Liquimat II (Kontron, Paris, France) with two 4 mm I.D. glass columns filled with DC-6a resin (Durrum, Palo Alto, CA, U.S.A.) to a bed height of 30 cm, was employed. The apparatus has the advantage that, while an analysis is in progress on one column, the other column is regenerated and then equilibrated in preparation for the second analysis. Two valves are switched automatically to ensure that the samples are injected on to alternate columns and that the effluent of the analysis column is directed to the fluorimeter. A two-column system is most efficient when short assay times are used since the proportion of time spent in regeneration and re-equilibration compared to analysis becomes significant using a single column.

Sample injection was made automatically by an APE-100 (Kontron) fitted with a 100-μl loop. Detection was carried out with the aid of a Fluoromonitor (American Instruments Co., Silver Spring, MD, U.S.A.). A flow cell of 70 μl was employed, with a Corning Glass 7-51 filter for the fluorescence excitation (340 nm) and a Wratten 2A for the emission (440 nm).

Analysis

Separation was made with a single eluting buffer (lithium citrate, 0.668 *M* Li^+, pH adjusted to 4.60 with HCl) using a flow-rate of 30 ml/h and column temperature of 54°C. Regeneration of the column was made after each analysis by passing lithium hydroxide (0.3 *M*) for 16 min. To form the fluorophore, the *o*-phthalaldehyde (OPA) reagent (17 ml/h) was mixed with the column eluent by means of a simple "T" connector and the reaction mixture passed immediately to the fluorimeter. The reagent consisted of 200 mg of OPA dissolved in 3 ml of methanol added to 1 l of 0.4 *M* (pH 10.4) potassium borate solution containing 3 ml of BRIJ and 1 ml of mercaptoethanol. The programme for the two columns is outlined in Table I. It shows an analysis time of 40 min for each column.

TABLE I

PROGRAMME FOR TWO-COLUMN ANALYSIS OF α-DIFLUOROMETHYLORNITHINE

Time (min)	Column 1	Column 2
0	Injection + eluting buffer	LiOH
16	Recorder and OPA* "on"	Re-equilibration
40	Analysis end, recorder and OPA "off"	Re-equilibration
40.01	LiOH	Injection + eluting buffer
56	Re-equilibration	Recorder and OPA "on"
80	Re-equilibration	Analysis end, recorder and OPA "off"
80.01	Repeat	Repeat

*OPA = *o*-phthalaldehyde.

Plasma extracts

Human or animal plasma and serum samples were diluted with one half volume of 20% trichloroacetic acid, allowed to stand for 30 min at 0°C to complete precipitation of the proteins, and then centrifuged for 2 min in an Eppendorf 3200 centrifuge.

The supernatant was further diluted with 0.2 *M* (pH 2.2) lithium citrate dilution buffer as required.

Urine

DFMO was excreted very rapidly in early urines collected after drug administration, so that a large dilution of urine samples could be made and injected directly on to the column without removing ammonia. Daily collections were made in polyethylene bottles, to which 1 ml of 6 *M* hydrochloric acid was added. An aliquot of 1 ml was then mixed with 250 μl of 20% trichloroacetic acid solution. After centrifugation 500 μl of the supernatant were further diluted to 10 ml with sample dilution buffer, before injection on to the column.

Organ extracts

DFMO has been measured in extracts of brain, liver, spleen, prostate and various tumours. Tissues were homogenised in 9 volumes of 0.2 *M* perchloric acid or 20% trichloroacetic acid and the supernatants were diluted and analysed.

Cell extracts

These were made in a similar fashion. Cells from rat hepatoma tissue culture (HTC) were centrifuged, washed with phosphate-buffered saline, and then sonicated with 0.9 ml of 0.1 *M* hydrochloric acid. Further dilution was then made by addition of 0.1 ml of 2 *M* perchloric acid.

Measurement

A 100-μl volume of sample extract was injected on to the column and the peak height of DFMO measured (retention time 30 min). The concentration

was calculated by reference to a calibration curve constructed from known standard solutions.

In the case of plasma samples, it was practical to prepare reference DFMO solutions in plasma, since slight differences in the slopes of the calibration curves obtained from plasma and aqueous samples were sometimes obtained (Fig. 1). Fluorescence was linear over the range 0.2—2 nmol per 100 μl injected.

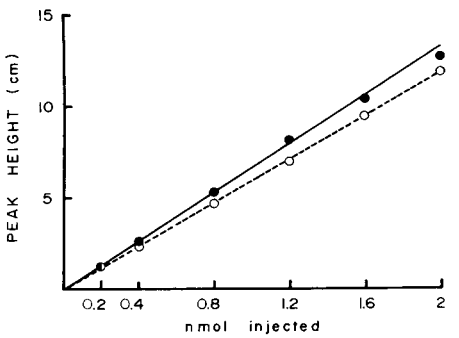


Fig. 1. Calibration plots of the peak height against the amount of DFMO injected. (•), DFMO in plasma; (○), DFMO in water.

Recovery

DFMO was added to plasma samples from different human volunteers to give a final concentration of 20 nmol/ml. They were then analysed by the procedure described for plasma samples. A recovery of 97.6% ± 2.9 (S.D.) (n = 6) was obtained. Similar recoveries were obtained when various tissue extracts were carried through the procedure. No significant change in the DFMO content of samples has been observed after storage at −20° C for several months.

RESULTS AND DISCUSSION

Fluorophore formation

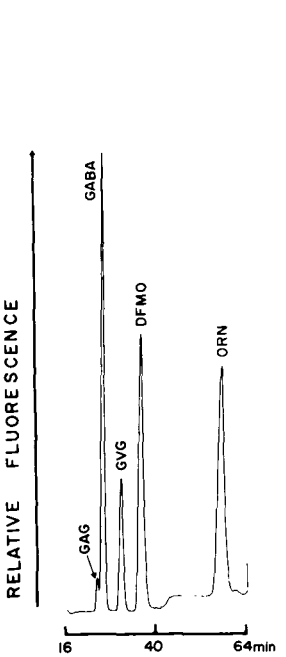
The reaction of *o*-phthalaldehyde and primary amines in the presence of mercaptoethanol was first reported by Roth [6] and subsequently applied to automated amino-acid analysis [7]. The identity of the fluorophores has been established recently by Stoney Simons and Johnson [8] as 1-alkythio-2-alkyl-isoindoles. It seemed evident that this reaction might be applied to α-difluoromethylornithine, and indeed the fluorescence quantum yield obtained was similar to that of ornithine. As mentioned above the mixing of column effluent and OPA solution was carried out without temperature control and the tube length (i.e. reaction time) between the mixing "T" and the fluorimeter was kept to a minimum, since this gave optimum response for the fluorescence of DFMO. Analysis was always made within the range of the calibration curve, i.e. 0.2—2 nmol per 100 μl injected, although a linear relative fluorescence/concentration relationship was found up to 10 nmoles

injected. Special precautions were not found to be necessary to avoid degradation of the OPA reagent if the volume prepared was used up within 2—3 days.

Chromatography

Short programmes for the separation of a few selected amino acids have been recently discussed by Olek et al. [9]. By eluting with a single buffer only a certain portion or "window" of the amino acids normally encountered in a physiological analysis are measured. The choice of the eluting buffer in the present assay was determined by our interest in inhibitors of both γ-aminobutyric acid (GABA) and ornithine metabolism. Fortunately, DFMO elutes between these two acids (Fig. 2), and indeed two GABA transaminase inhibitors, γ-acetylenic GABA (RMI 71.645) and γ-vinyl GABA (RMI 71.754), can also be separated with the present method. It is probable, therefore, that this assay can be used in studies involving these other inhibitors.

Fig. 3 shows chromatograms of human plasma carried through the procedure from a volunteer before and 4 h after ingestion of 10 mg/kg DFMO. Similar chromatograms have been obtained with plasma, or sera, from mouse, rat, rabbit, monkey and dog. Even haemolysed samples and extracts from sonicated



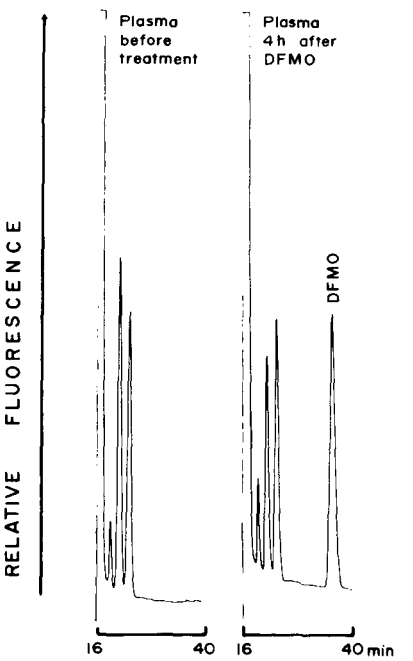


Fig. 2. Separation of γ-acetylenic GABA (GAG), GABA, γ-vinyl GABA (GVG), DFMO and ornithine (ORN) using the lithium citrate buffer for elution and OPA fluorophore formation for detection, as described in the Methods section, but using a longer elution time. One nanomole of each acid was injected.

Fig. 3. Chromatograms of human plasma prepared as described in the text. Left: blank plasma, i.e. before administering DFMO. Right: plasma sample from the same individual 4 h after taking DFMO 10 mg/kg orally.

red blood cells give "clean" chromatograms. The same remarks are applicable to all organ extracts so far analysed.

Fig. 4 shows a chromatogram of aliquots of human urine from samples collected for 24 h before and after ingestion of 10 mg/kg DFMO. Again no interference is evident, although if the drug concentration is low, as in later urine collections, some minor peaks can occur when a smaller dilution is made.

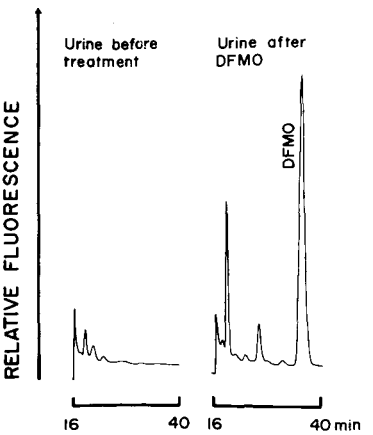


Fig. 4. Chromatograms of human urine prepared as described in the text. Left: blank urine from a 24-h sample before taking DFMO. Right: urine sample of the same individual from a 24-h collection after taking DFMO 10 mg/kg.

Application to biological samples

The assay has been used in pharmacological studies, particularly in a comparison of plasma half-lives of the drug in various species. It is sufficiently sensitive to enable the drug concentration profile to be followed even in individual rats. A catheter inserted in the rat femoral artery enabled blood samples (200 μl) to be taken at intervals for measurement of DFMO concentration. The drug could be measured for 6 h after giving the rats an oral dose of 200 mg/kg DFMO (Fig. 5A) and for 20 h in the monkey after 10 mg/kg orally (Fig. 5B). A comparison of the plasma half-life and urinary excretion of DFMO in various species is given in Table II.

It has also been important in several studies to demonstrate the presence of DFMO in the target organ. DFMO has been shown to enter the principal organs, to cross the blood—brain barrier and to enter tumours whose growth it retards [5]. The concentration of DFMO responsible for different biological effects in various animal models has been compared. For example, the concentration of DFMO that reduced the appearance of new tumours in rats treated with dimethylbenzanthracene and that exhibited contragestational effects in several species was around 10^{-4} *M*.

Similarly, in an evaluation of DFMO using cell-culture preparations, its ability to inhibit the target enzyme ornithine decarboxylase and consequently to affect polyamine biosynthesis has been compared to other inhibitors. In this respect, the inhibition of ornithine decarboxylase depends on the facility with which the inhibitor enters the cell. Investigations of the extra-

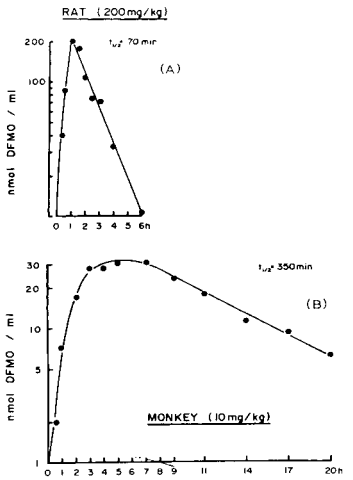


Fig. 5. Comparison of the plasma concentrations of DFMO in (A) rat after oral administration of 200 mg/kg, and (B) monkey after an oral dose of 10 mg/kg.

TABLE II

PLASMA HALF-LIFE OF DFMO AND ITS URINARY EXCRETION BY VARIOUS SPECIES

Species	Oral dose (mg/kg)	Half-life (min ± S.D.)	Amount of DFMO excreted in urine (0—24 h) (% ± S.D.)
Cynomologus monkey (n = 3)	10	353 ± 55	22.2 ± 3.5
Beagle (n = 2)	10	108, 132	26.9, 57.9
New Zealand white rabbit (n = 3)	10	121 ± 30	—
Sprague—Dawley rat (n = 4)	200	83 ± 30	82.5 ± 5.0*

*In the rat < 1% was excreted during 24—48 h.

and intra-cellular distribution of DFMO as a function of the medium concentration have therefore been made. Fig. 6 shows (A) the amount of DFMO found in the cells as a function of volume of cell culture taken for analysis with different concentrations of DFMO in the medium, and (B) the linear correlation between intra-/extra-cellular concentration of DFMO. The results indicate that there is passive diffusion of DFMO.

In summary, the method described has been used continuously for over a year for the measurement of DFMO in a variety of tissue extracts and biological fluids from several species. Interfering products which might invalidate the assay have never been encountered, even when DFMO concentrations were in the sub-nanomole range.

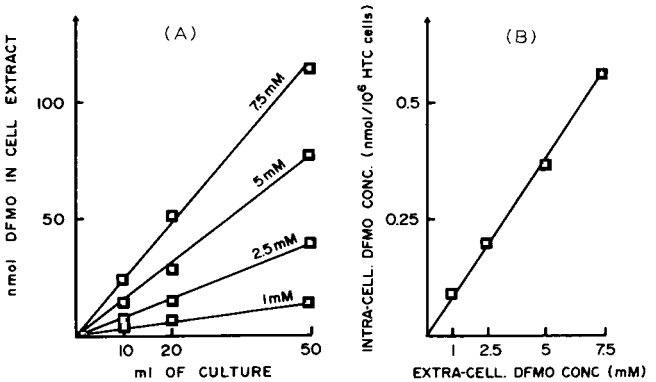


Fig. 6. Intracellular incorporation of DFMO by cultured rat hepatoma cells. (A) Amount of DFMO found within the cells from different volumes of culture with various extracellular DFMO concentrations in the medium. Number of cells per ml of culture was $3 \cdot 10^5$. (B) Relationship of intra- and extra-cellular DFMO content of cultured rat hepatoma cells after 48 h incubation.

ACKNOWLEDGEMENTS

We would like to thank Christian Gerst, Mrs. Marie-Louise Part and Miss Marlyse Nussli for their skilled technical assistance, and Miss Wendy Carter for secretarial help.

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Journal of Chromatography, 223 (1981) 417—420
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 802

Note

Simple and rapid high-performance liquid chromatographic method for the quantification of 3-methylhistidine

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The amino acid 3-methylhistidine (3-MeHis) is a non-reutilised amino acid present in the myofibrillar proteins actin and myosin [1]. The amino acid is quantitatively excreted in the urine in proportion to the rate of degradation of the myofibrillar proteins [1], thus providing a non-invasive technique for measuring the rate of myofibrillar protein breakdown in man [2]. The low concentration of 3-MeHis in urine, 0.05—0.20 m*M*, particularly relative to that of other amino acids, has led to the development of specific analytical techniques [3]. These methods have made use primarily of conventional automatic amino acid analysers (see ref. 4): however, recently high-performance liquid chromatographic (HPLC) methods have become available [5—7].

The present report describes an HPLC method which is both simple and rapid yet sensitive and which does not require the use of fluorimetric detection [6, 7].

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0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Equipment

A Model 6000A solvent delivery system with a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) was used in the present studies. Chromatographic separations were performed using a stainless-steel HPLC column (300 × 4 mm I.D.) packed with μBondapak C_{18}, particle size 10 μm (Waters Assoc.). A guard column (0.5 ml capacity) was used containing Bondapak Phenyl/Corasil, particle size 35—50 μm (Waters Assoc.).

The absorbance of the column eluent was monitored at 405 nm using a Model 440 absorbance detector (Waters Assoc.) fitted with a 405-nm filter and aperture plate. Absorbance was recorded on a linear chart recorder (Omniscribe B-5000; Houston Instruments, Austin, TX, U.S.A.) and quantified by measuring the peak heights.

Materials

All reagents were of the highest purity available. Amino acids and *o*-phthalaldehyde (OPT) were obtained from Sigma (St. Louis, MO, U.S.A.). Other reagents were purchased from BDH Chemicals (Poole, Great Britain) or from Waters Assoc.

Methods

Ninhydrin—*o*-phthalaldehyde (ninhydrin—OPT) reagent was prepared by adding OPT (5 g l^{-1}) to ninhydrin reagent prepared according to the description of Spackman et al. [8] using titanium trichloride as the reducing agent [9]. The reagent was stored under nitrogen in a dark bottle.

Urine samples were collected using glacial acetic acid as preservative and stored at —20°C to await analysis.

Samples were prepared by adding 0.5 ml of the ninhydrin—OPT reagent to 1 ml of filtered urine or a solution of 3-MeHis. The solution was mixed thoroughly and allowed to react at 40°C for 10 min in a water-bath. To this solution were added 4.0 ml of ethyl acetate; the solution was then mixed thoroughly and cooled in ice. The solutions were centrifuged at low speed to separate the aqueous and organic phases. Aliquots, normally 25 μl, of the aqueous phase containing 3-MeHis were injected directly into the chromatograph. The eluent was water—methanol (60:40), degassed with helium. The eluent flow-rate was 1 ml min^{-1}.

Results are expressed as mean ± standard error of the mean (number of determinations).

RESULTS AND DISCUSSION

Calibration curves for 3-MeHis prepared using standard 3-MeHis solutions were linear for the range up to 5.0 μmol ml^{-1} 3-MeHis assayed, thus providing an adequate range for the concentration of 3-MeHis in urine. The colour yield, determined spectrophotometrically, was $6.97 \cdot 10^3$ l mol^{-1} cm^{-1} at 405 nm for 1.0 *M* solution of 3-MeHis. Blank absorbance was 0.077 ± 0.003 (n=6) units. The precision of determination was good; replicate samples of 0.15 μmol

ml^{-1} 3-MeHis analyzed yielded a coefficient of variation of 7.0% (n=8).

A typical chromatogram obtained for the analysis of 3-MeHis is shown in Fig. 1. An excellent separation of 3-MeHis from other urinary components was obtained. Recovery of 3-MeHis added to urine was 100.4 ± 1.7% (n=8). Characterization of the 3-MeHis peak in urine samples was confirmed by co-chromatography with authentic 3-MeHis using different elution solvent mixtures. Using the present procedure the daily 3-MeHis excretion by healthy children, approximately 25 kg in body weight and receiving a meat-free diet, was 15.6 μmol 3-MeHis $mmol^{-1}$ creatinine 24 h^{-1}, a value similar to those obtained using ion-exchange chromatography (12.6 μmol $mmol^{-1}$ 24 h^{-1}) [2] and fluorescence HPLC (14.9 μmol $mmol^{-1}$ 24 h^{-1}) [7].

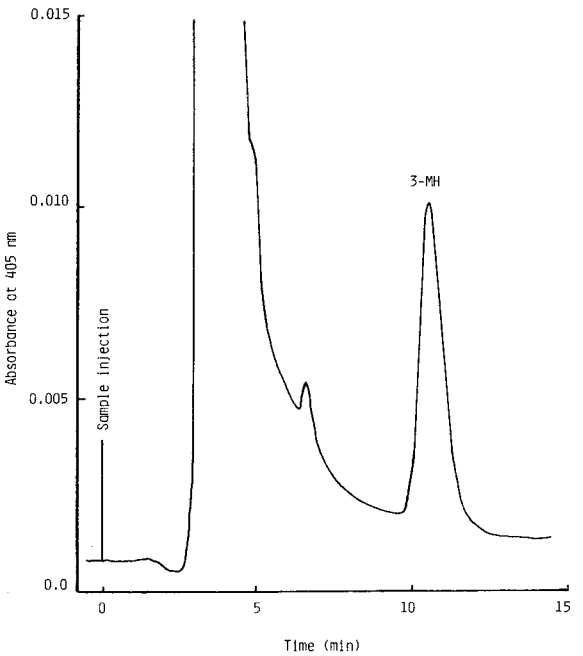

Fig. 1. Chromatographic separation of 3-methylhistidine (3-MH) in urine. The chromatogram was recorded using 1 ml of urine prepared as described in the text. 3-Methylhistidine represents 1.97 nmol. Other reaction products are rapidly eluted with the sample solvent front.

The analysis of 3-MeHis in the presence of many other components in urine requires special analytical methods [3]. The reaction mechanism between 3-MeHis and ninhydrin—OPT and the nature of the chromogenic product formed are not yet known. The reagent, previously shown to be semi-specific for 3-MeHis, is, however, an effective chromatographic reagent for the detection and quantification of 3-MeHis by ion-exchange chromatography [3]. The adaptation of this method to HPLC considerably simplifies and increases the sensitivity of the analytical technique when compared with conventional ion-exchange methods. The method is rapid and quantitative and requires only a basic model liquid chromatograph suitable for isocratic elution with spectrophotometric detection at 405 nm.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the expert technical assistance of Mr. L. Carrington and Waters Associates (Brisbane, Australia). This study forms part of a major study partially funded by the Cystic Fibrosis Foundation of Australia in conjunction with Drs. R. Shepherd, W.G. Cooksley and B. Thomas, whose participation is gratefully acknowledged.

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Journal of Chromatography, 223 (1981) 421—425
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 830

Note

Determination of 5-aminolaevulinic acid dehydrase activity in erythrocytes by high-performance liquid chromatography

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(Received December 3rd, 1980)

5-Aminolaevulinic acid dehydrase (ALA dehydrase, ALA-D), the second enzyme of the haem biosynthetic pathway, catalyses the condensation of two molecules of ALA to form the monopyrrole, porphobilinogen (PBG). This enzyme is inhibited by heavy metals [1—8] and by ethanol [9, 10]. The determination of ALA-D is used to differentiate between heavy metal poisoning and iron-deficiency anaemia, to study the effect of chemicals [11—13], drugs [14], and hormones [15] and to investigate disorders associated with haem biosynthesis such as the porphyrias and various types of anaemia.

This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of ALA-D activity in human erythrocytes. ALA is used as the substrate and the product (PBG) formed is determined by reversed-phase ion-pair chromatography on Hypersil-SAS (short-chain alkylsilica) with methanol—water (22:78, v/v) in the presence of PIC B7 (0.005 *M* 1-heptanesulphonic acid buffered at pH 3.5) as the mobile phase. PBG is separated effectively from ALA and the internal standard 2-methyl-3-carbmethoxy-4-(3-propionic acid)-pyrrole. The method has been used to establish a reference range for ALA-D activity in normal subjects.

EXPERIMENTAL

Materials and reagents

ALA hydrochloride and PBG were obtained from Sigma (London, Great Britain). Glacial acetic acid, methanol, sodium acetate trihydrate, sodium dihydrogen phosphate dihydrate, disodium hydrogen orthophosphate dodecahydrate, trichloroacetic acid and methylacetoacetate were from BDH (Poole,

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Great Britain). Except for methylacetoacetate, all reagents were of AnalaR grade. The ion-pair reagent PIC-B7 (1-heptanesulphonic acid) was purchased from Waters Assoc. (Milford, MA, U.S.A.) and was prepared by diluting one bottle (20 ml) of the reagent to 1 l with water.

