ISSN 0378-4347

VOL. 181 NO. 1 JANUARY 11, 1980
(Biomedical Applications, Vol. 7, No. 1)

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	The publication schedule for further issues will be published later.									
Chromatographic Reviews			184/1										
Biomedical Applications		181/1	181/2	181/3	181/4	182/1	182/2	182/3	182/4	183/1	183/2	183/3	183/4

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Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 22 volumes in 1980. The subscription price for 1980 (Vols. 181–202) is Dfl. 2838.00 plus Dfl. 352.00 (postage) (total *ca.* US$ 1556.00). The subscription price for the *Biomedical Applications* section only (Vols. 181–183) is Dfl. 399.00 plus Dfl. 48.00 (postage) (total *ca.* US$ 218.00). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 180) are available at Dfl. 140.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

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JOURNAL
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CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

VOL. 7 1980

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands

Journal of Chromatography, 181 (1980) 1—10
Biomedical Applications

CHROMBIO. 434

MODIFICATION OF ASSAYS FOR THE ROUTINE ANALYSIS OF 3-METHOXY-4-HYDROXYPHENYLGLYCOL IN URINE BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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(First received May 7th, 1979; revised manuscript received September 13th, 1979)

SUMMARY

Urinary 3-methoxy-4-hydroxyphenylglycol has been reliably assayed on a routine basis using an electron-capture detector method. Modifications of previous procedures include simplification of extraction and derivatization of urine, inclusion of an internal standard, prevention of losses during concentration, use of each urine as its own standard, and better chromatographic resolution by lengthening of columns and programming temperature. The assay shows a coefficient of variation of 3.1%.

INTRODUCTION

3-Methoxy-4-hydroxyphenylglycol (MHPG) was first measured with electron-capture gas chromatography by using trifluoroacetyl derivatives that enhanced the sensitivity and resolution of the analysis by Wilk et al. [1]. Their use of an AG 1-X4-chloride ion-exchange column removed freed phenolic acids, but introduced a 30% loss of MHPG. Dekirmenjian and Maas [2] simplified the procedure, and investigated many of the variables that contributed to the irreproducibility of the assay. Karoum et al. [3] utilized tryptophol as a ubiquitous internal standard with 3-methoxy-4-hydroxyphenethanol (MOPET) and MHPG added to one aliquot of a duplicate sample. Sharpless [4] has analyzed MHPG as the heptafluorobutyrate derivative and determined that there is no significant difference between MHPG in acidified and non-acidified urine that had been repeatedly thawed and refrozen during 10 months of storage.

The present assay is based on the method developed by Dekirmenjian and Maas [2]. Modifications were made in the initial sample size, transfer and derivatization procedure, use of an internal standard, and addition of isoamyl alcohol to prevent loss of MHPG during evaporative concentration. Each

urine sample is made to serve as its own standard curve, compensating for variations in extraction, hydrolytic and derivative losses, and instrument response.

EXPERIMENTAL

Materials

The di-MHPG piperazine salt, 3-methoxy-4-hydroxyphenethanol (MOPET) and β-glucuronidase—aryl sulfatase enzyme mixture (Cat. No. 34742) were obtained from Calbiochem (Los Angeles, Calif., U.S.A.). The (3-methoxy-4-sulfonyloxyphenyl)-glycol potassium salt (MHPG-SO_4) was obtained from Tridom Chemical (Hauppauge, N.Y., U.S.A.).

"Nanograde" ethyl acetate obtained from Mallinckrodt (St. Louis, Mo., U.S.A.) was distilled just prior to use and stored in the dark. Trifluoroacetic anhydride (TFAA) was obtained from Pierce (Rockford, Ill., U.S.A.). A solution of 1.0 *M*, pH 6.0 acetate buffer was mixed with a solution of 2% EDTA in a ratio of 2:1.

The gas chromatograph was a Model 5713A Hewlett-Packard equipped with an electron-capture detector. An on-column injection configuration 12 ft. × 2.0 mm I.D. glass column was used, packed with 1.5% OV-225 on 100—120 mesh Chromosorb G AW DMCS from Applied Science Labs. (State College, Pa., U.S.A.). A Hewlett-Packard automated sample injector Model 7671A was added to the chromatograph, allowing the overnight use of extended temperature programming from 130° to 190° at 1°/min. The injection port was maintained at 250°, and the detector at 350°. The carrier gas was 5% methane in argon, flow-rate 30 ml/min. The electron-capture detector contains 15 mCi of internally plated ^{63}Ni.

Methods

Portions of the measured urine samples (20 ml) were stored frozen at −20° in glass screw-capped vials (scintillation vials) without any added preservative. For assay, four 1-ml aliquots of a urine sample are placed in 30-ml tubes with PTFE-lined screw caps. MHPG standard solution (2 mg/100 ml, as the free form) is added to give final concentrations of 0, 1, 2, and 4 μg/ml of urine. MOPET internal standard (200 μl of a 3 mg/100 ml solution) is added to each tube. Each tube also receives 500 μl of buffer-EDTA and 60 μl of enzyme. The final volumes are made constant by adding 200, 150, 100, and 0 μl of saline to the four tubes. A saline standard curve is prepared by substituting 1 ml of saline for the urine sample. The samples are mixed briefly and incubated at 37° for 24 h.

The incubated samples are extracted once with 12 ml of ethyl acetate by reciprocal shaking for 45 min, and briefly centrifuged. Ten milliliters of the ethyl acetate is transferred to a 50-ml round-bottomed flask (14/20 taper) and 1.2 ml of isoamyl alcohol is added. The sample is evaporated just to dryness on a Rinco rotary evaporator connected to a double-trapped vacuum pump. TFAA (0.4 