Acetate buffer, pH 4.6 was made by diluting 57 ml of glacial acetic acid with 700 ml of water. Sodium acetate trihydrate (136 g) was added and the mixture was made up to 1 l with water.

Phosphate buffer, 0.2 *M*, pH 6.8 was prepared by mixing sodium dihydrogen phosphate dihydrate and di-sodium hydrogen orthophosphate dodecahydrate. A pH of 6.7—6.8 appears to be optimal for the measurement of ALA-D activity by PBG production [16] rather than pH 6.4 which is used in the European Standard Method [17]. ALA substrate (20 m*M*) was made by dissolving 335.2 mg of ALA—HCl in 100 ml of water. The final ALA concentration in the reaction mixture was 4 m*M*, which is optimal or greater by the method of Bonsignore et al. [18] and is the recommended final ALA concentration in the European Standard Method [17].

Preparation of 2-methyl-3-carbmethoxy-4-(3-propionic acid)-pyrrole (internal standard)

ALA-HCl (2.0 mg) was dissolved in 10 ml water to which 10 ml of acetate buffer was added. Methylacetoacetate (1 ml) was added and the mixture was heated in a water bath at 100°C for 20 min. Aliquots (1.0 ml) of the mixture were dried under reduced pressure, stored at 4°C, and redissolved in 10 ml of water prior to use.

Construction of calibration curve

A standard solution of PBG was made up by weight to approximately 100 μM and the exact concentration measured by the method of Mauzerall and Granick [19]. This solution was diluted to give a range of PBG solutions of concentrations 5—50 μM. A 0.5-ml aliquot of each PBG solution was mixed with 0.1 ml of the internal standard and 20 μl were injected into the chromatograph. The calibration curve was constructed by plotting PBG concentration against the ratio of the peak heights of PBG:internal standard. The curve was linear over the range tested.

Preparation of enzyme solution and incubation procedure

ALA-D was measured on blood collected into lithium heparin. Packed cell volume (PCV) was measured using a Coulter counter. The blood samples were used immediately or stored in an ice bath at 1—2°C until the enzyme activities were measured (within 2 h). The whole blood (0.5 ml) was haemolysed in water (3.25 ml) and 1.5 ml of the haemolysate (corresponding to 0.2 ml of whole blood) was used immediately as the enzyme solution. ALA substrate (0.5 ml) and phosphate buffer (0.5 ml) were added to each of three 10-ml test tubes. Trichloroacetic acid (100 g/l) was added to one of the tubes to act as a blank. All the tubes were placed in a constant-temperature water bath at 37°C for 5 min and the haemolysate (1.5 ml) was then added to each of the tubes and mixed thoroughly. The mixture was incubated for 1 h at 37°C and the reaction was terminated by adding 1 ml trichloroacetic acid solu-

tion. After centrifugation for 5 min at 1500 *g*, 0.5 ml of the supernatant was withdrawn into a vial containing 0.1 ml of the internal standard and 20 μl of the well mixed solution were injected into the chromatograph.

High-performance liquid chromatography

A Shandon Southern (Runcorn, Great Britain) liquid chromatograph with a variable-wavelength UV detector set at 240 nm and 0.02 a.u.f.s. was used. Injection was via a Rheodyne 7120 injection valve fitted with a 20-μl loop. A 5-μm particle Hypersil-SAS (C_1) column (10 cm × 5 mm I.D.) was used with methanol—PIC B7 in water (22:78, v/v) as the eluent. The pressure was set at 80 bar and the flow-rate was 1.2 ml/min.

Calculation

The PBG concentration in the incubation mixture was read off the calibration curve and the enzyme activity expressed as μmol of PBG formed per min per l of erythrocytes (RBC) at 37° C. Thus

$$\text{ALA-D activity} = \frac{X \times 3.5}{60 \times 0.2} \cdot \frac{100}{\text{PCV}}$$

where X is the concentration of PBG, 3.5/0.2 is the dilution factor and PCV is the percentage packed cell volume.

RESULTS AND DISCUSSION

HPLC has been used for the separation of ALA and PBG [20]. The present system, using ion-pair chromatography on a short-chain alkylsilica (C_1) column, improves the resolution and shortens the analysis time. With methanol—water containing the pairing ion 1-heptanesulphonic acid as the eluent effective separation of ALA, PBG and the internal standard is achieved (Fig. 1). The system is also satisfactory for the resolution of PBG and the internal standard from the endogenous impurities (Fig. 2a, b). When treated with Ehrlich's reagent (*p*-dimethyl-aminobenzaldehyde) the peak corresponding to PBG isolated from the incubation mixture by HPLC gave a typical absorption spectrum with an absorption maximum at 553 nm. This confirms the identity of

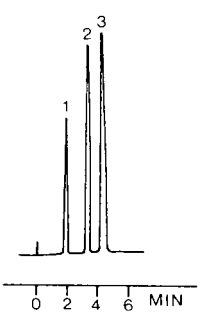

Fig. 1. Separation of ALA (1), PBG (2) and internal standard (3). Stationary phase, Hypersil-SAS; mobile phase, methanol—PIC-B7 in water (22:78, v/v); pressure, 80 bar; flow-rate, 1.2 ml/min; detection, 240 nm.

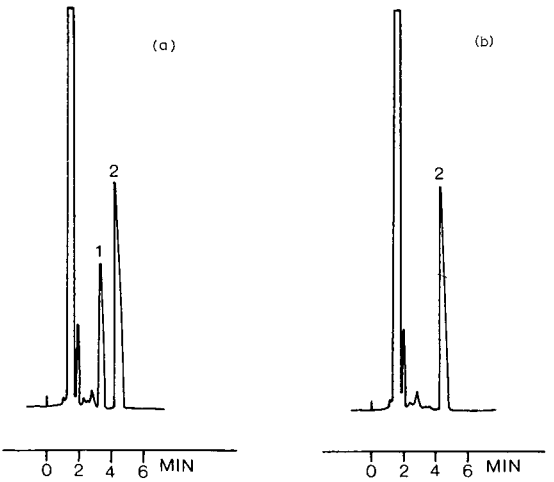

Fig. 2. Separation of PBG (1) and internal standard (2) in incubation mixture. (a) Test; (b) blank. Chromatographic conditions as Fig. 1.

the PBG peak in addition to evidence from capacity ratio measurement. This peak is absent (Fig. 2a) in the incubation mixture pretreated with trichloroacetic acid.

Using this method measured PBG production in 25 normal volunteers was found to be 17.50 ± 3.84 μmol/l RBC/min (Fig. 3). This is almost identical to the values given by Bonsignore et al. [18] (16.7 ± 3.3 μmol/l RBC/min after conversion from enzyme units) and by Tomokuni [4] using the method of Bonsignore et al. [18] (17.4 ± 3.2 μmol/l RBC/min), but is lower than that obtained by Burch and Siegal [21] using a different buffer system (29.3 ± 7.2 μmol/l RBC/min after conversion from enzyme units). The reproducibility of the method was shown by determination of ALA-D activity in one subject on six different occasions. This gave values of 19.25 ± 0.39 (S.D.) μmol PBG/l RBC/min (C.V. 2.03%).

The published methods for measuring ALA-D in terms of PBG production have not attempted to make any correction for loss of PBG, although this compound is unstable on incubation. It is obviously difficult and time consuming to perform an accurate correction for PBG loss in individual cases since in an unknown sample it is not known what concentration of PBG will be achieved in the incubation mixture and the rate of loss will depend at least partly on this. The European Standard Method [17] converts directly from measured PBG production to ALA consumption by multiplying by 2; in the present case this would give a mean of 35.0 ± 7.7 μmol ALA consumed/l RBC/min (Fig. 3).

In two alcoholic patients PBG production was markedly reduced, giving ALA-D activities of 4.9 and 5.4 μmol/l RBC/min, confirming that the present method is able to detect activity of the enzyme.

CONCLUSION

ALA-D activity in erythrocytes can be satisfactorily determined by mea-

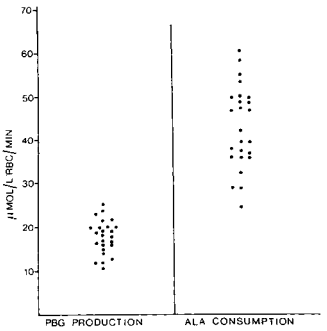

Fig. 3. ALA-D activity in 25 normal subjects determined by HPLC. Results are expressed as μmol/l RBC/min of PBG produced and ALA consumed.

suring the amount of PBG formed on a reversed-phase column with methanol—aqueous 1-heptanesulphonic acid (22:78, v/v) as the mobile phase. ALA was used as the enzyme substrate and 2-methyl-3-carbmethoxy-4-(3-propionic acid)-pyrrole was used as the internal standard. The mean value obtained by this method in 25 normal subjects was found to be 17.50 μmol/PBG produced/l RBC/min (S.D. ± 3.84, observed range 10.3—25.0). This was well correlated with existing methods for ALA-D determination.

ACKNOWLEDGEMENT

This work was partly supported by a grant from the Arthritis and Rheumatism Council.

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Journal of Chromatography, 223 (1981) 426—431
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 810

Note

Simultaneous assay of methylphenobarbital and phenobarbital in plasma using gas chromatography—mass spectrometry with selected ion monitoring

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(First received October 3rd, 1980; revised manuscript received December 7th, 1980)

Methylphenobarbital (mephobarbital; MPB) has been used as an anticonvulsant since 1932 [1]. The clinical pharmacokinetics and metabolic fate of the drug have been incompletely documented [2]. It has been long recognized that the drug is metabolically N-demethylated to phenobarbital (PB) [3]. There has been some study of the plasma levels of MPB [4] and PB [4, 5] which occur in patients taking MPB, and it is known that steady-state plasma levels of PB exceed those of MPB by a factor of 7—10 [6] or even more [4].

Plasma levels of barbiturates are frequently determined by gas—liquid chromatography (GLC) with on-column methylation [7] or by a homogeneous enzyme immunoassay technique [7]. Neither of these methods discriminates between MPB and PB. Specific assays for MPB and PB have been achieved by GLC involving on-column butylation [8], by direct chemical ionization mass spectrometry (MS) [9], and by a selected ion monitoring gas chromatography (GC)—MS technique [4]. Although there have been several reports of the use of high-performance liquid chromatographic techniques for barbiturate analysis (e.g. ref. 7), we are aware of only one report in which both MPB and PB were included [10]. None of these methods fulfilled the requirements of our proposed pharmacokinetic studies with MPB [11], for which a simultaneous assay for MPB and PB with good precision, low detection limits and convenience for coping with large sample numbers was desired. The present method met these criteria.

MATERIALS AND METHODS

Chemicals and reagents

MPB and PB were purchased from Applied Science Labs. (State College, PA, U.S.A.) and the internal standard, 5-ethyl-5-(4-methylphenyl)-barbituric acid

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(*p*-tolylbarbituric acid; TB) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Stock solutions of each barbituric acid in methanol were prepared at a concentration of 50 mg l^{-1}. N,N-Dimethylacetamide and tetramethylammonium hydroxide were from Sigma (St. Louis, MO, U.S.A.) and 1-iodopropane from Aldrich. Chloroform was analytical reagent grade, and was distilled before use.

Extraction and derivatization

Exactly 100 μl of the methanolic internal standard (TB) solution were dispensed into the assay tube (Pyrex tube, 150 × 20 mm with Teflon-lined screw cap), and the methanol was evaporated with a nitrogen stream. Plasma (0.1 —1.0 ml) was added, followed by 0.3 ml hydrochloric acid (0.2 *M*) and 5.0 ml chloroform. After shaking (2 min) and centrifuging (2 min at 1000 *g*), the aqueous layer was aspirated to waste, and the chloroform phase poured carefully into a clean test tube with a conical bottom. The chloroform was evaporated to dryness (nitrogen stream and water bath at 60°C), and the residue derivatized 10—15 min prior to chromatography. Derivatization was based on the method of Greeley [12], and was effected by taking the residue up in N,N-dimethylacetamide (40 μl), adding tetramethylammonium hydroxide (5 μl of a 20% w/v solution in methanol) and 1-iodopropane (10 μl). After 10—15 min standing, the precipitate (tetramethylammonium iodide) was centrifuged to a pellet, and 1—5 μl of the clear supernatant was injected into the GC—MS system.

GC—MS analysis

A Finnigan Model 3300F GC—MS system with a Model 6110 data system was used in these studies. The gas chromatograph was fitted with a 1.5 m × 2 mm I.D. glass column packed with 3% OV-101 on 80—100 mesh Gas-Chrom Q (Applied Science Labs.). The injector, glass jet separator and glass-lined transfer line were all at 250°C, and the column oven at 200°C. Helium flow-rate was 25 ml min^{-1}. The mass spectrometer was operated in the electron impact ionization mode, and the ions at *m/z* 146 and 160 were monitored. The ion source filament was left on throughout the run, to maintain source conditions as stable as possible.

Quantitation

Standard calibration graphs were obtained by the analysis of 1-ml aliquots of drug-free human plasma, to which had been added known amounts of MPB and PB. Peak heights were measured in preference to peak areas, as we attained better precision by this approach, in agreement with the recommendations of Millard [13]. Precision and detection limits were assessed by standard procedures. Extraction recovery was assessed by comparing the peak height ratios obtained for a set of plasma standards (5 mg l^{-1}) with those applying when 5.0 μg of each analyte were added to a tube containing TB which had been extracted from blank plasma. This procedure compared the extracted and unextracted analytes, both relative to the same quantity of (extracted) internal standard.

RESULTS

The electron impact spectra of the propylated derivatives of the three barbi-

turic acids are shown in Fig. 1. Similar spectra have been reported and interpreted by others [14,15]. The ions chosen for selected ion monitoring were the base peaks in each spectrum.

Typical selected ion chromatograms for blank plasma and for a 1.0 mg l^{-1} plasma standard are shown in Fig. 2. It was possible to inject samples at approximately 6-min intervals, as no responses for the monitored ions were observed after the TB peak had eluted. Processing of plasma standards yielded excellent linear calibration curves for each barbiturate, the correlation coefficient

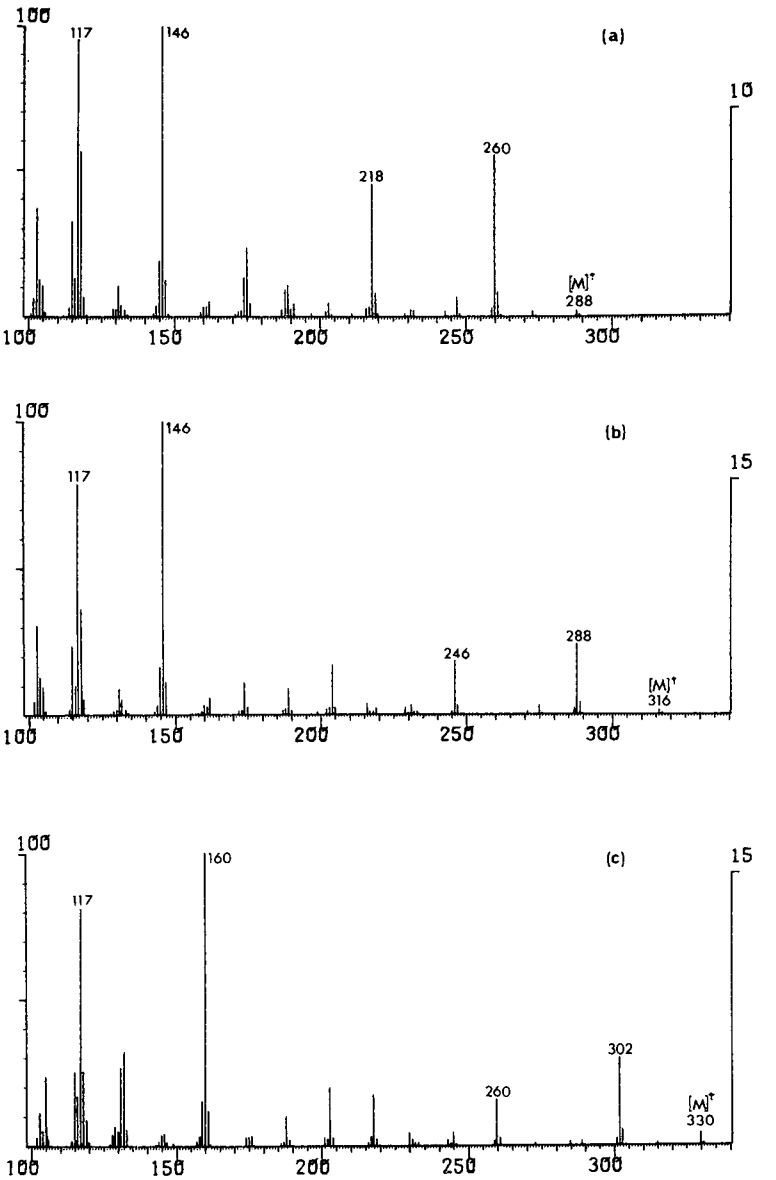

Fig. 1. Electron impact mass spectra of the N-propylated derivatives of (a) methylphenobarbital, (b) phenobarbital and (c) *p*-tolylbarbituric acid.

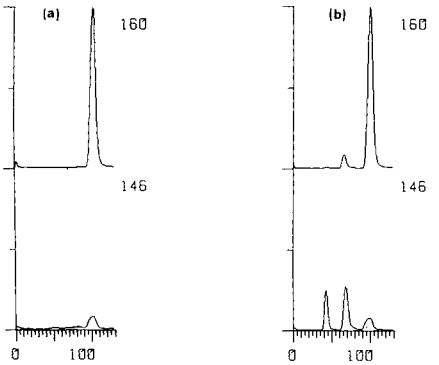

Fig. 2. Selected ion profiles for *m/z* 146 and 160 for (a) blank plasma and (b) plasma standard with both drugs at 1 mg l^{-1}. The scale on the abscissa represents points at which the ion current was sampled (actually 2-sec intervals).

always being greater than 0.99. The within-batch precision was very good, giving a coefficient of variation of less than 4% for each drug, with a standard error of the mean of 2% (n = 9). Thus assay of a 5 mg l^{-1} standard gave a value of 5.0 ± 0.18 (S.D.; n = 9), while at 1 mg l^{-1} the result was 1.0 ± 0.04 (S.D.; n = 9). However it was noted that minor variations in the slope of the calibration graph did occur from day to day. This effect has also been documented by Millard [13], and is a function of mass spectrometer tuning and ion source stability. Knowing that the calibrations were consistently linear, it was deemed preferable to measure concentrations in a day's assays by reference to a single point (5 mg l^{-1}) standard, multiples of which were extracted with the batch. As a control on instrumental stability, an injection was made from one of these standards after every three sample injections. Concentrations of MPB and PB were then calculated by reference to the mean of at least 9 such standard injections. The precision figures quoted above were obtained in this manner, indicating good instrument stability over the several hours necessary to collect the data.

Extraction recovery was calculated as 97% for MPB and 92% for PB. The lower limit of detection for both drugs, using a 1.0-ml plasma sample, was approximately 20 ng ml^{-1}.

An illustration of the application of the method to the single-dose pharmacokinetics of MPB is given in Fig. 3, which shows the time course for both MPB and PB following a single 100-mg intravenous dose of MPB in one volunteer. MPB (0.16 mg l^{-1}) and PB (0.17 mg l^{-1}) were still measurable after 9 days, and the elimination half-life of MPB was calculated as 64.2 h.

DISCUSSION

The use of GC—MS with selected ion monitoring for the determination of these barbiturates has been reported recently by Kupferberg and Longacre-Shaw [4]. Our method differs from theirs principally in regard to the derivatization technique, and the choice of ions for monitoring. The alkylation procedure of Kupferberg and Longacre-Shaw [4], which involved heating the samples for 30 min seemed less satisfactory than the milder method of Greeley [12]. While the former method is desirable for derivatization of relatively non-acidic

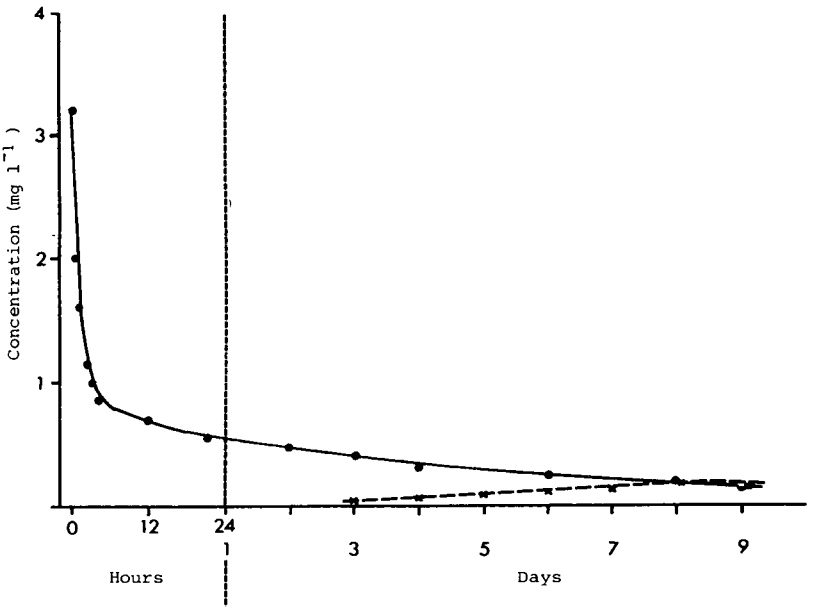

Fig. 3. Plasma concentrations of MPB (•——•) and PB (x— — —x) following a 100-mg intravenous dose of MPB in a volunteer. The vertical dotted line marks a change in the abscissa scale from hours to days. No PB was detected before day 3.

protons (such as that at N^1 in the hydantoin anticonvulsants [16], it may cause problems with the more hydrolytically labile barbiturate ring, the base catalysed fission of which has been carefully studied [17]. Although we sought to standardize the time from derivatization to chromatographing at 15 min, no significant degradation was apparent until more than two hours had passed. Unwanted hydrolytic reactions were also reduced by keeping the quantity of tetramethylammonium hydroxide catalyst to a minimum. Our selection of the base peak ions (*m/z* 146 and 160) for monitoring was based on our need for a highly sensitive assay. We observed that the $(M-28)^+$ fragment was the base peak for N-methyl and N-ethyl barbiturates, in agreement with Kapetanović and Kupferberg [18]. However for the N-propyl derivatives which we used on account of their satisfactory chromatographic resolution, the $(M-28)^+$ ion was only 25—50% of the base peak (Fig. 1). The observation that increasing chain length in the N-alkyl substituent can promote alternative fragmentation processes in the mass spectra of barbiturates has been documented previously [19]. Although the ions which we selected may present greater potential for interference [13] than the higher mass, but less abundant, ions, we have not encountered interfering compounds in biological samples to date. These problems could presumably be largely overcome by monitoring quasi-molecular ions in the chemical ionization mode [9], but we lacked the facilities to investigate this question.

The present method was developed primarily for the study of single-dose pharmacokinetics of MPB. Its application is illustrated here in the case of a single 100-mg intravenous dose to a volunteer. The method has also been ap-

plied to a more detailed investigation [11] of the kinetics of MPB than was achieved [6] with our earlier GLC technique [8]. The method is also applicable to studies in neonates, or for analysis of trace quantities of MPB and PB in specimens such as saliva and breast milk, for which similar methods have recently been reported for the related anticonvulsant, primidone [20].

ACKNOWLEDGEMENT

The authors wish to acknowledge the generosity of the Utah Foundation, who provided funds for the purchase of the GC—MS system.

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Journal of Chromatography, 223 (1981) 432—437
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 822

Note

Antipyrine determination in human plasma by gas—liquid chromatography using nitrogen—phosphorus detection

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(First received October 13th, 1980; revised manuscript received December 23rd, 1980)

Antipyrine, used as a marker of human hepatic oxidative drug metabolism, has been measured spectrophotometrically since 1949 [1]. The need for a more specific method, particularly in subjects with renal disease [2], has led to development of techniques using gas—liquid chromatography (GLC) with flame ionization detection [2—4], high-performance liquid chromatography [5], and radioimmunoassay [6]. Heterocyclic nitrogens in the antipyrine molecule suggest that GLC coupled to a nitrogen—phosphorus detector (NPD) may offer a specific, sensitive method of antipyrine quantitation in plasma [7].

Here we describe such a method which, when coupled with an automated injection system, permits analysis of up to 100 samples per 24 h. Extraction is simple and requires minimum technical time (1—1.5 h) for complete sample preparation. Comparison of this method to the spectrophotometric method is made and application to a single-dose pharmacokinetic study is demonstrated.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytic instrument is a Hewlett-Packard Model 5840A gas chromatograph equipped with a nitrogen—phosphorus detector and electronic integrator, coupled to a Hewlett-Packard Model 7672A automatic injection system. The column is coiled glass, 1.83 m × 2 mm I.D. packed with 3% SP-2250 on 80—100 mesh Supelcoport (Packing 1-1767, Supelco, Bellefonte, PA, U.S.A.). The carrier gas is ultra high pure helium (Matheson Gas Products, Gloucester, MA, U.S.A.) at a flow-rate of 30 ml/min. The detector purge is ultra high pure hydrogen (Matheson) at a flow-rate of 3 ml/min mixed with dry air (Matheson) at 50 ml/min. Operating temperatures are: injection port, 310°C; column,

230°C; detector, 275°C. Before being connected to the detector, a new column is conditioned at 285°C for 48 h with a carrier flow-rate of 30 ml/min.

At the beginning of each working day, the column is primed by injection of 5 μg asolectin in benzene. Use of this phospholipid as a priming agent gives greater peak resolution, presumably by coating surface-active sites on the column.

Reagents

The following reagents are used: pesticide grade ethyl acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), analytical reagent grade toluene (Mallinkrodt, St. Louis, MO, U.S.A.), certified isoamyl alcohol (Fisher), analytical reagent grade methanol (Fisher), analytical reagent grade benzene (Mallinkrodt), and analytical reagent grade sodium hydroxide (Mallinkrodt). Isoamyl alcohol is glass distilled prior to use; other solvents are used without further distillation.

Reference standards

Pure antipyrine is obtained from Aldrich (Milwaukee, WI, U.S.A.). Pure diazepam was kindly supplied by Hoffmann-LaRoche (Nutley, NJ, U.S.A.). The antipyrine standard is prepared by dissolving 100 mg in 100 ml methanol. Sequential dilutions to 100 μg/ml are made. Diazepam is dissolved in 100 ml benzene, with sequential dilutions in benzene to 100 μg/ml. These solutions are stored in amber bottles at 4°C and are stable for at least six months.

Preparation of samples

Diazepam is used as the internal standard. A 100-μl volume of stock solution (100 μg/ml), containing 10 μg diazepam, is added to a series of 15-ml glass culture tubes with PTFE-lined screw top caps. A 1-ml sample of unknown plasma is added to each tube. Calibration standards are prepared by adding 1, 2.5, 5.0, 10, 25, and 50 μg antipyrine to consecutive tubes. Drug-free control plasma is added to each of the calibration tubes. One blank sample, taken from the subject prior to drug administration, is analyzed with calibration standards and each set of unknown samples.

Extraction procedure

One ml 1 *M* sodium hydroxide is added to each tube. To this 6 ml ethyl acetate are added and the tubes are agitated gently in the upright position on a vortex mixer for 10 min. The samples are centrifuged at room temperature for 10 min at 400 *g* (Portable Refrigerated Centrifuge Model PR-2; head No. 269, International Equipment, Boston, MA, U.S.A.). The organic layer is transferred to a conical 13-ml centrifuge tube. The organic extracts are evaporated to dryness at 40°C under conditions of mild vacuum. The final dry residue is redissolved in 1 ml toluene—isoamyl alcohol (85:15), of which 6 μl is injected into the chromatograph.

Clinical pharmacokinetic study

A healthy 24 year old female volunteer participated after giving written informed consent. A single 1.2-g dose of antipyrine was administered by constant intravenous infusion over 5 min. Mutiple venous blood samples were drawn

over the following 24 h. Concentrations of antipyrine in plasma were determined spectrophotometrically [1,8], and by the method described above.

Plasma antipyrine concentrations determined by each method were analyzed by iterative weighted non-linear least-squares regression analysis as described in detail elsewhere [9]. After correction for the time of infusion [10], the following pharmacokinetic variables were determined: distribution half-life, elimination half-life, total volume of distribution, and total clearance.

RESULTS

Evaluation of method

Under the described conditions the retention times of antipyrine and diazepam are 2.35 and 12.35 min, respectively (Fig. 1). The relation between antipyrine plasma concentration and antipyrine-to-diazepam peak areas is linear to at least 50 μg/ml. Analysis of 26 standard curves constructed on different days over a 3-month period indicated that the correlation is always 0.99 or greater. Day-to-day coefficient of variation in the slopes of the calibration curves was 6.0%.

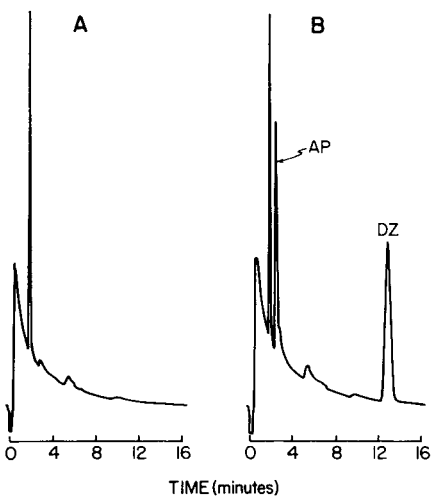

Fig. 1. Gas—liquid chromatogram of (A) extract of 1 ml of drug-free blank plasma and (B) the same plasma after addition of 5 μg/ml antipyrine (AP) and 10 μg/ml diazepam (DZ), the internal standard.

The sensitivity limit of the method is 1 μg per ml of the original sample. Coefficients of variation for identical samples were: at 50 μg/ml, 3.7% (n=6); at 25 μg/ml, 2.7% (n=6); at 10 μg/ml, 3.2% (n=6); at 5 μg/ml, 2.6% (n=5); at 2.5 μg/ml, 5.7% (n=6); and at 1 μg/ml, 6.6% (n=6). Residue analysis indicated that extraction of antipyrine is greater than 95% complete.

A series of 72 plasma samples from pharmacokinetic studies was analyzed by both GLC—NPD and spectrophotometric methods to establish comparability of methods (Fig. 2). The correlation coefficient between methods was 0.98, and the slope of the regression was 1.07.

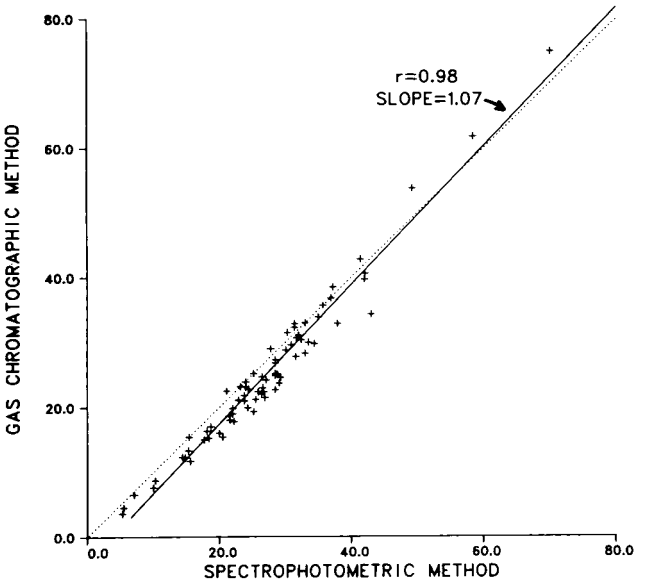

Fig. 2. Comparison of antipyrine determination using spectrophotometric and GLC—NPD methods on 72 plasma samples obtained from pharmacokinetic studies. The solid line was determined by linear regression analysis; the dotted line is the line of identity ($y=x$).

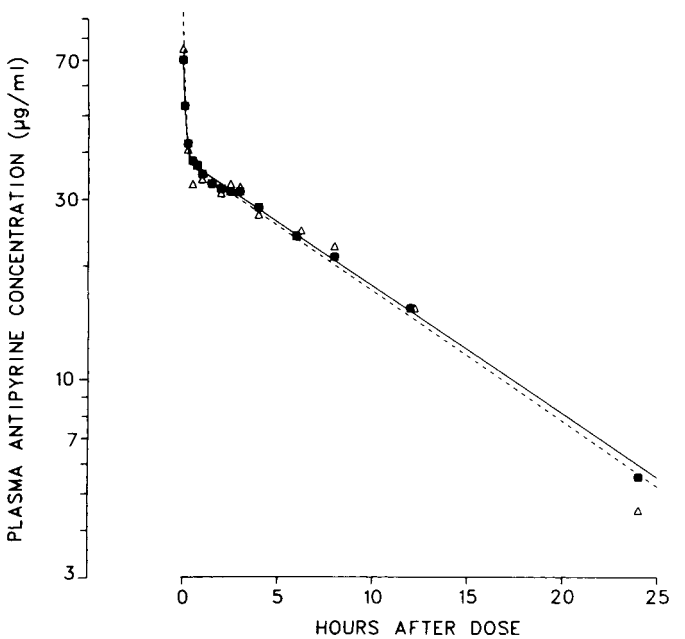

Fig. 3. Plasma antipyrine concentrations and pharmacokinetic functions from single-dose study of a 24 year old female with analysis of each sample using both spectrophotometric (■——■) and GLC—NPD (△ — — — △) methods. See Table I for kinetic parameters.

Pharmacokinetic study

Fig. 3 shows plasma antipyrine concentrations and pharmacokinetic functions for the subject as determined by each method. Derived pharmacokinetic parameters obtained from each method of analysis were nearly identical (Table I).

TABLE I

COMPARISON OF PHARMACOKINETIC PARAMETERS DERIVED FROM ONE STUDY* WITH PLASMA ANTIPYRINE DETERMINED BY SPECTROPHOTOMETRIC AND GLC—NPD METHODS

Parameter	Methods	
	GLC-NPD	Spectrophotometric
Distribution half-life (min)	4.0	4.9
Elimination half-life (h)	8.7	8.9
Volume of central compartment (l/kg)	0.22	0.29
Total volume of distribution (l/kg)	0.53	0.52
Total metabolic clearance (ml/min/kg)	0.70	0.68

*See Fig. 3.

DISCUSSION

This report describes a rapid, specific, and sensitive method for quantitation of antipyrine in plasma using GLC—NPD. A single basic extraction from plasma, evaporation of the organic phase, and injection of the redissolved residue directly into the chromatograph is the method utilized. Blank plasma samples are consistently free of contaminants in the areas corresponding to retention times of antipyrine and diazepam. Structural analogues of antipyrine were evaluated as potential internal standards. However, their retention times were similar to contaminant peaks attributable to endogenous plasma constituents or to blood collection artifacts appearing with the NPD system. Although the retention time of diazepam is long, it is well separated from contaminants and has excellent chromatographic properties.

Injection of the known major metabolites of antipyrine (4-hydroxy, 3-hydroxymethyl, and N-desmethyl derivatives) yielded no chromatographic peaks under the described conditions. Thus the method is highly specific for intact antipyrine. Furthermore, the high degree of comparability between GLC and spectrophotometric methods suggests that the latter technique, widely used for pharmacokinetic studies of antipyrine, also is essentially specific for the intact drug in the healthy persons with normal renal function that we have

studied to date. The value of the GLC method is in the short sample preparation time, its sensitivity and reproducibility, and the ease of adapting it to automated techniques for analysis of more than 100 samples per 24 h.

ACKNOWLEDGEMENTS

Supported in part by Grants MH-34223 and GM-07611 from the United States Public Health Service, and by Grant 77-611 from the Foundations' Fund for Research in Psychiatry.

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Journal of Chromatography, 223 (1981) 438—444
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 823

Note

Assay of triazolam in plasma by capillary gas chromatography

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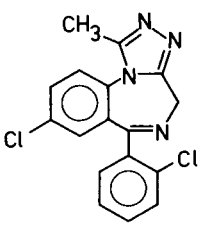
(Received November 7th, 1980)

Triazolam is a triazolobenzodiazepine derivative (Fig. 1) which was recently



TRIAZOLAM

Fig. 1. Structure of triazolam.

introduced as a short-acting hypnotic. A few procedures for the determination of this drug in plasma have been described, such as radioimmunoassay [1] and high-performance liquid chromatography [2]. However, owing to a lack of sensitivity and/or specificity, these methods cannot be used to study the pharmacokinetics of this highly potent drug following administration of therapeutic doses (0.25—0.5 mg).

In this paper a gas—liquid chromatographic (GLC) method for the assay of triazolam is described that is sufficiently sensitive to be applied for pharmacokinetic studies in man. The use of a combination of solid injection, a support-coated open-tubular (SCOT) column and electron-capture detection has proved to be very suitable for this purpose.

The applicability of the method is illustrated by a preliminary study of the pharmacokinetics of triazolam in healthy volunteers.

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Chemicals

Triazolam as a reference substance and also triazolam tablets (0.5 mg; Halcion) were kindly supplied by the Upjohn Company (Kalamazoo, MI, U.S.A.). Clonazepam was a gift from Dr. I.C. Dijkhuis (Apotheek Haagse Ziekenhuizen, The Hague, The Netherlands). For preparing the standard solutions, ethanol (p.a. grade) (Merck, Darmstadt, G.F.R.) was used; the other organic solvents were freshly distilled (J.T. Baker, Deventer, The Netherlands).

Extraction procedure

To 2.0 ml of plasma in a centrifuge tube were added 50.0 μl of ethanol containing 5.0 ng of clonazepam (internal standard) and 0.5 ml of 0.2 *M* borate buffer (pH 9.0). After homogenization, the sample was extracted twice with 5 ml of *n*-pentane—dichloromethane (4:3) for 15 sec on a whirlmixer. After centrifugation (5 min at 2000 *g*) the upper organic layer was transferred to a silanized conical evaporation tube and evaporated to dryness at 50°C under a gentle stream of dry nitrogen. Finally, the residue was dissolved in 40 μl of ethyl acetate and 4—5 μl of this solution were brought on to a glass-lined needle of the solid injection system [3]. After evaporation of the ethyl acetate, the residue was injected into the gas chromatograph.

Apparatus and chromatographic conditions

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a ^{63}Ni pulse-modified electron-capture detector and a solid injection system, was used. A SCOT column, (10 m × 0.4 mm I.D.) made of Duran 50 glass was used. The support layer was Tullanox (silanized fumed silica), particle size < 10 μm (Cabot Corp., Boston, MA, U.S.A.) and the stationary phase was 0.5% PPE-21 (Chrompack, Middelburg, The Netherlands) and 3% OV-17 (Chrompack). The operating temperatures were as follows: injection port, 350°C; column, 250°C; and detector, 350°C. The flow-rate of the carrier gas (helium) was 10 ml/min and that of the auxiliary gas (argon—methane, 95:5) was 25 ml/min.

For mass spectrometry (MS), an LKB-2091 combined gas chromatograph—mass spectrometer equipped with a PDP-11 computer system was used.

Preparation of calibration graphs

The concentration of triazolam in plasma was calculated with the aid of calibration graphs prepared by adding known amounts of triazolam to 2.0 ml of blank plasma. These standard samples were analysed by the described procedure and the ratio of the peak height of triazolam to that of the internal standard was plotted against the known concentrations of triazolam. The same procedure was followed for determining the extraction yield of triazolam from plasma at various concentrations, except that clonazepam (5.0 ng) was added after extraction as an external standard. The ratios obtained were compared with those for standard amounts of the drug.

RESULTS AND DISCUSSION

Assay procedure

Fig. 2 shows gas chromatograms of extracts of plasma samples spiked with 2.5 and 0.25 ng/ml of triazolam and with internal standard, and the gas chro-

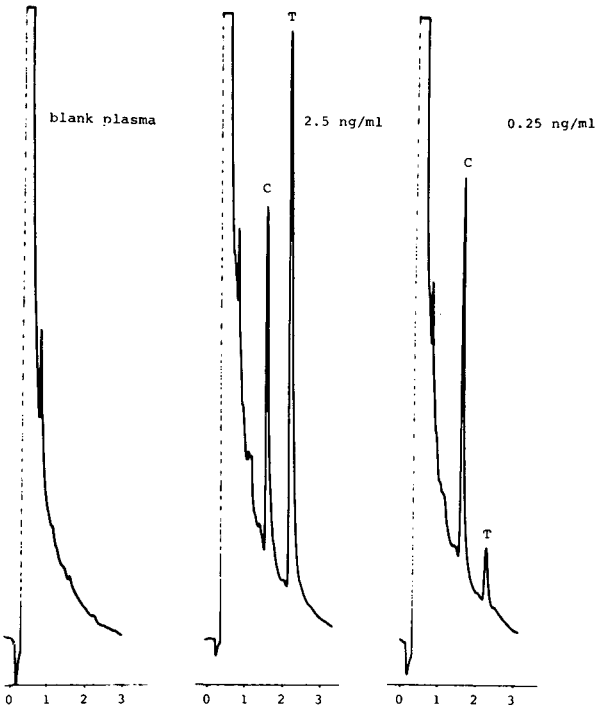


Fig. 2. Gas chromatograms obtained after extraction of 2.0 ml of blank plasma (left), 2.0 ml of plasma spiked with 2.5 ng/ml of triazolam (centre) and 2.0 ml of plasma spiked with 0.25 ng/ml of triazolam (right); the internal standard is clonazepam (C, 5.0 ng).

matogram of a blank extract. From the blank it appears that no interfering substances are co-extracted and that the retention times are short (1.5 and 2.2 min for the internal standard and triazolam, respectively). Clonazepam appears to be suitable as an internal standard, because the two peaks are well separated.

The mass spectrum of triazolam obtained from the analysis of the GLC peak was identical with the direct inlet mass spectrum (Fig. 3; mass spectrum of triazolam). In a previous study by De Boer et al. [3], it was shown that clonazepam also leaves the column unchanged. Thus both compounds appear to be determined in intact form. According to the standard curve (Fig. 4) there is good linearity between the detector response (peak height of triazolam/peak height of clonazepam) and the concentration of triazolam in the range 0.1—25 ng/ml. The correlation coefficient of such curves was not less than 0.999. Extraction yields determined in the same concentration range appeared to be constant and linear with concentration, with a mean value of 80% (the relative standard deviation at each concentration was 10% or less for $n = 3$, except for a

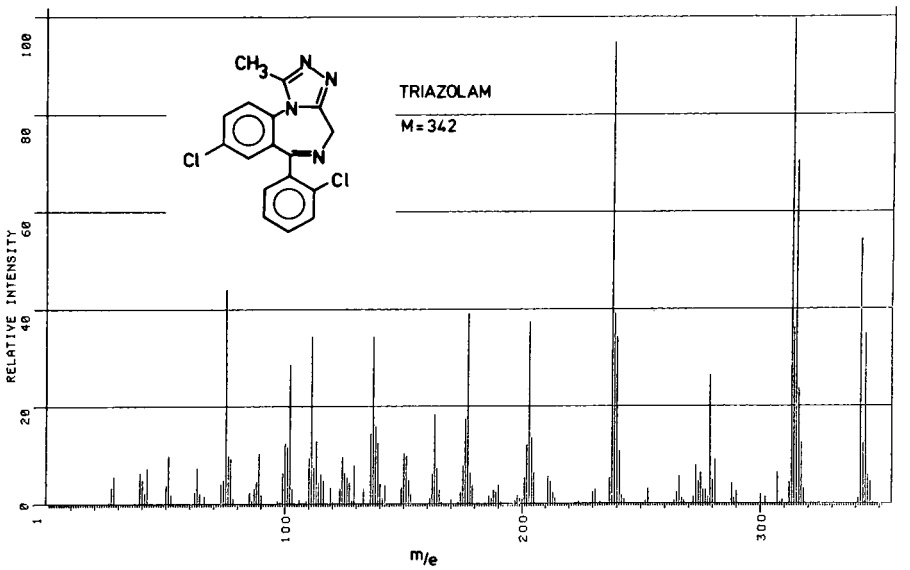


Fig. 3. Mass spectrum of triazolam obtained with the direct inlet system.

concentration of 0.1 ng/ml, where the relative standard deviation was 16.5%). Fig. 5 shows the plasma calibration graph, consisting of five individual calibration graphs for eight concentrations each. In the concentration range 0.5—25 ng/ml the relative standard deviations were below 8.5% (n = 5). The relative standard deviations for concentrations of 0.25 and 0.1 ng/ml were 17% and 16%, respectively. The correlation coefficient for each individual curve was greater than 0.999, illustrating the good linearity of the method. The lowest measurable concentration is about 0.05 ng/ml in plasma when 2.0-ml samples are used.

Human experiments

The utility of the present method for the assay of triazolam in pharmacokinetic studies in man was demonstrated by a preliminary study with healthy volunteers. Four healthy male volunteers swallowed a 0.5-mg tablet with 150 ml of tap water after an overnight fast. No food, fluid or tobacco was allowed for 3 h after drug administration. Blood samples were taken after about 20, 40, 60, 80 and 100 min and 2, 3, 4, 6, 9 and 12 h, from a forearm vein by means of a flexible venous cannula for the first 4 h and subsequently by venous puncture. Blood clotting was prevented by adding a small drop of heparin solution (5000 I.U./ml) to the samples. After separation, the plasma samples were stored at −20°C until taken for analysis, which was performed according to the procedure described above. The plasma concentration profile obtained for one volunteer is shown in Fig. 6.

Triazolam appeared to be absorbed rapidly from the tablet formulation and the elimination rate was also rapid, as shown by the short elimination half-life of about 2 h. The results are given in Table I.

The values of t_{max} (peak time), c_{max} (maximum concentration) and $t_{½}$ (elimination half-life) were similar to those found by Eberts et al. [4].

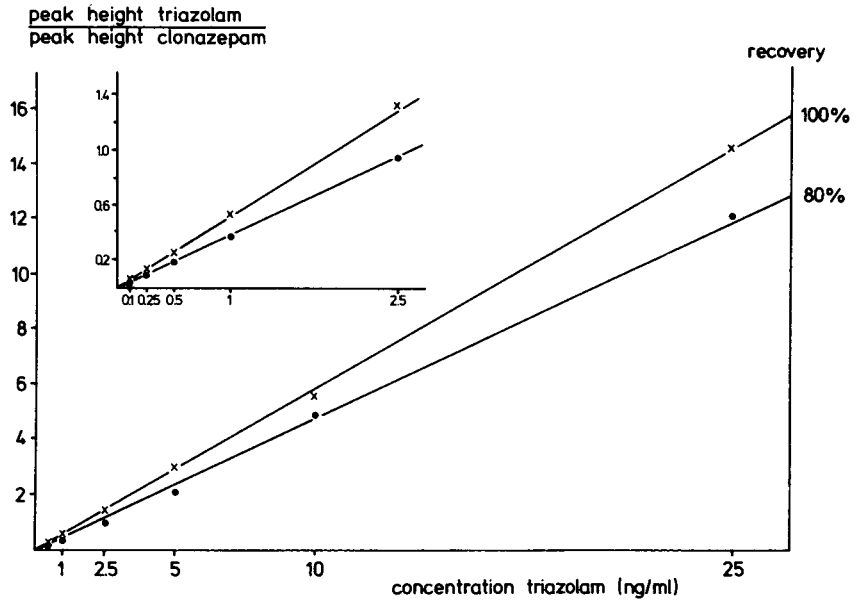


Fig. 4. Standard curve for triazolam and the calibration graph obtained after extraction from plasma using clonazepam as external standard (determination of recovery). The inset indicates on an expanded scale the recovery at low concentrations of triazolam.

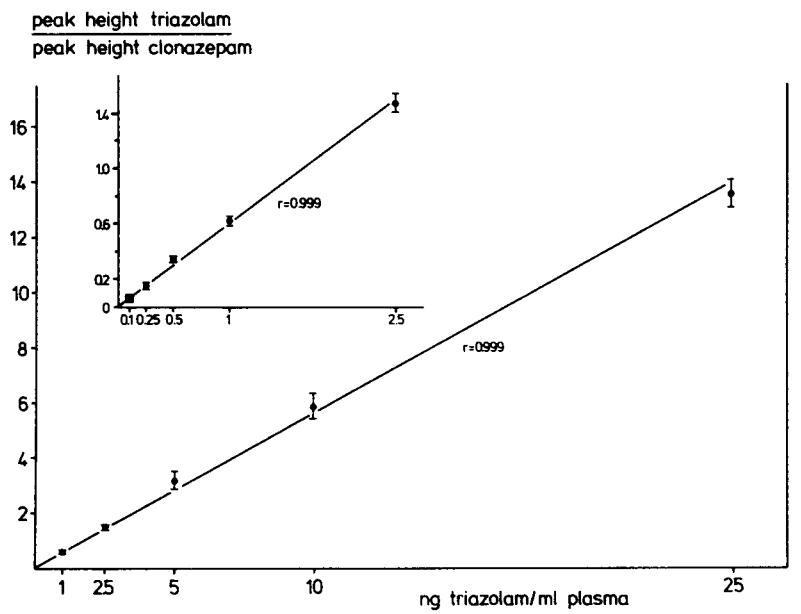


Fig. 5. Calibration graph for triazolam in plasma in the concentration range 0.1—25 ng/ml. Each point represents the mean ± S.D. of five observations. The inset indicates on an expanded scale the relationship between detector response and triazolam concentration at low concentrations of triazolam.

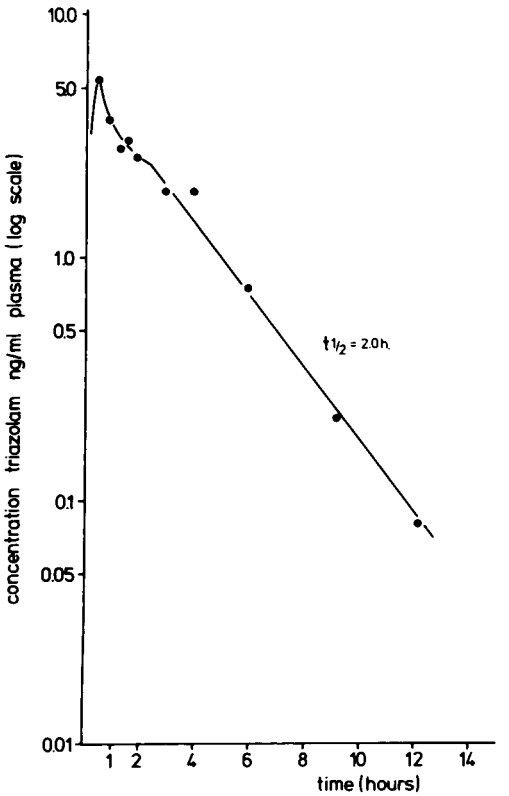


Fig. 6. Plasma concentration curve on a semi-logarithmic scale for triazolam in a healthy volunteer (F.S.) following administration of a 0.5-mg tablet (Halcion).

TABLE I

PHARMACOKINETIC PARAMETERS OF TRIAZOLAM FOLLOWING THE ADMINISTRATION OF A 0.5-mg TABLET (HALCION)

Subject	t_{max} (h)	c_{max} (ng/ml)	$t_{½\,el}$ (h)
R.G.	2	4.3	2.1
A.G.J.	1	4.4	2.3
A.N.	0.8	7.0	1.8
L.K.	1	2.5	2.0
Mean ± S.D.	1.2 ± 0.5	4.3 ± 1.9	2.1 ± 0.2

CONCLUSIONS

The procedure described for the quantitative determination of triazolam in plasma is rapid and precise. Further, with a lowest measurable concentration of about 0.05 ng/ml in plasma, it is sensitive enough for measuring the plasma concentration for at least three times the elimination half-life after therapeutic dosing.

From the results of the preliminary pharmacokinetic study in man it appears that triazolam is a benzodiazepine which is eliminated from the body very rapidly in comparison with many other benzodiazepines [5]. For a hypnotic, such a rapid elimination should be considered an advantage, because a limited duration of action may thereby be achieved [6].

ACKNOWLEDGEMENTS

The authors thank Dr. C.J. van Boxtel, Department of Clinical Pharmacology, Wilhelmina Gasthuis, Amsterdam for supervising the human studies, Dr. N.P.E. Vermeulen for performing the mass spectrometric analysis, Mr. C.J. van der Poel and co-workers for constructing the glass-lined needless, Mr. L. Bontje and co-workers for drawing the capillary columns and Mr. L.W. Gerrése and co-workers for constructing the solid injection system.

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Journal of Chromatography, 223 (1981) 445—448
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 815

Note

Ion-pair high-performance liquid chromatographic assay of levamisole in biological fluids

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(First received October 13th, 1980; revised manuscript received December 15th, 1980)

The discovery and early development of the broad-spectrum anthelmintic drug tetramisole (2,3,5,6-tetrahydro-6-phenylimidazo[2,1-*b*] thiazole) was described in 1966 [1, 2]. The compound was found to be effective against numerous gastrointestinal and pulmonary nematodes in a variety of domestic animals and against at least two nematodes infesting the intestinal tract of man [2].

Levamisole is the laevo isomer of tetramisole and has fewer side-effects [3]. Methods for its determination in plasma have been described, based on polarographic assay [4], thin-layer chromatography [5] and gas chromatography [6]. Recently a high-performance liquid chromatographic (HPLC) method has been proposed for controlling veterinary anthelmintic preparations [7]. The aim with the method described here was to cover the same concentration range in biological samples as with the gas chromatographic method, but with a simpler sample handling procedure. The described HPLC method requires a single extraction step using a small volume of serum (0.5 ml), followed by ion-pair chromatography on a reversed phase, and is specifically designed for pharmacokinetic studies.

EXPERIMENTAL

Apparatus

A Model 202 chromatography with a U6K injector and a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) was used, equipped with a Schoeffel GM 770 variable-wavelength UV detector and a 250 × 4.6 mm I.D. column packed with μBondapak C_{18} (particle size 10 μm) (Waters Assoc.).

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Reagents

The reagents were of analytical grade (Prolabo, Paris, France). Levamisole was purchased from Specia (Paris, France).

Standards

A stock standard solution containing 1 mg/ml of levamisole in methanol was prepared. The solution was diluted with mobile phase in order to obtain 1, 0.1 and 0.01 μg in a constant injection volume (10 μl). Standard solutions were stored at 4°C until use.

Operating conditions

The mobile phase was 0.2% acetic acid in water—methanol—heptane sulphonic acid (55:45:2) and the flow-rate was 2 ml/min (inlet pressure 200 bar). Before use the mobile phase was degassed by applying a vacuum to the solvent reservoir for approximately 5 min.

Detection was effected at 225 nm. The system was operated at ambient temperature (18—20°C) and the retention time for levamisole was 3.8 min. A typical chromatogram for 100 ng of levamisole injected directly into the column with a detection sensitivity of 0.01 a.u.f.s. is shown in Fig. 1.

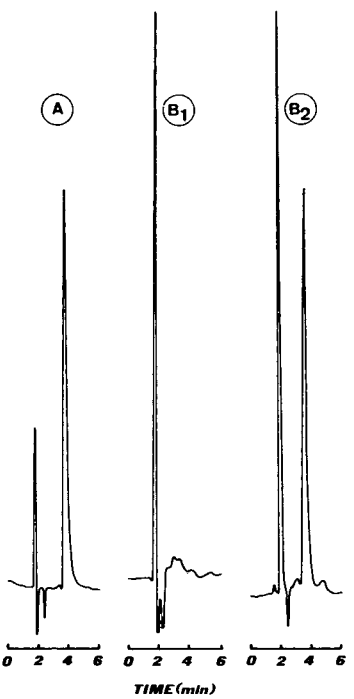

Fig. 1. Typical chromatograms: (A) corresponding to an injection of 0.1 μg of levamisole (0.01 a.u.f.s.) and (B) obtained on analysis of sheep blank plasma sample (B_1) and of plasma from sheep administered levamisole (B_2).

Extraction procedure

One millilitre of 0.1 *N* sodium hydroxide solution and 10 ml of chloroform were added to 0.5 ml of plasma, urine or mucus. The tubes were shaken for 10 min and centrifuged at 8400 *g* for 10 min. The organic phase was separated by filtration on phase-separating paper (Whatman 1PS) and evaporated to dryness under a stream of nitrogen gas to prevent oxidation. The residue was dissolved in 100 μl of eluent and the entire solution was injected into the column.

Calibration graphs

The recovery of levamisole added to sheep serum or mucus in the concentration range 0.1—1 μg was 70 ± 4%.

Analyses of the standards with or without extraction showed in both instances a high correlation between the concentration (x) and peak height (y). The equation of a typical calibration graph from 0.1 to 1 μg/ml was $y = 79.51x + 0.76$ and the correlation coefficient was 0.999.

When calibration graphs were constructed on five different days for a levamisole range of 0.1—1 μg/ml an excellent linear relationship was obtained each time. The slopes of the calibration graphs were reproducible, with a coefficient of variation of 3.5%.

RESULTS AND DISCUSSION

Ion-pair chromatography was performed by adjusting the pH of the mobile phase so that the sample was present in its ionic form (pH 3.5). A strongly ionic counter ion with a strong lipophilic group attached (heptanesulphonic acid; Waters Assoc.) was used.

The procedure described permits the rapid and selective determination of levamisole in biological fluids. Extraction with chloroform results in a clear extract that can be injected directly into the liquid chromatographic column without further purification. No interference from endogenous substances was observed. 4-Hydroxylevamisole, the major metabolite of levamisole, was also separated with a retention time of 2.8 min.

The method has been utilized in pharmacokinetic studies of levamisole in sheep. Preliminary results for plasma and mucus levels were obtained for intramuscular administration in two sheep. Each animal received a dose of 15 mg/kg and samples of blood were taken for 6 h and 2, 3, 4 and 6 h after the administration. Nasal mucus was collected on cotton plugs, which were weighed, rinsed and analysed for levamisole. A peak concentration (5.08 μg/ml) in plasma was observed 40 min after treatment (Fig. 2). The mean half-life of levamisole was about 1.8 h, and after 24 h a mean concentration of 0.05 μg/ml was still measurable.

Maximal nasal mucus levels were obtained 2 and 3 h after administration of the drug, and were 5—10 times greater than plasma concentrations. This demonstrates the ability of levamisole to diffuse from the blood into the pulmonary and bronchial secretions in which the drug exerts its anthelmintic activity against pulmonary worms. In the mucus fluid levels are effectively higher than the lethal concentration (2—6 μg/ml) as determined against selected strains of Trichostrongyles [8] or Ostertagia [9].

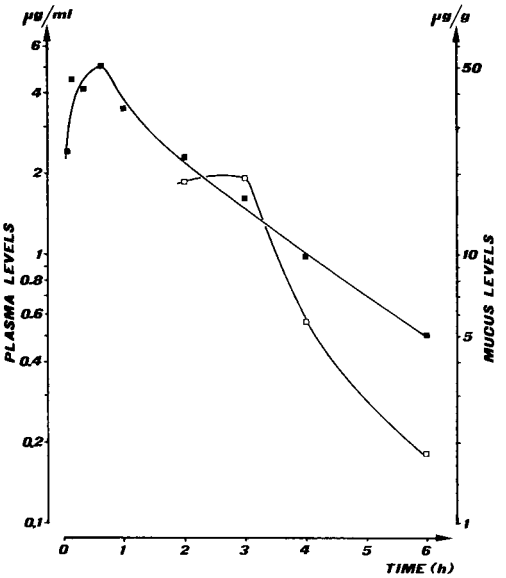

Fig. 2. Plasma (▪) and mucus (□) levels of levamisole after intramuscular administration of 15 mg/kg of levamisole to a sheep.

The lowest concentration of levamisole that can be determined in 0.5 ml of serum with acceptable precision was 0.05 μg/ml. Approximately 20—25 samples can be conveniently assayed in 1 day by one analyst.

ACKNOWLEDGEMENT

The authors thank Mr. Marcel Caussette for preparing the figures.

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Journal of Chromatography, 223 (1981) 449—453
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 817

Note

High-performance liquid chromatographic determination of haloperidol in plasma

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(First received October 23rd, 1980; revised manuscript received December 5th, 1980)

It has been recognized [1—3] that haloperidol is a drug for clarifying the correlation between plasma drug concentrations and clinical effects of psychotropic drugs. Several methods [4—7] for the assay of plasma levels of haloperidol have been described, including gas chromatography [4—6] using an electron-capture detector and radioimmunoassay [7]. Because it has a low sensitivity [4,5] and is time consuming [6], procedures based on gas chromatography are unsuitable for routine monitoring of plasma levels in man. Reagents that are not readily available are required in radioimmunoassay [7].

Bianchetti and Morselli [3] described a method based on gas chromatography using a nitrogen—phosphorus selective detector. We have developed a more rapid and convenient method based on high-performance liquid chromatography (HPLC) using a reversed-phase column and a UV detector.

EXPERIMENTAL

Materials and reagents

Haloperidol, 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl)-1-(4-fluorophenyl)-1-butanone, was kindly supplied by Dainippon Pharmaceutical Co., Osaka, Japan. The internal standard, diphenylamine, was obtained from Koso Chemical Co., Tokyo, Japan, and a solution containing 1.0 μg/ml in the mobile

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phase [methanol—water (63:37) containing 0.2 *M* ammonium acetate] was prepared. All other chemicals were of reagent grade and were used without further purification.

Apparatus and chromatographic parameters

A Hitachi 635A liquid chromatograph equipped with a multi-wavelength UV detector (Hitachi 635-0900) and a high-pressure sampling valve (Hitachi 635-5101, 1 μl to about 2 ml) was used. The column was heated at 55° C using a constant-temperature water-bath circulator. Peak heights were calculated using a Takeda digital integrator (Tr 2221 A). Separation was accomplished on a 25 × 0.4 cm microparticulate reversed-phase column (Nucleosil C18, 5 μm: Macherey, Nagel & Co., Düren, G.F.R.).

The mobile phase flow-rate was 0.5 ml/min, and the UV detector was set at 250 nm and was operated at 0.005 a.u.f.s.

Extraction procedure

Aliquots of plasma or serum (1.0 ml) were transferred into 10-ml glass-stoppered centrifuge tubes (1.5 × 10.5 cm) and acidified with 3.5 ml of 0.1 *N* hydrochloric acid. Diethyl ether (4 ml) was added and the mixture was shaken vigorously for 5 min, then centrifuged at 800—1000 *g* for 5 min. The upper, ether layer was aspirated off, then 4 ml of the aqueous layer were transferred into test-tubes containing 1 ml of 1 *N* sodium hydroxide solution and 4.5 ml of chloroform were added. The mixture was shaken gently for 10 min and then centrifuged at 1000 *g* for 5 min.

The aqueous layer was aspirated off then the tubes were shaken by hand or with a Vortex mixer and centrifuged again to obtain the clear chloroform layer. Four millilitres of the chloroform layer were transferred into 10-ml evaporating tubes and dried in vacuo in a water-bath at 30° C. The dry residue was dissolved in 100 μl of elution solvent containing the internal standard, and 30—40 μl of these samples were then chromatographed.

Calibration graph

Standard solutions containing 10, 20, 30, 40, 60 and 80 ng/ml of haloperidol in 0.1 *N* hydrochloric acid were prepared by dilution of a 1.0 μg/ml methanolic stock solution. This stock solution was stable for several weeks if stored at 4° C and protected from light. One millilitre of standard solution and 2.5 ml of 0.1 *N* hydrochloric acid were added to 1.0 ml of drug-free plasma and the samples were processed as described above. The ratios of the peak height of haloperidol to that of diphenylamine were used to construct a calibration graph.

RESULT AND DISCUSSION

HPLC mobile phase

The elution behaviour of haloperidol was examined by using methanol—water containing various amounts of ammonium acetate as the mobile phase. An ammonium acetate-free mobile phase was undesirable as elution of haloperidol did not occur until 35 min, whereas in the presence of ammonium acetate haloperidol eluted rapidly as a sharp, symmetrical peak. The peak height of

haloperidol increased with increasing concentration of ammonium acetate. However, it is well known that a reversed-phase column such as Nucleosil is unstable if the pH of the mobile phase is greater than 7.5; the pH values of methanol—water that contained 0.2 and 0.3 *M* ammonium acetate were 7.3 and 7.5, respectively. In addition, the amount of methanol was carefully controlled, as variations produced changes in the retention time of haloperidol. From the results, methanol—water (63:37) containing 0.2 *M* ammonium acetate was selected as the mobile phase.

Selectivity

Fig. 1 shows the chromatograms obtained for a plasma blank and a plasma sample to which 20 ng/ml of haloperidol had been added. The separation of haloperidol and diphenylamine from endogenous substances was good.

A graph of the ratio of the peak height of haloperidol to that of the internal standard against drug concentration was linear and passed through the origin, with a correlation coefficient of 0.9997. The coefficient of variation of the method at 20 ng/ml was 2.4% (n=8).

The recovery of haloperidol from a plasma sample in comparison with an aqueous sample that contained distilled water instead of plasma was approximately complete. The recoveries of haloperidol from human plasma to which 10 and 20 ng/ml had been added were 102.2 ± 2.9% (n=4) and 100 ± 3.9% (n=4), respectively.

The sensitivity of this method was 5 ng/ml of haloperidol. If a sample of 2 ml or more of plasma was used, it was possible to detect 2—3 ng/ml of haloperidol.

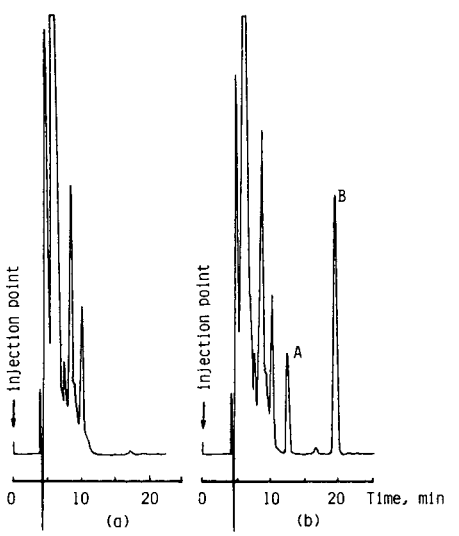

Fig. 1. High-performance liquid chromatograms of (a) human plasma blank and (b) haloperidol in human plasma. Peaks: A = haloperidol; B = internal standard (diphenylamine).

Haloperidol plasma levels in patients

Table I shows the plasma levels in patients undergoing chronic haloperidol treatment. Although only a few results were obtained, it can be seen that the plasma levels increased in proportion to the dose, and these levels of haloperidol were within the range measured by Itoh et al. [8].

TABLE I

PLASMA LEVELS OF HALOPERIDOL IN PATIENTS UNDERGOING CHRONIC TREATMENT

Case	Dose (mg/day)	Plasma level* (ng/ml)
1	6.0	4.6
2	8.0	9.0
3	18.0	22.9

*Plasma levels were determined 3.0 h after oral administration.

Application

In order to examine the application of this method to analogues of haloperidol such as methylperidol and spiroperidol, the retention times and sensitivities for these two drugs were determined by the above method, and the results are given in Table II.

The retention times of these two analogues were similar to that of haloperidol and, as shown in Fig. 1, the peaks of the analogues were not subject to interference from that of drug-free plasma. In addition, there was no marked difference in the peak heights of the three drugs. It therefore seems that the method is applicable to the determination of methylperidol and spiroperidol in plasma samples.

According to our results, the method appears to be suitable for monitoring haloperidol plasma levels during chronic treatment, and also for investigation of the correlation between haloperidol plasma levels and pharmacological effects.

TABLE II

RETENTION TIMES AND RELATIVE PEAK-HEIGHT RATIOS OF HALOPERIDOL, METHYLPERIDOL AND SPIROPERIDOL

Compound	Retention time (min)	Relative peak-height ratio*
Haloperidol	12.5	1.0
Methylperidol	11.6	0.7
Spiroperidol	10.9	0.8

*Ratio of the peak height of the compound to that of haloperidol.

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Journal of Chromatography, 223 (1981) 454—459
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 829

Note

High-performance liquid chromatographic analysis of lorcainide and its active metabolite, norlorcainide, in human plasma

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(Received November 11th, 1980)

Lorcainide is a new antiarrhythmic drug which is effective for the treatment of premature ventricular contractions [1] and ventricular tachycardia [2]. It is effective following both oral and intravenous administration. Lorcainide undergoes a saturable presystemic elimination following oral administration, and an active metabolite, norlorcainide, accumulates in the plasma to concentrations greater than those of the parent drug [3]. Norlorcainide has been shown to possess antiarrhythmic properties similar to those of lorcainide [4]. Due to the variability in the first-pass extraction and the accumulation of the active metabolite, it is desirable to measure lorcainide and norlorcainide plasma concentrations during clinical trials and as a therapeutic aid.

A gas chromatographic method for the analysis of lorcainide and norlorcainide has been published previously [5]. The method employed electron-capture detection and was sensitive to a lower limit of 10 ng/ml of lorcainide or norlorcainide in plasma. The method described here is a high-performance liquid chromatographic procedure using a reversed-phase system with UV absorbance detection. Our method is sensitive to a lower limit of detection of 5 ng/ml of lorcainide or norlorcainide in plasma. The internal standard employed is the calcium antagonist D-600 which is structurally dissimilar from lorcainide (see Fig. 1), but has similar solubility and chromatographic and absorptive characteristics.

EXPERIMENTAL

Chemicals and reagents

Lorcainide HCl {N-(4-chlorophenyl)-N-[1-(1-isopropyl)-4-piperidinyl] benzeneacetamide hydrochloride} and norlorcainide [N-(4-chlorophenyl)-N-(4-

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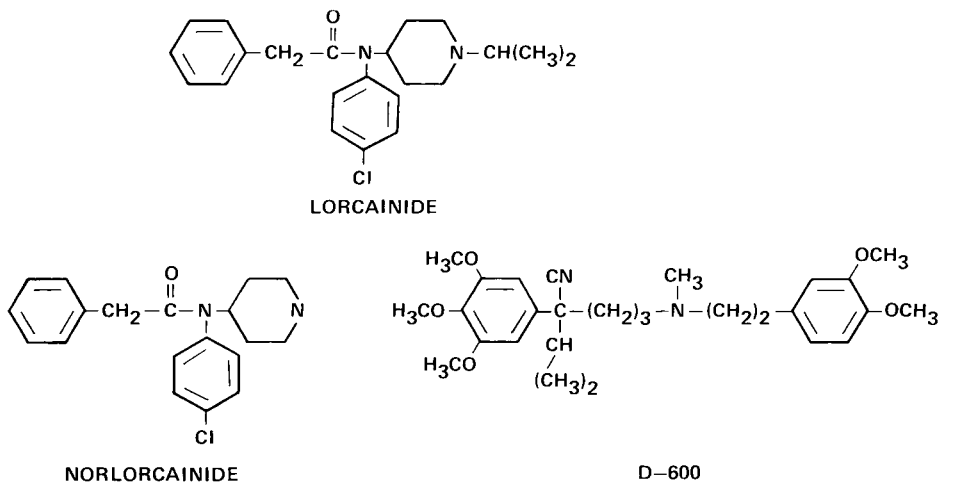

Fig. 1. Structures of lorcainide, norlorcainide, and D-600.

piperidinyl) benzeneacetamide] were provided by Janssen Pharmaceutical (New Brunswick, NJ, U.S.A.). The internal standard D-600 {α-isopropyl-α-[(N-methyl-N-homoveratryl)-α-aminopropyl]-3,4,5-trimethoxyphenylaceto-nitrile hydrochloride} was obtained from Knoll (Ludwigshafen, G.F.R.). Glass distilled acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other solvents were of reagent grade.

Instrumentation and chromatographic conditions

A Varian Model 8500 high-pressure liquid chromatographic pump was employed to pump solvent through a Waters Assoc. (Milford, MA, U.S.A.) μ-Bondapak phenyl reversed-phase column (30 cm × 3.9 mm I.D.; particle size 10 μm). The flow-rate was 80 ml/h which produced a precolumn pressure of 68 atm (1000 p.s.i.). The detector was a Schoeffel Model SF 770 variable-wavelength absorbance detector. The detector was set for absorbance at a wavelength of 196 nm. A dual-pen Houston-Omniscribe recorder was used at 10 and 20 mV outputs. Chart speed was 0.5 cm/min.

Mobile phase preparation

The mobile phase consisted of acetonitrile—phosphate buffer (2:3). The phosphate buffer was an 0.02 *M* solution of KH_2PO_4 with the pH adjusted to 2.3 by the dropwise addition of approximately 1.9 ml of H_3PO_4 per l of buffer. The mixture was filtered and degassed prior to use.

Extraction procedure

The extraction procedure is outlined in Fig. 2. PTFE-lined, screw-capped tubes are initially rinsed and wetted with heptane—isoamyl alcohol (95:5). Plasma (0.2—2.0 ml) and 100 μl of aqueous internal standard solution (200 ng) are added to tubes. The plasma is made basic with the addition of 200 μl of 2 *N* NaOH, which is 67% saturated with NaCl, and 5 ml of a mixture of heptane—isoamyl alcohol (95:5) is added. The samples are mixed by gentle rocking on a

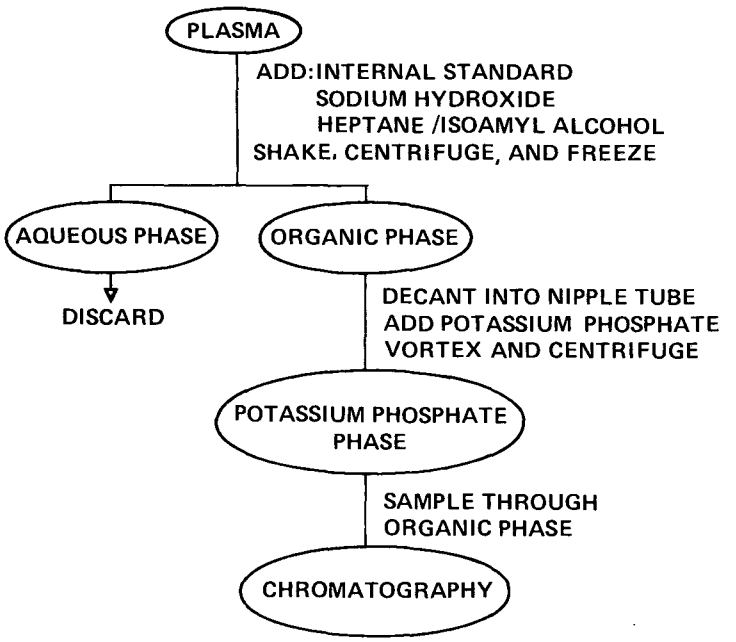

Fig. 2. Schematic outline of the sample preparation used in the analysis of lorcainide and norlorcainide in plasma.

mechanical shaker for 10 min and then centrifuged for 15 min. The aqueous phase is frozen by placing the tubes in a mixture of acetone—dry ice for a minute, and the organic phase is decanted into a clean tube with an elongated cone at its base of approximately 50 μl capacity. To this tube are added 20 μl of 0.02 *M* KH_2PO_4, which has been adjusted to a pH of 1.5 with H_3PO_4. The sample is shaken on a vortex mixer for 90 sec and centrifuged to separate the aqueous and organic layers. An aliquot (10 μl) of the aqueous phase is injected into the chromatograph using a 25-μl syringe.

Preparation of calibration standards

Aqueous stock solutions containing lorcainide and norlorcainide at concentrations ranging from 5 to 514 ng per 100 μl are used for preparing standard curves. These are stored at 4° C with no detectable decomposition of lorcainide or norlorcainide over a three-month period.

RESULTS AND DISCUSSION

The retention times for norlorcainide, lorcainide and the internal standard are 4.3, 6.0 and 7.7 min, respectively. Chromatograms of an extract of plasma from a subject not taking lorcainide (A) and an extract of a plasma sample from a patient who was taking lorcainide (B) are shown in Fig. 3. A structural analog of lorcainide, N-(4-chlorophenyl)-N-[1-(3-methylbutyl)-4-piperidinyl] benzeneacetamide, was also evaluated for use as an internal standard; it eluted with a retention time of over 10 min. There also appeared to be more variation in the analysis of repeated samples when this compound was employed. Due to

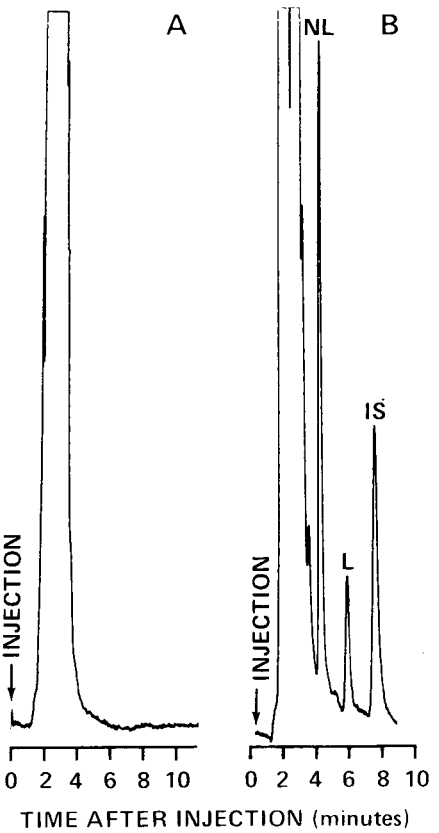

Fig. 3. Chromatograms of extracted patient plasma (A) not taking lorcainide and (B) 8 h after taking 100 mg of lorcainide orally. Peaks: L, lorcainide, measured 32 ng/ml; NL, norlorcainide, 123 ng/ml and IS, internal standard, D-600.

the slow elution and the problem with variability, D-600 was employed as a more desirable internal standard. The chromatogram shown in Fig. 3B was from a blood sample drawn 8 h following administration of a 100-mg oral dose of lorcainide. The measured concentrations of lorcainide and norlorcainide were 32 ng/ml and 123 ng/ml, respectively.

The chromatography was sensitive to slight changes in the mobile phase composition. Increasing the molarity of the phosphate buffer decreased the retention times and subsequently the separation of the three components. The acidity of the phosphate buffer also greatly influenced the retention times, with the retention times decreasing with a decrease in pH. Since a pH of 2 is the lowest recommended pH for a bonded-phase column, a pH of 2.3 was employed. This pH maximized the chromatography, producing sharp symmetrical peaks with good baseline separation. Under these conditions, samples could be injected every 9 min.

Rinsing the tubes and caps used for extraction with the organic solvent was found to improve the precision of the assay. When they were not rinsed with the heptane—isoamyl alcohol solvent, poor reproducibility was observed with coefficients of variability of greater than 10%. The extraction efficiencies of

lorcainide and norlorcainide were determined by comparing peak heights of extracted and directly injected ssmples. The recovery of lorcainide was 38 ± 0.6%, and for norlorcainide it was 41 ± 1.0%.

Since patients receiving antiarrhythmic drug therapy often are given other medications concurrently, interference from other compounds with the analysis of lorcainide was evaluated. Several drugs were added to plasma samples in quantities representative of therapeutic or higher concentrations. The following drugs were evaluated: lidocaine, quinidine, propranolol, metroprolol, digoxin, verapamil, chlorothiazide, and furosemide. A peak at 3.8 min was observed with propranolol, but this did not cause any major interference. Verapamil, however, eluted as a peak at 7.0 min, which was not totally separated from the internal standard. In the event that lorcainide plasma concentrations need to be measured in the presence of verapamil, either the chromatography can be slowed down to produce a better separation, or the methyl-butyl analog of lorcainide can be utilized as an internal standard.

Standard curves were prepared by adding known amounts of drug and metabolite to blank blood or plasma samples and determining the peak height ratios (lorcainide or norlorcainide/internal standard). The standard curves were linear over a range of from 5 to 500 ng/ml and extrapolated through the

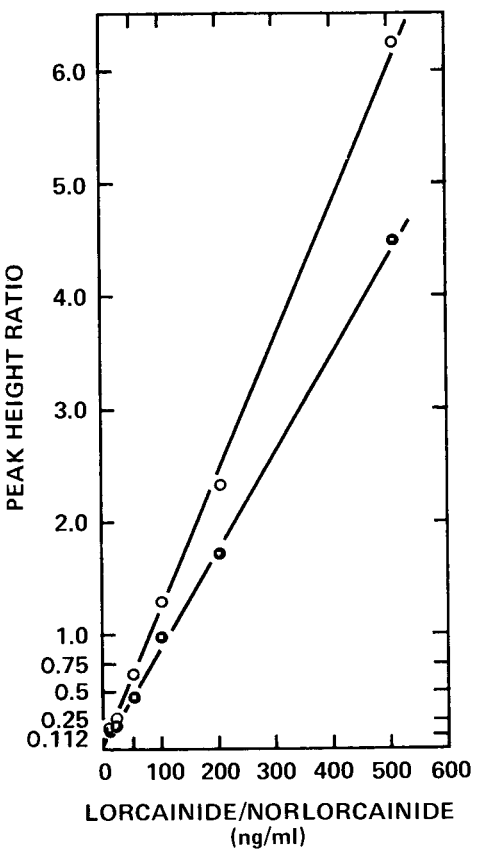

Fig. 4. Standard curve for lorcainide and norlorcainide extracted simultaneously was linear over a range from 5 to 500 ng/ml of plasma and extrapolated through the origin.

origin. A typical standard curve for lorcainide and norlorcainide analyzed simultaneously from plasma is shown in Fig. 4. The average coefficient of variation from eight standard curves was 6.03 ± 1.14% for lorcainide and 5.16 ± 2.34% for norlorcainide.

The lowest concentration standard which was routinely employed for the calibration curve was 11 ng/ml for both lorcainide and norlorcainide. Only on rare occasions were samples containing less than 10 ng/ml encountered. The lower limit of sensitivity of this method is 5 ng/ml.

Reproducibility was evaluated by extracting and analyzing replicate plasma samples containing 11, 51, and 514 ng/ml of lorcainide and norlorcainide. These data are summarized in Table I. The coefficients of variation (C.V.) for lorcainide at 11, 51, and 514 mg/ml were 5.21, 1.09, and 2.37%, respectively. The C.V. values for norlorcainide at each concentration were 4.51, 8.80, and 3.59%, respectively.

TABLE I

REPRODUCIBILITY AT GIVEN PLASMA CONCENTRATIONS $N = 5$

Concentration (ng/ml)		C.V. (%)
Lorcainide	Norlorcainide	
11	—	5.21
51	—	1.09
514	—	2.37
—	11	4.51
—	51	8.80
—	514	3.59

Since it may be desirable to extract plasma samples on a day prior to chromatographic analysis, the stability of lorcainide and norlorcainide was investigated when left in the acid phase after completing the extraction. Samples left in acid at 4°C or room temperature for up to seven days showed no signs of decomposition, suggesting that lorcainide, norlorcainide and the internal standard are stable under these conditions for at least a week.

The method described here for the quantitative determination of lorcainide and norlorcainide in plasma is simple and rapid and allows the daily analysis of 25—30 plasma samples.

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Journal of Chromatography, 223 (1981) 460—465
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 831

Note

Simultaneous determination of sulfinpyrazone and four of its metabolites by high-performance liquid chromatography

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(Received November 14th, 1980)

Sulfinpyrazone (SO) (Fig. 1), 1,2-diphenyl-4-[2-(phenylsulfinyl)-ethyl]-3,5-pyrazolidinedione, Anturane®, was originally introduced as a uricosuric drug [1]. New investigations [2] concerning the drug have shown it to be effective in the suppression of platelet function in patients suffering from thrombo-embolic disorders. Recently [3], a significant reduction in the incidence of sudden death after myocardial infarction has been reported.

Attention was drawn to the metabolism of SO by the finding of a prolonged and biphasic effect of SO in the rabbit [4]. This led to the identification of two new and very active metabolites, the sulfide (S) and the *p*-hydroxylated sulfide (SOH), in rabbit [5] and in man [6]. Quantitative determination of the new metabolites was carried out by gas chromatography—mass spectrometry. Several papers [7—10] have dealt with the determination of sulfinpyrazone itself and its well-known metabolites — the sulfone (SO_2) and the *p*-hydroxylated sulfinpyrazone (SOOH) — but none of these methods are directly applicable to determination of the new metabolites. This paper presents a rapid, selective and sensitive high-performance liquid chromatographic (HPLC) method for the simultaneous determination of SO and its four metabolites (SO_2, S, SOH and SOOH) in human plasma.

MATERIALS AND METHODS

Chromatography

A Waters Assoc. Model 600 liquid chromatographic pump was equipped with a U6K injection system. Separation was achieved with a Knauer® 250 mm × 4.6 mm I.D. steel column packed with 5 μm Spherisorp ODS particles. The absorbance was measured at 254 nm by a Waters 440 dual-channel photometer. The solvent system was methanol–0.02 *M* phosphate

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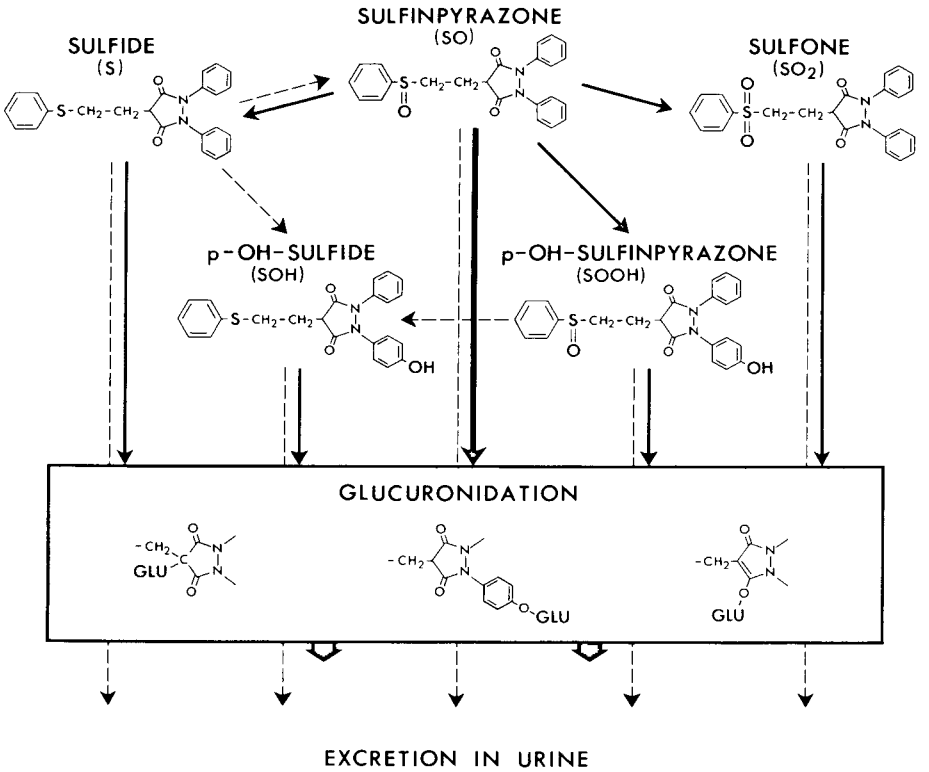

Fig. 1. Reductive and oxidative metabolic pathways of sulfinpyrazone.

buffer (pH 7.0) (40:60) at a flow-rate of 1.3 ml/min. The chromatograms were recorded on an OmniScribe (Houston Instruments) dual-channel recorder.

Reagents

Sulfinpyrazone (SO, 1,2-diphenyl-4-[-2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione) and its metabolites, the sulfone (G 31 442, SO_2), the *p*-hydroxylated sulfinpyrazone (G-32 642, SOOH), the sulfide (G 25671, S) and the *p*-hydroxylated sulfide (G 33378, SOH) were obtained from Ciba-Geigy, Basel, Switzerland. 1-Chlorobutane (Fluka, Bachs, Switzerland), methanol, and dichloromethane (Merck, Darmstadt, G.F.R.) were of analytical grade. The internal standard, naproxen (Nap), was obtained from Astra-Syntex, Södertälje, Sweden. Sodium sulfite (Merck) in water (100 mg per 5 ml) was prepared just before use.

Procedure

A 0.5—1 ml sample of plasma, to which a suitable amount of internal standard was added, 0.5 ml of sodium sulfite and 1 ml of 1 *N* HCl in a 12-ml glass stoppered centrifuge tube were extracted for 30 min on a horizontal shaker with 8 ml of a mixture (1:3) of dichloromethane and 1-chlorobutane.

After centrifugation 7.0 ml of the organic phase were transferred to another glass stoppered tube and washed with 2 ml of 0.05 *M* phthalate buffer (pH 5.0) by vortex mixing for 60 sec. After centrifugation, 6 ml of the organic phase were evaporated to dryness under nitrogen in a tapered centrifuge tube and redissolved in 100 μl of 1 *N* NaOH, 100 μl of sodium sulfite and 200 μl of hexane. After vortex mixing and centrifugation, 25 μl of the aqueous phase were injected on the chromatograph.

Preparation of standard curves

Standard curves were constructed by the addition of 0.5—20 μg of the compounds per milliliter of plasma (internal standard, naproxen: 5 μg/ml). Samples were treated as described under Procedure and the ratios of the peak heights of sulfinpyrazone and its four metabolites to that of the internal standard were plotted against the concentration of sulfinpyrazone and metabolite.

Application

Two healthy male volunteers were given a single oral dose of 200 mg of sulfinpyrazone. The time courses of the plasma levels of SO and metabolites were followed for 48 h. Treatment was then continued with 200 mg of sulfinpyrazone twice for one day (at 8 a.m. and 12 a.m.), followed by 200 mg four times a day during the next five days (at 8 a.m., 12 a.m., 6 p.m. and 10 p.m.). Heparinized blood samples were drawn just before the first morning dose (10 h after the previous dose) on days 4, 6 and 8 (subject 1) or on days 4, 7 and 8 (subject 2). Thereafter the decline of SO and the metabolites was followed for 48 h (subject 1) and 24 h (subject 2). Plasma samples were kept frozen at −20°C until analysis.

RESULTS AND DISCUSSION

The extraction of sulfinpyrazone (SO) and its metabolites was carried out using a mixture (3:1) of 1-chlorobutane and dichloromethane, which gave satisfactory recovery for five of the six compounds (Table I). Extraction with 1-chlorobutane alone did not give sufficient recovery of the polar compounds, while extraction with dichloromethane alone gave low recovery of the very lipophilic sulfide metabolite (S) [6]. Addition of dichloromethane to 1-chlorobutane provided a polar and efficient extraction medium with a density lower than that of plasma, and was thus more easy to handle. The recovery from plasma of the hydroxylated sulfinpyrazone (SOOH) was very low, but it could be increased using the extraction procedure described by Bjornsson et al. [10].

Oxidation of the compounds in the 4-position of the dioxopyrazolidine ring [11] or S-oxidation of the sulfide is possible during the extraction procedure. Initial experiments gave a low recovery for sulfide concentrations of less than 2 μg/ml, but addition of sodium sulfite to the plasma before extraction increased the recovery of the sulfide metabolite considerably, indicating that the sulfite protected the metabolite against oxidation. A small but constant part (about 4%) of the sulfide was converted to sulfinpyrazone during the

extraction procedure. Reduction of the extraction time did not diminish this fraction but reduced the recovery. The fraction could, however, easily be calculated from the standard curves making a correction of the concentration calculations possible. The sulfite did not reduce the sulfone or sulfinpyrazone.

Addition of sulfite to the final sodium hydroxide solution also protects the sulfide metabolite from oxidation, making this solution stable for several days at room temperature. Back-extraction from the extraction mixture into 1 *N* NaOH, as used by Bjornsson et al., in the extraction of SO and the sulfone metabolite (SO_2) was not applicable since the sulfide was not extracted. Instead evaporation to dryness under nitrogen and redissolving the residue in 1 *N* NaOH was preferred. To obtain complete dissolution and a clear liquid to inject into the chromatograph, it was necessary to wash the sodium hydroxide phase with hexane. No loss of compounds was seen during this final step. Also the washing with pH 5 buffer caused no loss, and better chromatograms were obtained when this step was added to the extraction procedure.

Sulfinpyrazone and its four metabolites together with the internal standard could be separated by reversed-phase HPLC using a 10 μm LiChrosorb RP-18 or a 5 μm Spherisorp ODS column. The latter was preferred since the retention time for the less-polar metabolite, the sulfide (S), could be reduced to 11 min without losing separation of the polar compounds; 20—25 min were necessary with the LiChrosorb column.

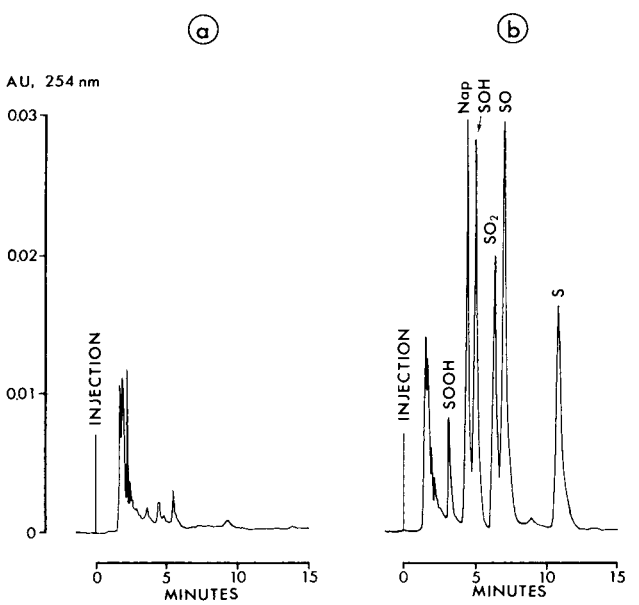

Fig. 2. Chromatograms of HPLC of (a) a plasma blank and (b) a plasma sample spiked with sulfinpyrazone (SO, 2.5 μg/ml), and metabolites (SOOH, 2 μg/ml; SOH, 2 μg/ml; SO_2, 2.5 μg/ml; S, 2.5 μg/ml), with naproxen (Nap, 5 μg/ml) as internal standard. The plasma samples were treated as described under Procedure.

Fig. 2 shows chromatograms of a plasma blank (a) and of a plasma sample spiked with sulfinpyrazone, its four metabolites and the internal standard (b). A small blank peak appears at the same retention time as that of the hydroxylated sulfide metabolite (SOH) equivalent to about 150 ng/ml of this metabolite. Reducing the methanol content in the eluent to 36% separated this interfering peak from the SOH metabolite.

Standard curves for SO and the metabolites were linear ($r > 0.995$) up to 20 μg/ml and with the exception of the SOH metabolite the straight lines intercepted the ordinate very near the origin. The slopes of standard curves, minimum detectable concentrations, extraction recoveries and precision of the analysis are given in Table I.

TABLE I

SLOPES OF STANDARD CURVES, RECOVERY, DETECTION LIMITS AND PRECISION OF THE ANALYSIS FOR SULFINPYRAZONE AND METABOLITES

Compound	Slope of standard curve*	Recovery** (%)	MDC*** (ng/ml)	C.V. (%)§	
				a	b
SO	0.380	85	30	4.5	10.8
SO_2	0.286	85	30	3.9	9.0
S	0.242	88	30	3.2	8.3
SOOH	0.108	16	100	5.4	15.2
SOH§§	0.442	99	100	4.8	12.3

*Ratio of peak height of 1 μg/ml compound to that of 5 μg/ml naproxen (internal standard).
**Recovery corrected for loss of solvent during the extraction procedure.
***MDC = minimum detectable concentration, defined as peak height = two times baseline noise.
§Within-run coefficient of variation ($n = 6$) for 5 μg/ml (a) and 0.5 μg/ml (b).
§§Blank peak subtracted.

Fig. 3 shows the plasma concentrations of sulfinpyrazone and the metabolites after treatment with SO as described under Application. After a single oral dose of SO only small amounts of the metabolites were found. The concentrations of the *p*-hydroxylated metabolites, SOOH and SOH, were below the detection limit of the method. Continuous treatment with sulfinpyrazone causes the level of the metabolites to increase considerably. In vitro the sulfide is more than ten times as active as SO and the sulfone is about five times as active as SO in inhibiting platelet aggregation [12]. Taking these data into consideration the beneficial effect of long-term treatment with sulfinpyrazone upon sudden death after acute myocardial infarction [3] could be assigned to the presence of these two metabolites. The rate of disappearance of the compounds from plasma after cessation of treatment is shown in the right-hand parts of the graphs in Fig. 3. Further investigation of the metabolism and pharmacokinetics of sulfinpyrazone during long-term treatment is in progress.

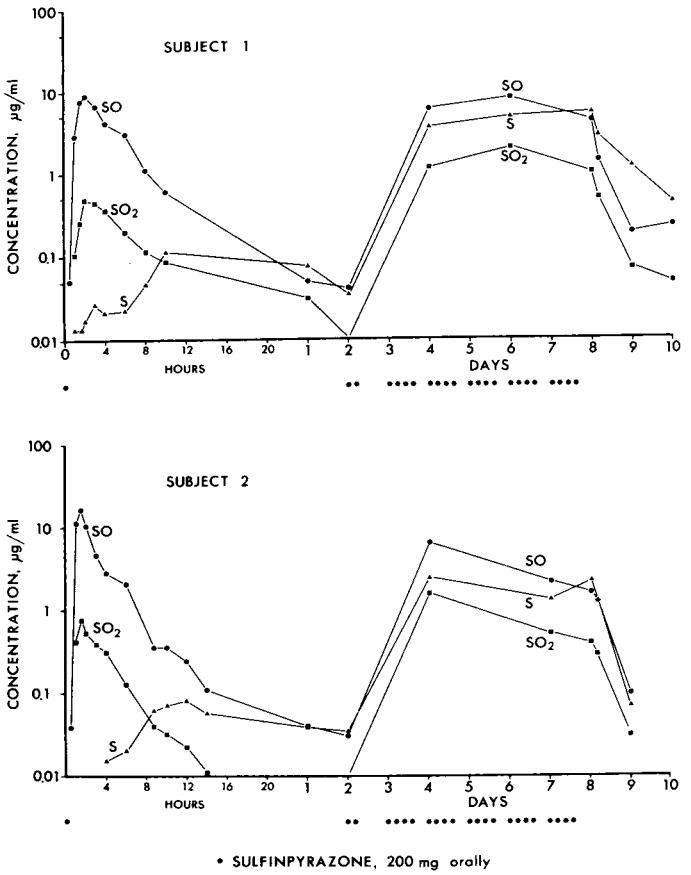

Fig. 3. Plasma concentrations of sulfinpyrazone and metabolites in two subjects after a single dose followed by continuous oral administration of sulfinpyrazone as described under Application.

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Journal of Chromatography, 223 (1981) 466—472
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 832

Note

Determination of trichlormethiazide in human plasma and urine by high-performance liquid chromatography

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(First received October 27th, 1980; revised manuscript received December 31st, 1980)

Trichlormethiazide is a thiazide diuretic drug which is orally administered at relatively low doses of 4—8 mg. Studies of the time-course of the drug in biologic fluids of humans have not been published. However a colorimetric method, based on the Bratton and Marshall technique, has been utilized in quantitating the drug in plasma and urine specimens obtained from dogs [1]. Other methods, which have been employed in more recent studies involving other thiazide diuretics, include gas chromatography [2—4] and high-performance liquid chromatography (HPLC) [5—9]. In two HPLC assays for hydrochlorothiazide in serum [8] or serum and urine [9], trichlormethiazide has been used as an internal standard, although the trichlormethiazide was not added initially and carried through the entire assay procedure. A HPLC procedure has also recently been developed for the purpose of screening urine specimens for the presence of all currently used thiazide drugs [10].

The HPLC assays described below are simple, rapid, sensitive and specific for trichlormethiazide in plasma and in urine obtained from humans receiving the drug. The methods are suitable for single- or multiple-dose studies of the pharmacokinetics or bioavailability of this drug in man.

EXPERIMENTAL

Materials

Trichlormethiazide was provided by Schering (Kenilworth, NJ, U.S.A.) and bendroflumethiazide was provided by E.R. Squibb & Sons (Princeton, NJ, U.S.A.). The diethyl ether and acetonitrile were from Burdick & Jackson

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Labs. (Muskegon, MI, U.S.A.) and the 2-propanol and methanol were HPLC grade from Fisher Scientific (Fairlawn, NJ, U.S.A.). Water is deionized and all other chemicals were of reagent grade.

All glass tubes employed in the extraction and evaporation procedures were placed in a 500°C kiln for 1 h to eliminate any contaminants remaining after washing with detergent, rinsing with deionized water and drying at 100°C overnight. The 15-ml conical tubes (Pyrex No. 8060) were silanized with 4% trimethylchlorosilane (Alfa Products, Danvers, MA, U.S.A.) in dry toluene. After silanization, the tubes were rinsed with methanol and dried at 100°C overnight.

Extraction

Plasma samples. Standard curves for trichlormethiazide in plasma were prepared with pooled human plasma. Standard solutions of trichlormethiazide were made in methanol from an initial stock solution containing 1.2 mg/ml of drug in methanol. The aqueous spiking solutions were then prepared using 1 ml of the appropriate methanol standard, diluted to 100 ml with 0.01 *N* sodium hydroxide, to yield final concentrations of 60, 120, 240, 360 and 450 ng/ml of drug. A solution of bendroflumethiazide in 0.01 *N* sodium hydroxide was prepared at a concentration of 2 μg/ml, to be used as an internal standard. These stock solutions were stable for at least one week when stored at 4°C.

To 3 ml of plasma in a 50-ml PTFE-lined screw-cap centrifuge tube were added 1 ml of one of the trichlormethiazide standard solutions, 1 ml of the bendroflumethiazide solution and 0.2 ml of 0.01 *N* hydrochloric acid, to provide a final pH of 6.9—7.2. Blank plasma samples were prepared using 2 ml of 0.01 *N* sodium hydroxide in place of the drug and internal standard solutions. The assay of plasma samples obtained from a subject who had received the drug involved the addition of 1 ml of internal standard and 1 ml of 0.01 *N* sodium hydroxide to 3 ml of plasma.

The plasma was extracted with 10 ml of diethyl ether for 15 min, with gentle mixing on a platform shaker. Following centrifuging, 2200 *g* at −10°C for 15 min, the tubes were placed in a dry ice—acetone mixture until the aqueous phase was frozen. The ether was then decanted into a 15-ml conical tube. In some instances a small yellowish droplet formed at the bottom of the tube due to the transfer of a small portion of the aqueous phase. In such cases the tube was again frozen, and the ether decanted into a fresh 15-ml conical tube. The ether was evaporated under a stream of nitrogen at 70°C in a water bath for 45 min. The residue was reconstituted with 50 μl of 0.01 *N* sodium hydroxide, with vortexing for approximately 25 sec. A 20-μl aliquot was then injected into the chromatograph.

Urine samples. Standard curves were prepared for trichlormethiazide using freshly voided human urine. Standard solutions of drug were prepared similarly to those for the plasma assay, beginning with a stock solution of 1 mg/ml in methanol. The final spiking solutions contained 0.2, 0.6, 1.0 and 2.0 μg/ml of drug in 0.01 *N* sodium hydroxide. The same internal standard solution was used for the urine and plasma assays. Standard curves were prepared by adding 1 ml of drug solution and 1 ml of internal standard solution to 2 ml of

freshly voided human urine. Urine samples obtained from subjects receiving the drug were assayed after the addition of 1 ml of internal standard solution and 1 ml of 0.01 *N* sodium hydroxide. Blank urine samples were assayed after the addition of 2 ml of 0.01 *N* sodium hydroxide to 2 ml of urine. The urine samples were extracted by adding 2 ml of urine to a 50-ml PTFE-lined screw-cap centrifuge tube containing 500 mg of sodium bicarbonate. Following the addition of the trichlormethiazide, internal standard and/or 0.01 *N* sodium hydroxide solution, 10 ml of ether was added, and the samples were extracted and treated as given in the plasma assay. The residue remaining after evaporation of the ether was dissolved in 100 μl of methanol. A 20-μl aliquot was then injected into the chromatograph.

Chromatography

The chromatograph consisted of a M6000 pump fitted to a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm, particle size 10 μm) from Waters Assoc. (Milford, MA, U.S.A.). The urine samples were injected with an automatic sampler (WISP) and the plasma samples utilized a U6K injector from Waters Assoc. A Schoeffel SF 770 Spectroflow variable-wavelength UV detector, set at 269 nm, at a range of 0.02 a.u.f.s. was used for the plasma assays. A Waters Assoc. 440 fixed-wavelength detector, set at 280 nm, at a range of 0.02 a.u.f.s., was employed for the urine assays. The mobile phase for the plasma assays consisted of 17% 2-propanol and 1% acetic acid in deionized water. A mobile phase of 35% methanol, 5% acetonitrile and 1% acetic acid in deionized water was used for the urine assays. The flow-rate was 2 ml/min (pressure approximately 138 bar) for both assays.

Quantitation

Standard curves were prepared from pooled human plasma and urine. An unweighted least-squares regression was employed to fit plots of peak height ratio (drug/internal standard) versus drug concentration in plasma and urine.

Stability studies

Plasma samples (3 ml) were spiked with 1 ml of trichlormethiazide solutions containing 0, 60, 120, 240, 360 and 450 ng/ml of drug. Similarly 2-ml urine specimens were spiked with 1 ml of trichlormethiazide solutions containing 0.1, 0.4, 0.8 and 1.6 μg/ml of drug. A portion of the plasma and urine samples was assayed immediately, and the remaining portion was frozen for five weeks until the time of assay.

Sample collection

A healthy human subject received two 4-mg tablets of trichlormethiazide (Schering). Urine samples were collected before dosing and over the time intervals 0—2, 2—4, 4—6, 6—8, 8—12, 12—16, 16—24, 24—36 and 36—48 h after dosing. Also, 15-ml blood samples were taken just before dosing and 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h following drug administration. The resulting plasma and urine specimens were stored frozen until assay.

RESULTS AND DISCUSSION

The detection wavelength and composition of the mobile phase were not the same for the urine and plasma assays. The changes were required because common parameters for both assays could not be found which permitted resolution of drug and internal standard from interfering constituents of the plasma or urine samples.

The recovery of internal standard added to ten plasma and eight urine samples averaged (± S.D.) 85.7 ± 4.7% and 89.1 ± 7.0%, respectively. The recovery of trichlormethiazide from ten plasma samples prepared in duplicate over a concentration range of 20—150 ng/ml averaged 70.9 ± 4.1%. The recovery of drug from eight urine samples prepared in duplicate over a concentration range of 0.05—0.8 μg/ml averaged 82.8 ± 4.6%. The recoveries were essentially the same at both extremes of the plasma and urine concentration ranges. The precision of the plasma assay was evaluated by assaying ten samples containing 20 ng/ml of drug and ten others containing 80 ng/ml. The relative standard deviations for the 20 ng/ml and 80 ng/ml samples were 9.1% and 3.9%, respectively.

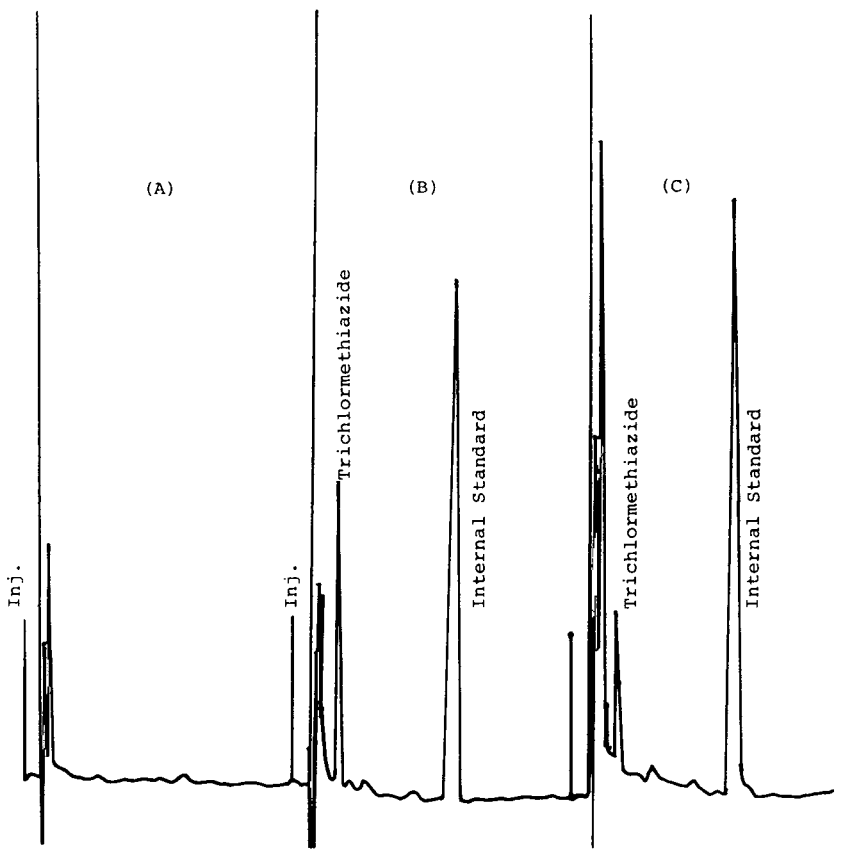

Fig. 1. Chromatograms of (A) predose control plasma, (B) plasma obtained from a subject 1 h after receiving 8 mg of trichlormethiazide and containing internal standard and (C) pooled plasma containing internal standard and 40 ng/ml of trichlormethiazide.

Standard curves for duplicate plasma concentrations, over a concentration range of 20—150 ng/ml, prepared on five different days, exhibited excellent linearity, with correlation coefficients of at least 0.995 for each curve. The intercepts did not differ significantly from zero and the mean slope forced through zero was 0.00491 ± 0.00007. The mean relative standard deviation for the concentration-normalized peak height ratios was 7.5 ± 1.2%. Similarly, five standard curves for duplicate urine concentrations, over a concentration range of 0.05—0.5 μg/ml were linear, with correlation coefficients of at least 0.998 for each curve. The intercepts were not significantly different from zero and the mean slope forced through zero was 0.6461 ± 0.0114. The mean relative standard deviation for the concentration-normalized peak height ratios was 3.6 ± 2.6%. The frozen plasma and urine specimens did not exhibit any evidence of degradation after five weeks of storage. Based on a minimum detectable peak in the chromatogram of 5 mm, the lower limit of sensitivity for the plasma and urine assays was 10 ng/ml and 50 ng/ml, respectively. Attempts were not made to increase the sensitivity of the urine assay, since it was anticipated that the drug levels in urine would be higher than those in plasma. Fig. 1 illustrates the chromatograms obtained from plasma

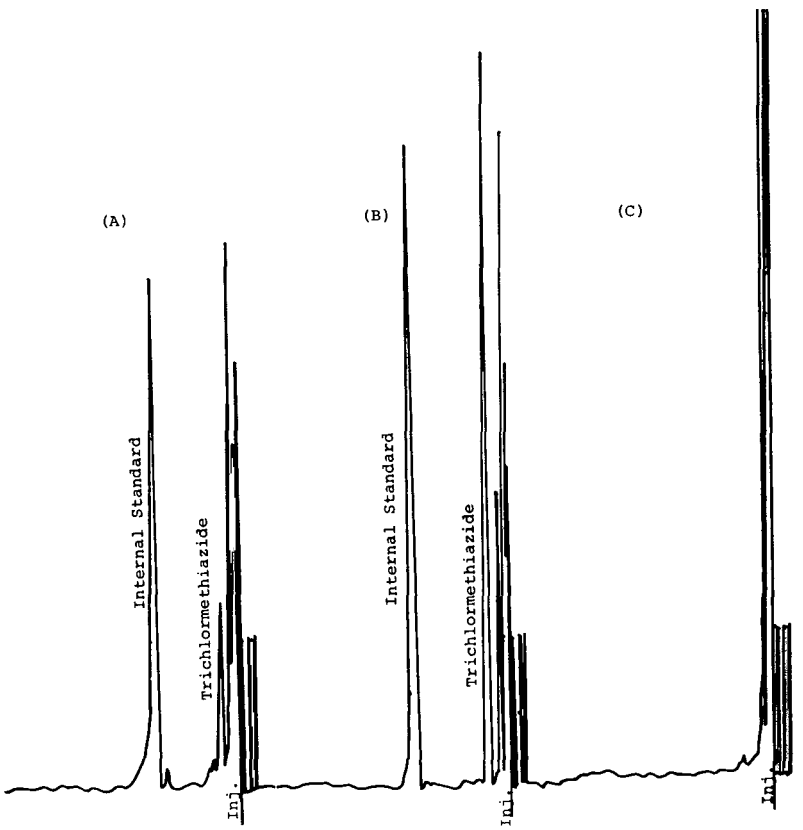

Fig. 2. Chromatograms of (A) pooled urine containing internal standard and 0.25 μg/ml of trichlormethiazide, (B) urine obtained from a human subject 4 h after an 8-mg dose of trichlormethiazide and containing internal standard and (C) predose urine obtained from the human subject.

extracts from the subject who received an 8-mg dose of trichlormethiazide. Also illustrated is a plasma sample spiked with 40 ng/ml of drug. The retention times for the drug and internal standard were 3.7 and 14.4 min, respectively. Fig. 2 illustrates the chromatograms for urine extracts from the same subject, as well as a pooled urine sample spiked with 0.25 μg/ml of drug. The retention times for the drug and internal standard were 3.7 and 10.7 min, respectively. These procedures have several advantages over the recently published HPLC urine screening method for thiazide drugs [10]. The screening method was not developed as a quantitative assay. Further, the present approach, which is applicable to both urine and plasma, requires fewer extraction steps.

No interferences were noted in the chromatograms for pre-dose plasma or urine extracts. However, preliminary studies did indicate the potential for a preservative in heparin solution to interfere with the quantitation of trichlormethiazide. When plasma samples were assayed which were obtained from whole blood exposed to heparin solution, a large peak appeared in the chromatogram which overlapped the drug peak. This interference was traced to benzyl alcohol which was present in a concentration of 1% in the heparin solution. The interference was eliminated by evaporating the ether extracts

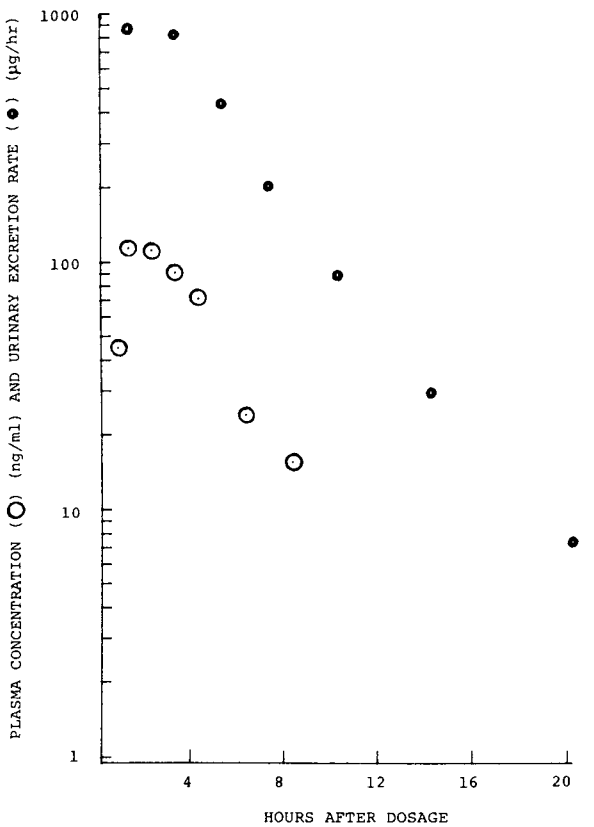

Fig. 3. Semi-logarithmic plot of plasma trichlormethiazide concentrations (○) and urinary excretion rates (●) for a human subject who received an 8-mg dose of trichlormethiazide.

and any residual benzyl alcohol for at least 45 min, at 70°C, under a stream of nitrogen.

Fig. 3 illustrates a semi-log plot of the plasma concentration and urinary excretion rate profiles for trichlormethiazide in the subject who received an 8-mg dose. There was no drug detectable in the plasma samples obtained after 12 h. The total urinary recovery of trichlormethiazide was 4.97 mg (62% of the dose) after 48 h, with 4.66 mg being excreted within the first 12 h. There are no known metabolites of trichlormethiazide, and no evidence for the presence of a metabolite was observed in the chromatograms for samples obtained from this subject.

ACKNOWLEDGEMENTS

The work was supported in part by the U.S. Food and Drug Administration (Contract No. FDA 223-77-3011) and the Tennessee Department of Public Health.

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Journal of Chromatography, 223 (1981) 473—478
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 833

Note

High-performance liquid chromatographic determination of bromural in serum upon hemoperfusion

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(Received November 13th, 1980)

Patients suffering from acute intoxication caused by bromural (BU) have usually been treated by symptomatic therapy. More positive therapy has been needed medically, and the application of hemoperfusion has been tried. There are few detailed reports on the effectiveness of hemoperfusion in intoxication [1—6], and none describing a comprehensive study of BU in serum upon hemoperfusion. This paper reports a rapid, precise and simple high-performance liquid chromatography (HPLC) method for the routine determination of BU in serum upon hemoperfusion.

EXPERIMENTAL

Reagents

Acetonitrile (HPLC grade), methanol (analytical grade), ethanol (analytical grade) and 3-nitro-*p*-anisidine (technical grade, 3NPA) were obtained from Wako (Osaka, Japan), and used without further purification. BU was purchased from Iwaki (Tokyo, Japan) and recrystallized from ethanol (m.p. 153—155°C).

Stock solutions

Bromural stock solution (10 mg per 100 ml) was prepared by dissolving BU in acetone. Internal standard stock solution (13 mg per 100 ml) was prepared by dissolving 3NPA in ethanol. Ten-fold and fifty-fold dilutions of these two solutions, respectively, were made before use.

Apparatus

An Hitachi 635 T liquid chromatograph equipped with a variable-wavelength detector, and also a liquid chromatograph consisting of a Kyowa Seimitsu Mini Micro Pump Type KHU 16 and a Kyowa Seimitsu variable-wavelength detector Type KLC-200, were used in this work. A Kyowa Seimitsu Universal Injector Type KHP-130 was used to introduce samples into the chromatographic system.

Column preparation

LiChrosorb RP-18 (Merck, Darmstadt, G.F.R.; particle size 5 μm) was packed into a stainless-steel column (125 mm × 4 mm I.D.) using a balance density method via a 10-ml stainless-steel packer at the rate of 800 kg/cm^2 using a Kyowa Seimitsu Ultra High Pressure Pump Type KHW-20. The efficiency of the columns was tested with a standard mixture using sodium nitrate as an unretained marker. The solvent system was methanol—water (80:20, v/v). Under these conditions, the column efficiency was 22,000 theoretical plates for benzene and 20,000 for anthracene.

Hemoperfusion

A Shaldon catheter was inserted into the femoral vein of adult dogs. The perfusion circuit had a priming volume of about 200 ml of 0.9% saline containing 10 units of heparin per ml. The charcoal columns were designed and supplied by Asahi Medical Co. and contained, on average, 200 g of bead-type activated charcoal from petroleum pitch with a 0.5-μm thick collodion coating. BU was administered orally via a stomach tube. The blood flow-rate was maintained at 60 ml/min and perfusion time was 3 h (Fig. 1).

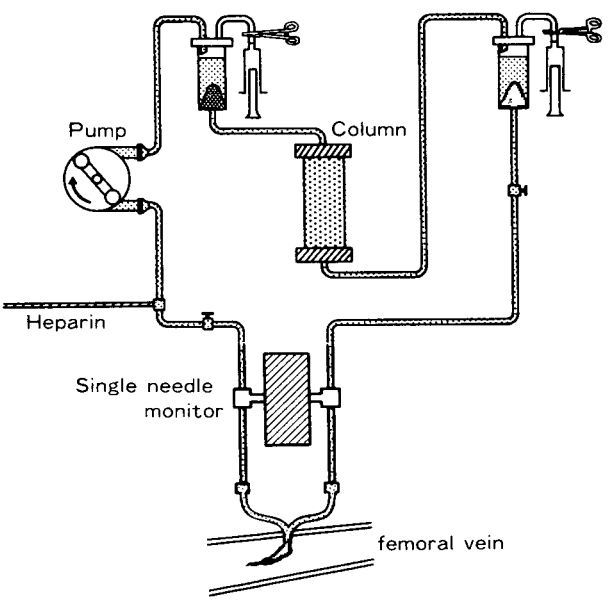

Fig. 1. The hemoperfusion circuit.

Procedure

Serum (0.1—0.5 ml) was transferred to a 10-ml glass-stoppered centrifuge tube and diluted with two volumes of ethanol containing the internal standard 3NPA (2.6 μg/ml) [7]. The serum was mixed with ethanol by stirring on a vortex mixer (60 sec) and ultrasonics (45 W, 38 kHz, 5 min). After centrifugation (1500 *g*, 5 min), a 50-μl aliquot of the upper phase was injected into the HPLC system.

A standard graph was prepared by adding BU to "blank" (no drug) dog serum so that the final concentration of BU in serum was 10—500 μg/ml.

RESULTS AND DISCUSSION

To determine the optimum extracting solvent for BU in serum, the chromatographic profile and the peak height ratio of BU/3NPA were measured on 5-μm LiChrosorb RP-18 columns with methanol, ethanol or acetonitrile. The peak height ratios for these solvents were 3.62, 3.78 and 2.42, respectively. Consequently, ethanol was chosen as the optimum extracting solvent for BU in serum.

A liquid chromatogram from dog serum 4 h after oral administration of BU is shown in Fig. 2, which corresponds to the experiment with 150 mg/kg BU in Fig. 3. BU and 3NPA eluted from the columns gave symmetrical peaks

Fig. 2. Liquid chromatogram of bromural (BU) extracted from dog serum. Operating conditions; packing, LiChrosorb RP-18 (5 μm); column, 125 mm × 4 mm I.D.; mobile phase, acetonitrile—water (1:3, v/v); column temperature, ambient; flow-rate, 0.38 ml/min (80 kg/cm²); detection, 210 nm; sensitivity, 0.04 a.u.f.s.; injection volume, 50 μl. Peaks: a = BU; b = 3-nitro-*p*-anisidine (I.S.).

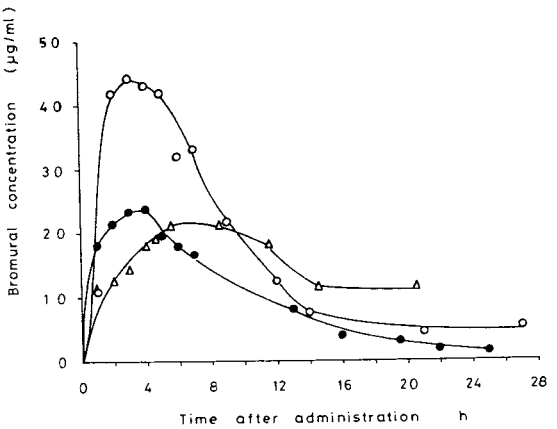

Fig. 3. Time courses of bromural (BU) concentration in serum after oral administration of BU to dog. ○——○, BU 220 mg/kg; △——△, BU 220 mg/kg; ●——●, BU 150 mg/kg.

with retention times of 11 and 22.5 min, respectively. Blanks were prepared from serum of BU-free subjects, and no interfering peaks were observed at the retention times of the compounds of interest.

The calibration curve of detector response vs. BU in serum was linear over the range 0.5—25 μg. The extraction of BU from serum was substantially complete within the 10—500 μg/ml range, and the reproducibility of the assay was good down to a concentration of 10 μg/ml of serum. The mean recovery from nine samples containing 10—500 μg of BU per ml was 98.3% (C.V. 1.85%).

In the experimental case without hemoperfusion, the serum level of BU in dog reached a maximum 4 h after oral administration and decreased for 24 h, except in the case of one dog administered 220 mg/kg (△ in Fig. 3), which died 21 h after the oral administration of BU.

In the experimental case with hemoperfusion, the serum level of Bu in dog was decreased dramatically during hemoperfusion (Fig. 4). A serum level rebound was observed after the end of the hemoperfusion. This suggests that the phenomenon is primarily a delayed gastrointestinal absorption.

Fig. 5 shows comparative chromatograms of BU extracted from a female patient suffering from acute BU intoxication.

CONCLUSION

The HPLC method described permits the rapid determination of BU in serum down to 10 μl/ml. The preparation of serum samples prior to chromatography is simple, only requiring a single-step extraction. No derivatization procedure is necessary. The precision of the method is good and no interfering peaks are seen with serum. Only a small sample size is needed for analysis, and this method is therefore suitable for the routine clinical monitoring of serum levels of BU in patients and for use in research studies involving pharmacokinetics and bioavailability.

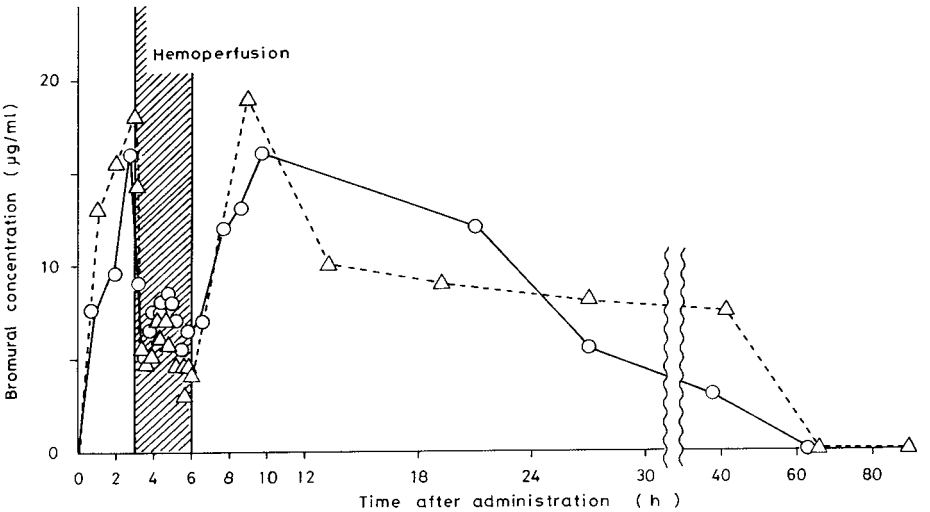

Fig. 4. Time courses of bromural (BU) concentration in serum upon hemoperfusion. △— — —△, BU 200 mg/kg; ○——○, BU 220 mg/kg.

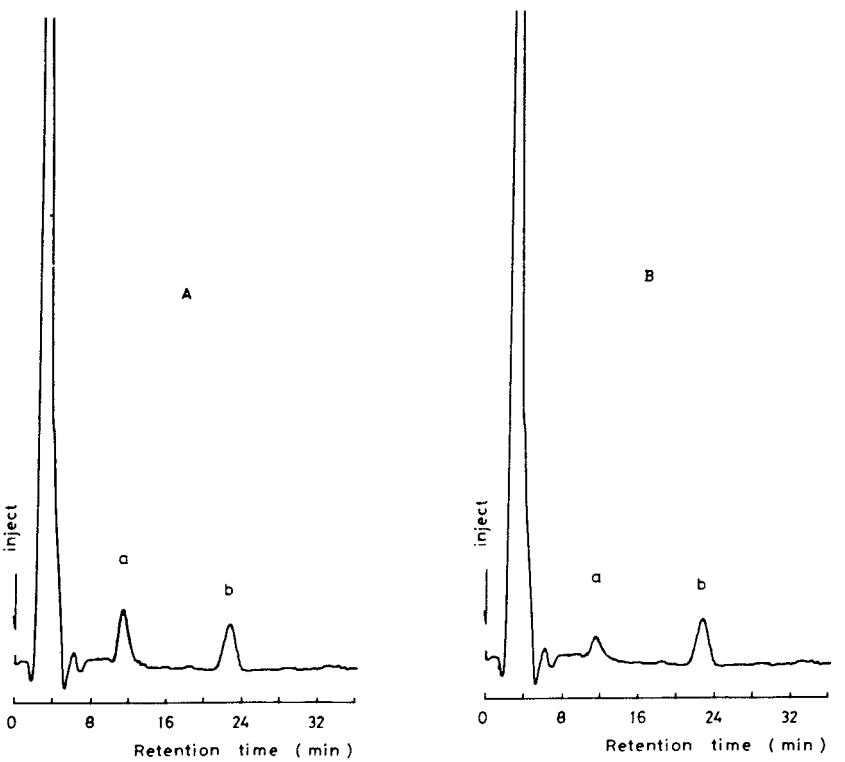

Fig. 5. Comparative chromatograms of bromural (BU) extracted from a female patient suffering acute intoxication. (A) during hemoperfusion, (B) after hemoperfusion. Peaks: a = BU, b = 3-nitro-*p*-anisidine (I.S.). Chromatographic conditions as shown in Fig. 2.

ACKNOWLEDGEMENT

The authors are grateful to Dr. H. Kishimoto for his valuable advice.

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Journal of Chromatography, 223 (1981) 479—483
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 809

Note

Rapid high-performance thin-layer chromatography of salicylic acid, salicylamide, ethoxybenzamide and paracetamol in saliva

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(First received September 23rd, 1980; revised manuscript received December 2nd, 1980)

The determination of salivary drug concentrations is gaining increasing interest for pharmacokinetic studies. For a number of drugs the saliva/plasma ratio is well established. The mechanism as well as determining factors of salivary excretion of drugs have been discussed in detail [1—9]. As salivary drug concentrations are usually lower than plasma concentrations the analytical procedure has to be especially sensitive and should be suitable for routine work.

A large number of papers report the determination of weak analgesics generally with the emphasis on separating the parent drug from its metabolites. Chromatographic methods include thin-layer chromatography (TLC) [10,11], gas chromatography [8], and high-performance liquid chromatography [12—14]. TLC has the advantage of simplicity and allows the simultaneous determination of several samples in one run. With the recent advent of high-performance TLC (HPTLC) a higher precision is attainable than with classical TLC—densitometry and analysis times are considerably shortened [15,16].

TABLE I

COMPARISON OF METHODS FOR ANALYSIS OF SALICYLIC ACID

Reference	Analytical method	Sample	Sensitivity	Reproducibility (%)
10	TLC—densitometry	Tablets	75 μg/spot	1.31
11	TLC—densitometry	Serum	111 μg/spot	4.50
13	HPLC	Plasma	200 μg/ml	3.82
This paper	HPTLC	Saliva	1 μg/ml	13.10
			= 40 ng/spot	5.30

Table I provides a comparison of recent work done on salicylic acid with our results. The HPTLC method proves to be more sensitive; however, the error increases proportionally at lower concentrations, especially in the nanogram range. Consequently, the final judgement should only be made on the basis of work done with saliva which is discussed later in the text.

EXPERIMENTAL

Apparatus and materials

The reference substances — salicylic acid, salicylamide, ethoxybenzamide, paracetamol — all analytical grade, were purchased from Merck (Darmstadt, G.F.R.). All the reagents (chloroform, acetone, ammonium sulfate, formic acid and dichloroethane) were of analytical grade (Merck).

Extraction procedure

The extraction of salicylic acid, salicylamide, ethoxybenzamide, and paracetamol was performed on a Vortex whirlmixer as follows. Two grams of ammonium sulfate and 1 drop of concentrated sulfuric acid were added to 2 g of saliva and mixed for 30 sec. Then 2 ml of chloroform were added; the tube was sealed by a glass stopper and shaken vigorously for 15 min. After centrifugation the aqueous layer was removed and the chloroform was pipetted into a 3-ml conical flask. The tube was washed twice with 0.5 ml of acetone and then added to the chloroform phase. The solvents were evaporated under a stream of nitrogen. A 0.1-ml volume of acetone was pipetted (enzyme pipette) into the flask, dissolving the residue with the aid of the mixer.

Chromatography

Chromatography was performed on 10 cm × 20 cm HPTLC plates coated with silica gel 60 (Merck). Standard and test solutions (2-μl volumes) were applied to the HPTLC plate using a Mikroliter Applicator (Merck) and 2-μl glass capillaries (Merck).

A stock solution in acetone and three dilutions in acetone were prepared weekly and kept cool and in the dark. The stock solution was 1 mg/ml for paracetamol and the dilutions 200, 100 and 25 μg/ml. The stock solutions for salicylic acid, salicylamide and ethoxybenzamide were each 100 μg/ml and the dilutions for each were 20, 10 and 2.5 μg/ml. All dilutions proved to be stable for at least one week. They were used for the standard curve and had to be chromatographed on each plate.

For each plate 19 spots were applied 1 cm apart in the sequence standard—test solution—standard; there were 3 spots for each standard concentration, leaving 10 spots for test solutions. The spot diameter was less than 2 mm. The starting point was kept constant at 1 cm from the edge of the HPTLC plate by means of the Mikroliter Applicator.

The chromatography solvent system consisted of concentrated formic acid—dichloroethane (1:10, v/v) and was suitable for all compounds. The inside of a tank for ascending chromatography (Camag, Muttenz, Switzerland) was lined with filter paper to accelerate saturation which was reached after 45 min.

The solvent system could be used for three plates on the same day at room temperature.

The plates were developed for 9 cm, which corresponds to 20 min, and were allowed to dry in the air for 15 min. The chromatograms were scanned in situ with a PMQ 3 densitometer (Zeiss, Oberkochen, G.F.R.). The spectrophotometer parameters were: slit length 7 mm, slit width 0.7 mm, scanning speed 120 mm/min. The scans were recorded on a Metrawatt RE 647; integration of spot areas (by product of peak height and width at one half the peak height) was performed using a Spectra-Physics Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Calculation of the concentration in each saliva sample was made from the standard curve obtained for each plate. The standard curves for all of the substances were linear, the correlation coefficient being at least $r = 0.998$.

Salicylic acid, salicylamide and ethoxybenzamide were measured by their fluorescence: excitation wavelength 314 nm, emission wavelength 390 nm, filter. Paracetamol was measured by its light absorption at 247 nm, using remission. The R_F values obtained were: paracetamol, 0.13; salicylamide, 0.43; ethoxybenzamide, 0.56; salicylic acid, 0.62.

Blank saliva from different subjects was chromatographed on the same plate with each substance. There was only one spot detectable by UV at 247 nm for an unknown salivary compound. The R_F value of the tiny peak was 0.06; the separation from paracetamol was sharp. While measuring fluorescence no detectable spot from blank saliva over the whole plate could be observed under our photometric conditions.

RESULTS AND DISCUSSIONS

Accuracy

The concentration range for the paracetamol standard curve was 400, 200, 50 ng/spot, 50 ng being the limit of practical sensitivity. The concentration range for salicylic acid, salicylamide, ethoxybenzamide standard curve was 40, 20, 5 ng/spot, 5 ng being the limit of practical sensitivity for salicylic acid, and 2.5 ng the limit of practical sensitivity for salicylamide and ethoxybenzamide.

The accuracy of the scanning measurement was determined as instrument error — one spot was measured three times — and as total instrument error — one standard concentration was measured on six different spots, three times. Table II presents the accuracy data for all standard curves, expressed as the relative standard deviation.

Recovery

Preliminary experiments had indicated the concentration ranges which were to be expected after intake of a particular drug. We prepared saliva samples with blank saliva obtained from different subjects, and added 0.1 ml of acetone containing the drug concentration of interest. To assess the recovery in routine work we decided to prepare and extract two samples each day over a period of six successive days. From twelve saliva samples we calculated our recovery data in the following ranges: paracetamol, 2.5 and 5 μg/

TABLE II

COMPARISON OF THE SCANNING MEASUREMENT TO THE TOTAL INSTRUMENT ERROR

	Concentration (ng/spot)	Instrument error (rel. S.D. %)	Total instrument error (rel. S.D. %)
Paracetamol	400	≤1.0	2.36
	200	≤1.6	2.07
	50	≤6.0	6.3
Salicylic acid	40	≤1.2	5.3
	20	≤3.0	4.0
	5	≤5.0	15.0
Salicylamide	40	≤0.7	1.0
	20	≤1.0	1.35
	5	≤3.0	6.8
Ethoxybenzamide	40	≤0.6	2.74
	20	≤1.2	4.3
	5	≤3.0	4.42

TABLE III

MEAN AND STANDARD DEVIATION OF THE EXTRACTION PROCEDURE FROM SALIVA

Substance	Concentration (μg/ml)	Recovery (%) (n = 12)
Paracetamol	5	$\overline{X}$ = 96.1 S.D. = 11.94 rel. S.D. = 12.4
	2.5	$\overline{X}$ = 84.0 S.D. = 20.88 rel. S.D. = 24.9
Salicylic acid	1	$\overline{X}$ = 74.6 S.D. = 9.8 rel. S.D. = 13.1
	0.5	$\overline{X}$ = 87.0 S.D. = 10.5 rel. S.D. = 12.0
	0.25	$\overline{X}$ = 77.5 S.D. = 15.5 rel. S.D. = 20.0
Salicylamide	0.25	$\overline{X}$ = 78.2 S.D. = 13.6 rel. S.D. = 17.4
Ethoxybenzamide	0.25	$\overline{X}$ = 80.15 S.D. = 6.4 rel. S.D. = 8.0

ml; salicylic acid, 0.25, 0.5, 1 μg/ml; ethoxybenzamide, 0.25 μg/ml; salicylamide, 0.25 μg/ml. The data obtained are presented in Table III. The extraction ranges given here correspond to the average concentrations which were obtained in preliminary experiments after therapeutic doses.

Comparison with data from earlier work done with saliva

While comparing our results with literature data we found that paracetamol had been extracted from saliva in the range 20—5 μg/ml but neither recovery data nor standard deviations were given [2,17].

To our knowledge ethoxybenzamide has not before been extracted from saliva.

Salicylamide has been studied in the range 25—1 μg/ml with an extraction yield of 83%, but no standard deviation was given [8]. Salicylic acid was analyzed in the range 1.2—0.08 μg/ml with standard deviations of 2—25% [1], which is in agreement with our results.

Pohto [7] determined salicylic acid in the range 1.2—0.5 μg/ml by UV measurement at 277 and 300 nm, with better reproducibility compared to our HPTLC method. However, his analytical procedure would be inconvenient for studying salivary drug concentrations after administration of tablets containing additional different drugs.

In conclusion, we believe that the method described here is convenient for bioavailability studies and in routine therapeutic monitoring.

ACKNOWLEDGEMENT

The authors wish to thank Mrs. A. Schulze Elfringhoff for her skillful technical assistance.

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Journal of Chromatography, 223 (1981) 484—485
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 811

Letter to the Editor

Comments to the article "Improved gas chromatographic method of determining diclofenac in plasma"

Sir,

We have been engaged in the measurement of diclofenac in human plasma, using the method of Geiger et al. [1], for over four years and were naturally interested in a recent claim by Ikeda et al. [2] of an improvement in the method. However, after a very careful study of the paper, we feel that the following comments are necessary:

(1) The "improvement" reported is based on a claim that a three-fold increase in sensitivity can be obtained by the addition of methanol to the derivatization medium, trifluoroethanol (TFE) containing 0.5% sulphuric acid. On the basis that this mixture resulted predominantly in the methyl ester rather than the indolone, they then completely replaced trifluoroethanol with methanol or ethanol in 0.1 or 0.5% sulphuric acid. Since Geiger et al. demonstrated almost 100% conversion to the indolone, we find the claim of Ikeda et al. difficult to understand. It is hard to imagine that the detector response to the ester should be three times that of the indolone.

We compared derivatization with TFE, TFE + methanol and methanol alone, each containing 0.5% sulphuric acid. The results are presented in Table I. An internal standard, CGP 4287, was used and this mimics closely the partition

TABLE I

MEAN PEAK HEIGHT RATIO (DICLOFENAC/CGP 4287) AND ABSOLUTE PEAK HEIGHT OF DICLOFENAC AFTER THE DERIVATIZATION PROCEDURES DISCUSSED IN THE TEXT

n = 10.

Derivatization procedure	Peak height ratio (mean ± S.D.)	Peak height of diclofenac (mm)
Trifluoroethanol	1.089 ± 0.14	70
Trifluoroethanol + methanol	0.954 ± 0.08	69
Trifluoroethanol	0.811 ± 0.07	90
Methanol	0.951 ± 0.12	92

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characteristics of diclofenac and is derivatized in the same way. For this reason we have presented our results as peak height ratios but we have also included the absolute peak height of diclofenac. Ikeda et al. use aldrin as a gas chromatography standard added immediately before injection. It can be seen that neither of the proposed modifications resulted in increased sensitivity.

(2) Ikeda et al. do not report measurements below 100 ng/ml while Geiger et al. claim that their method is capable of measuring diclofenac down to 2 ng/ml. After a 25-mg oral dose in man, measurement below 100 ng/ml is required for pharmacokinetic studies.

(3) The only modification we have found necessary during our four years' experience with the method of Geiger et al. is the substitution of *n*-heptane for benzene because of the carcinogenic hazard associated with the latter. We are surprised that Ikeda et al. did not consider this worthwhile.

In conclusion, we feel strongly that papers with the word "improved" in their titles should be scrutinized very carefully, before publication, in order to ensure that there is, indeed, a significant improvement before being allowed to become part of the established literature.

NOTE ADDED IN PROOF

After having considered additional information from Ikeda et al. and after further investigations of our own we should like to add the following to our comments:

(A) Derivatization of diclofenac with methanol in 0.1% sulphuric acid results in the methyl ester which, with our equipment, produces an increased response (66%) from the electron-capture detector.

(B) The use of "old", rather than fresh, potassium hydrogen carbonate, results in conversion of the ester to the indolone with a consequent reduction in peak height (under our conditions the retention times of methyl ester and indolone are identical). "Old" bicarbonate is considerably more alkaline than "fresh" due to the formation of carbonate on standing. We understand that Ikeda et al. now favour washing the methyl ester with water.

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(Received August 28th, 1980)

Journal of Chromatography, 223 (1981) 486—491
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 812

Letter to the Editor

Supplementary data for improved gas chromatographic method of determining diclofenac in plasma — Behavior of the methyl ester and the indolone derivative of diclofenac in gas—liquid chromatography with electron-capture detection

Sir,

Geiger et al. [1] reported a method for determining diclofenac, an anti-inflammatory and antirheumatic agent. Their method consists of derivatizing the compound into its indolone and analyzing the derivative by gas—liquid chromatography. We have presented a different method for determination of diclofenac [2], which utilizes derivatization of diclofenac into its methyl and ethyl esters, and have shown that the method has a three-fold higher sensitivity than the indolone method.

We have now made a precise comparison between these two methods in terms of the rate of derivatization, the efficiency of extraction of the derivative into organic solvents, and the stability of the derivatives during the analytical procedures.

METHODS

Synthesis of diclofenac derivatives

The methyl ester and the indolone derivatives of diclofenac were synthesized as follows.

For the methyl ester formation, diclofenac (200 mg) was mixed with 10 ml of methanol containing 0.5% sulfuric acid and the mixture was heated at 60°C for 1 h. For the indolone compound formation, diclofenac (200 mg) was added to 10 ml of trifluoroethanol (TFE) containing 0.5% sulfuric acid, and heated at 75°C for 75 min. After heating, each reaction mixture was added to 30 ml of water and the reaction products were extracted with *n*-hexane (3 × 50 ml for each compound). The *n*-hexane layers were dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residues were crystallized from methanol—water (2:1, v/v), and recrystallized from methanol. The ester and the indolone derivative proved to be analytically pure (white prisms and pale yellow prisms, respectively). Analytical data are summarized in Table I.

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TABLE I

PROPERTIES OF THE METHYL ESTER AND THE INDOLONE DERIVATIVE OF DICLOFENAC

		Methyl ester	Indolone derivative
m.p.*		107—108°C	127—128°C
R_F value**		0.78	0.33
Elemental analysis			
	C found	57.93%	60.51%
	calc.	58.09%	60.46%
	H found	4.06%	3.19%
	calc.	4.22%	3.26%
	N found	4.34%	4.96%
	calc.	4.52%	5.04%
m/e (M^+)		310	278

*The melting point of the methyl ester is reported as 101—102°C [3, 4] and 103—104°C [5], but that of the indolone derivative is not given [6].

**Thin-layer chromatography on silica gel was conducted using the solvent *n*-hexane—chloroform (4:1) and each spot was visualized by UV absorption.

Derivatization of diclofenac and gas—liquid chromatography of derivatives

Derivatization of diclofenac was conducted in the manner described in the previous paper [2] for the methylation, and according to the method of Geiger et al. [1] for the indolone formation. Briefly, a methanol solution (10 μl) of diclofenac (1 μg) was placed in an ampoule, and methanol was evaporated at about 40°C under a gentle stream of nitrogen. The dried residue was dissolved in 0.15 ml of TFE containing 0.5% sulfuric acid (method A), 0.15 ml of TFE containing 0.5% sulfuric acid and 0.05 ml of methanol (method B), or 0.15 ml of methanol containing 0.5% sulfuric acid (method C). After the ampoule was sealed, the mixture was reacted in a water bath at 75°C for 75 min for method A, or at 60°C for 1 h for methods B and C. After reaction, the ampoule was opened, and 0.4 ml of 25% potassium hydrogen carbonate solution and 2 ml of *n*-hexane were added. The mixture was shaken on a Vortex mixer, and a 1-μl aliquot was injected into the gas chromatograph, which was operated as previously described [2] except that the column oven temperature was maintained at 250°C instead of 260°C.

Rate of conversion to the derivatives and the extraction efficiency

The rate of conversion to the derivatives and the extraction efficiency by the above three methods were examined. Three different systems of experiments were conducted simultaneously. First, diclofenac (1 μg or 3.14 nmol) was derivatized by the above three methods and analyzed as described above. Second, a methanol solution (10 μl) of the authentic indolone derivative (1 μg or 3.60 nmol) was added to a mixture of TFE—0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium hydrogen carbonate (0.4 ml), and a methanol solution (10 μl) of the authentic methyl ester (1 μg or 3.23 nmol) was added to a mixture of TFE—0.5% sulfuric acid (0.15 ml), methanol (0.05 ml) and 25%

aqueous potassium hydrogen carbonate (0.4 ml), or a mixture of methanol–0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium hydrogen carbonate (0.4 ml). The derivatives in the mixtures were extracted with 2 ml of *n*-hexane, and 1-μl aliquots of the *n*-hexane layer were subjected to gas–liquid chromatography. Third, *n*-hexane solutions containing 500 ppb of the authentic indolone derivatives and the authentic methyl ester were prepared, and 1-μl aliquots were injected into the gas chromatograph.

The rate of conversion to the derivatives can be calculated by dividing the respective values obtained from the first experiment by the values from the second, which were multiplied by 3.14/3.60 for the indolone and 3.14/3.23 for the methyl ester.

The extraction efficiency can be calculated by dividing the values from the second experiment by the values from the third.

Stability of the derivatives in an alkaline medium

A methanol solution (10 μl) of the authentic methyl ester (1 μg) was added to a mixture of methanol–0.5% sulfuric acid (0.15 ml) and water (0.4 ml), a mixture of methanol–0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium hydrogen carbonate (0.4 ml), a mixture of methanol–0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium carbonate (0.4 ml), or a mixture of methanol–0.5% sulfuric acid (0.15 ml) and aqueous potassium hydroxide (0.4 ml). A set of the mixtures was prepared, and they were allowed to stand at 20°C. At desired periods, the mixtures were extracted with 2 ml of *n*-hexane, and 1 μl of the *n*-hexane layer was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Electron-capture detector response to the authentic derivatives

n-Hexane solutions containing 50, 100 and 200 ppb of the authentic methyl ester and the authentic indolone derivative were prepared, and 1-μl aliquots were injected into the gas chromatograph. Results obtained are shown in Table II, and indicate that the methyl ester gives 1.95 times (by peak height), or 2.25 times (by peak area), more sensitive responses than the indolone derivative. When the sensitivity is compared on a molar basis, the methyl ester is 2.2 times (by peak height), or 2.4 times (by peak area), more sensitive than the indolone derivative.

Rate of conversion to the derivatives and the extraction efficiency

Using the authentic derivatives, we examined the rate of conversion to the derivatives and the extraction efficiency under the standard derivatization conditions. The results are summarized in Table III, and indicate that diclofenac was quantitatively methylated by method C and in a 95% yield by method B, while the conversion to the indolone derivative occurred in a 76% yield by method A. On the other hand, the extraction efficiency with *n*-hexane was almost 100% by methods A and C, but was about 94% by method B.

By dividing the difference in the electron-capture detector response (2.2 by peak height) by the rate of conversion to the indolone (0.76), it is clear that

TABLE II

ELECTRON-CAPTURE DETECTOR RESPONSE TO THE AUTHENTIC METHYL ESTER AND THE AUTHENTIC INDOLONE DERIVATIVE

All values are expressed as mean ± S.D. (n = 5).

Compound	Concentration (ppb)	Peak area (cm^2)	Peak height (cm)
Methyl ester	50		18.5 ± 0.07* (20.6)***
	100		6.4 ± 0.03** (7.1)***
	200	2.0 ± 0.01** (2.23)***	14.0 ± 0.06** (15.6)
Indolone derivative	50		9.5 ± 0.2*
	100		3.3 ± 0.1**
	200	0.89 ± 0.02**	7.1 ± 0.2**

*The detector was operated with a pulse-rate of 2.5 kHz and the electrometer setting was kept at range 10^2 and attenuation 4.
**The detector was operated with a pulse-rate of 10 kHz and the electrometer setting was kept at range 10^2 and attenuation 8.
***The values were calculated on a molar basis; namely, the values marked * or ** were multiplied by 310/278.

TABLE III

THE RATE OF CONVERSION OF DICLOFENAC TO THE METHYL ESTER OR THE INDOLONE DERIVATIVE AND THE EXTRACTION EFFICIENCY OF THE DERIVATIVES

All values are expressed as mean ± S.D. (n = 5). The detector was operated with a pulse-rate of 10 kHz, and the electrometer setting was kept at range 10^2 and attenuation 16.

Method*	Peak height (cm)	Peak height (cm) of authentic samples (500 ppb)		Rate of conversion (%)	Rate of extraction (%)
		With extraction	Without extraction		
A	5.81 ± 0.46**	7.65 ± 0.09***	7.68 ± 0.10	76.0	99.6
B	13.8 ± 0.3**	14.5 ± 0.2***		95.2	94.2
C	14.8 ± 0.3**	15.3 ± 0.2***	15.4 ± 0.1	99.3	99.4

*Derivatization methods A, B and C are defined in the text (A, TFE—H_2SO_4; B, TFE—methanol—H_2SO_4; C, methanol—H_2SO_4).
**Each 1 μg of diclofenac was derivatized by method A, B or C, and analyzed as described in Methods. All values were corrected for molar concentration of the authentic samples.
***A methanol solution (10 μl) of the authentic methyl ester (1 μg) or the indolone derivative (1 μg) was treated according to methods B and C for the methyl ester or method A for the indolone, except that the heating at 60°C for 1 h or at 75°C for 75 min was omitted. Details are given in Methods.

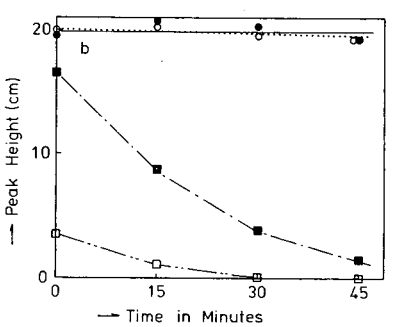
method C should be about three times more sensitive than method A. This figure completely agrees with the results demonstrated in the previous paper [2].

Stability of the derivatives in an alkaline medium

We incidentally noticed that the peak height of the methyl ester obtained by method C decreased with the passage of time when the ester was allowed to stand prior to extraction in 0.4 ml of 25% aqueous potassium hydrogen carbonate prepared a month previously. We were interested in this phenomenon, and examined the stability of the authentic methyl ester in an alkaline medium. The result is shown in Fig. 1, which indicates that the methyl ester apparently decomposed under alkaline conditions of potassium carbonate or potassium hydroxide, but not under alkaline conditions of potassium hydrogen carbonate freshly prepared. The indolone derivative formed by method A, however, was stable in an alkaline medium of potassium hydrogen carbonate whether the potassium hydrogen carbonate reagent was prepared a month previously or was freshly prepared (data not shown). Therefore, it is recommended that 0.4 ml of water instead of 25% potassium hydrogen carbonate in the previous report should be added to the reaction mixture in method B or C.

Measurements of diclofenac below 100 ppb

In the previous paper [2] measurements of diclofenac below 100 ppb were not reported. However, measurements below 100 ppb are required for pharma-



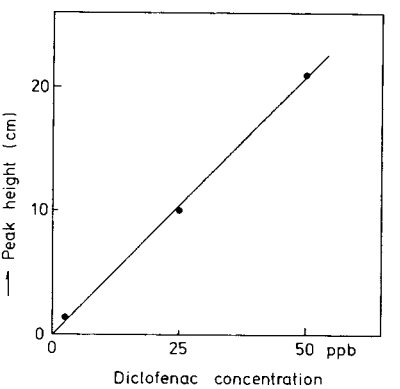


Fig. 1. Time course of decomposition of the authentic methyl ester under various alkaline conditions as described in Methods. (•) $KHCO_3$, (▪) K_2CO_3, (□) KOH, (○) water. The detector was operated with a pulse-rate of 10 kHz, and the electrometer setting was kept at range 10^2 and attenuation 8.

Fig. 2. Standard curve of diclofenac after derivatization with methanol—H_2SO_4. The standard curve was prepared as described in the previous paper [2] using 2.5, 25 and 50 ng of diclofenac, except that the derivative was extracted with 1 ml of *n*-hexane instead of 2 ml in the previous method [2] after the addition of 0.4 ml of water instead of 0.4 ml of aqueous 25% $KHCO_3$. A 1-μl aliquot of the *n*-hexane layer was injected into the gas chromatograph. The detector was operated with a pulse-rate of 2.5 kHz, and the electrometer setting was kept at range 10^2 and attenuation 4.

cokinetic studies after a usual 25-mg oral dose in man. Therefore, measurements below 100 ppb were attempted by method C. For this purpose, the detector was operated with a pulse-rate of 2.5 kHz instead of 10 kHz. The former gives about a three-fold increase in sensitivity over the latter. A typical standard curve is shown in Fig. 2. This shows that diclofenac below 1 ng in 1 ml of plasma can be measured by this method.

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(Received Novermber 24th, 1980)

Related articles published in *Journal of Chromatography*, Vols. 207 and 208

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Errata

J. Chromatogr., 222 (1981) 324—328
Page 324, authors' names, "Polona Vovopivec" should read "Polona Vodopivec".

J. Chromatogr., 222 (1981) 478—481
Page 478, authors' names, "R. Skobolo" should read "R. Skoblo".

Vol. 223, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETING

STRATEGY IN DRUG RESEARCH, THE 2nd NOORDWIJKERHOUT IUPAC–IUPHAR SYMPOSIUM

The 2nd IUPAC–IUPHAR symposium will be held in Noordwijkerhout, The Netherlands, at the Leeuwenhorst Congress Center on August 25–28, 1981. There will be 14 lectures spread over the four days of the symposium concerning the topics: Receptor Studies and Research Strategy; Lessons from Enzyme Chemistry; Toxicological Parameters as a Lead; Pharmacokinetics as a Starting Point; and Biological Measurements: Methods and Data Handling. The language of the program will be English. The proceedings will be published by Elsevier Scientific Publishing Company in the book series, Pharmacochemistry Library.

For further information contact the Symposium secretariat: c/o Merck Sharp & Dohme B.V., Professional and Government Liaison, Waarderweg 39, P.O. Box 581, 2003 PC Haarlem, The Netherlands. Tel: 023-31 93 30, extension 2217. Telex: 41019.

CALENDAR OF FORTHCOMING EVENTS

May 11–15, 1981 Avignon, France	**5th International Symposium on Column Liquid Chromatography** Contact: Professor G. Guiochon, Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, Route de Saclay, 91128 Palaiseau, France. (Complete program published in Vol. 208, No. 2)
June 16–17, 1981 Venice, Italy	**1st International Symposium on Chromatography in Biochemistry, Medicine and Environmental Research** Contact: Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546. (Further details published in Vol. 198, No. 3)
June 18–19, 1981 Venice, Italy	**8th International Symposium on Mass Spectrometry in Biochemistry, Medicine and Environmental Research** Contact: Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546.

June 22–26, 1981 Veldhoven, The Netherlands	**4th International Symposium on Affinity Chromatography and Related Techniques** Contact: Secretariat, 4th Int. Symp. on Affinity Chromatography and Related Techniques, Department of Organic Chemistry, Faculty of Sciences, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands, (Further details published in Vol. 222, No. 2).
June 28–July 3, 1981 Kiryat Anavim, Israel	**The Sixth International Symposium on Bioelectrochemistry and Bioenergetics** Contact: Aharon Katzir-Katchalsky Center, Weizmann Institute of Science, Rehovot, Israel.
July 22–24, 1981 Manchester, Great Britain	**Biochemical Society Annual General Meeting and Joint Society/Nucleotide and Nucleic Acid Group Colloquium on Biochemical Interactions of Plasmids with their Hosts** Contact: The Biochemical Society, 7 Warwick Court, High Holborn, London WC1R 5DP, Great Britain.
Aug. 25–28, 1981 Noordwijkerhout, The Netherlands	**2nd Noordwijkerhout IUPAC–IUPHAR Symposium "Strategy in Drug Research"** Contact: Secretariat 2nd Noordwijkerhout IUPAC/IUPHAR Symposium, c/o Merck Sharp & Dohme B.V., Professional and Government Liaison, Waarderweg 39, P.O. Box 581, 2003 PC Haarlem, The Netherlands.
Sept. 1–4, 1981 Siofok, Hungary	**3rd Danube Symposium on Chromatography** Contact: Hungarian Chemical Society, H-1368 Budapest, P.O.B. 240, Hungary. Tel. Budapest 427–343. (Further details published in Vol. 189, No. 2).
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749-2623. (Further details published in Vol. 222, No. 2)
Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC '81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 26 20 20.

MANUFACTURERS' LITERATURE

N-1552

BOOKLET ON PROTEIN AND PEPTIDE SEPARATIONS

The Varian Instrument Group has published a booklet showing the protein chemist how to make rapid protein and peptide separations using high-performance liquid chromatography. The separations are described in three modes: steric exclusion, ion-exchange and reversed-phase chromatography. The system configurations presented are based on the Varian Series 5000 liquid chromatographs.

N-1553

DATA SHEET ON HYDROLYZATE RUN

Beckman Instruments has published a data sheet entitled "New one-hour hydrolyzate run for the Model 119CL amino acid analyzer". This data sheet describes a 1-h single-column hydrolyzate method for the Beckman Model 119CL amino acid analyzer. It contains a sample chromatogram, the operating parameters, information about a new buffer and a reference to further technical information.

N-1558

SEPARATION NEWS, 7, 1980

This issue of Separation News, a publication of Pharmacia Fine Chemicals AB, discusses protein separation and characterization techniques and presents a step-by-step guide to planning and evaluating a protein purification scheme. The following items are discussed: aims and criteria, preliminary knowledge and planning, the initial purification step, selective purification steps, checking the progress of the purification, and further purification and optimization.

N-1559

REAGENZIEN, DIAGNOSTICA, CHEMIKALIEN 1981

Reagenzien, Diagnostica, Chemikalien 1981 is the title of the 1981 catalogue of E. Merck, Darmstadt. The catalogue gives information on the Merck products in the field of reagents, diagnostics and chemicals for general use. The catalogue contains subject numbers, names, the size of the package and the price in German marks, and information on ordering and delivery.

N-1561

QUICK TRIGLYCERIDE ANALYSIS

A new, simple HPLC procedure for analyzing mixtures of triglycerides is described in Bulletin 787, recently published by Supelco. Until recently, analysts who used HPLC had to separate triglyceride mixtures in two steps. Each sample had to be fractionated by degree of unsaturation through argentation chromatography or by acyl chain length through reversed-phase HPLC. The triglycerides in each fraction then were separated through the alternate process. The number of separations which had to be performed increased as the range in acyl chain length and degree of unsaturation increased in a triglyceride mixture. Now triglycerides can be separated by degree of unsaturation and acyl chain length in one step, by elution from Supelcosil LC-18 columns with a non-aqueous mobile phase. The quality of the separations achieved by with this method is equal to that obtained with the slower two-step method.

N-1562

NEW QUARTERLY BULLETIN

Marine Colloids has recently published the first issue of SeaNotes, an eight-page quarterly bulletin devoted to advances in electrophoresis, immunology, molecular biology, microbiology and related sciences. Along with features and articles prepared by specialists in their disciplines, SeaNotes will detail in each issue, eight or ten abstracts of the most significant papers and articles presented in each quarter.

N-1564

CBS 46, OCTOBER 1980

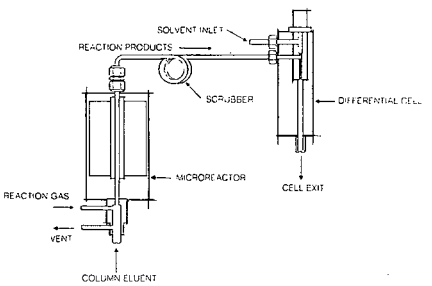
CAMAG's Bibliography Service, October 1980, has eight pages of literature references on the field of thin-layer chromatography. In the October 1980 issue CAMAG describes the Nanomat versatile sample application system, suitable for both conventional and

high-performance thin-layer chromatography, and a procedure for quantitative trace analysis by high-performance thin-layer chromatography.

N-1565

BROCHURE ON HALL DETECTOR

A brochure is available from Tracor Instruments, Inc. describing the 700A Hall Electrolytic Conductivity Detector for gas chromatography. This eight-page bulletin describes the principle of operation, the four operational modes with actual application samples for each mode. The detector can be made selective for either nitrogen-, halogen-, sulfur- or nitrosamine-containing compounds.



N-1566

HALL DETECTION OF NITROSAMINES

Zelda Penton, of the Instrument Division of Varian, has recently published a study "Analysis of Volatile Nitrosamines with the Hall Detector" in the series "GC at Work". This application note gives procedures for using the Hall electrolytic conductivity detector with a Varian Model 3700 gas chromatograph to measure nitrosamines. The minimum detectable quantity of N,N-dimethylnitrosamine using this procedure was found to be less than 40 pg.

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

NEW PRODUCTS

N-1556

PREPARATIVE ELECTROPHORESIS SYSTEM

The Macro-Page preparative electrophoresis system from Birchover Instruments Ltd., is now available with tank systems based on the designs of Brownstone for the separation of proteins and enzymes, and of Southern for the separation of DNA and RNA genetic material, using polyacrylamide or agarose gels in a wide range of concentrations.

Bands of separated material pass out from the gel into a sample collector chamber which is emptied and refilled with clean buffer on an intermittent, variable time collection base to prevent excessive dilution and to optimise resolution. In combination with a fraction collector the Macro-Page forms an automatic system for the collection of separated material.

N-1557

DOUBLE SLAB CELL

Bio-Rad's Protean double slab electrophoresis cell is now available with interchangeable 16- and 32-cm-high lower buffer chambers. The 16-cm buffer chamber offers the user the possibility to work with gel slabs 5, 12 or 16 cm long. The larger chamber allows the use of 32-cm slabs. Adaptions for agarose gel electrophoresis, preperative gel electrophoresis, DNA sequencing and other long-gel applications are available with the cell.

N-1563

LOW GELLING TEMPERATURE AGAROSE

SeaPlaque agarose is a low-gelling temperature agarose. A 1% solution will remain fluid at 37°C for at least 24 h. It will then set to a reasonably firm gel in about 10 min at 25°C and once gelled, will not remelt unless the temperature exceeds 65°C. SeaPlaque agarose offer practical advantages to workers in the fields of molecular biology, microbiology, cell biology, immunology and protein biochemistry.

PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	The publication schedule for further issues will be published later.					
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1						

INFORMATION FOR AUTHORS

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

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

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

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

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

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ELECTROPHORESIS

A SURVEY OF TECHNIQUES AND APPLICATIONS

Part A: Techniques

Z. DEYL, Czechoslovak Academy of Sciences, Prague (editor)
F. M. EVERAERTS, Z. PRUSÍK, and P. J. SVENDSEN (co-editors)

JOURNAL OF CHROMATOGRAPHY LIBRARY 18

This first volume in a two part set, deals with the principles, theory and instrumentation of modern electromigration methods. The second volume will be concerned with details of applications of electromigration methods to diverse categories of compounds, although a few applications are already discussed in Part A.

Some electromigration methods have become standard procedures because of their extensive use in analytical and preparative separations. These are discussed together with newer developments in the field. Hints are included to help the reader to overcome difficulties frequently arising from the lack of suitable equipment. Adequate theoretical background of the individual techniques is included. A theoretical approach to the deteriorative processes is presented in order to facilitate further development of a particular technique and its application to a special problem.

In each chapter practical realizations of different techniques are discussed and examples are presented to demonstrate the limits of each method. The mathematical and physicochemical background is arranged so as to make it as coherent as possible for both non-professionals such as post-graduate students, and experts using electromigration techniques.

1979 xvi + 492 pages US $83.00/Dfl. 170.00 ISBN: 0-444-41721-4

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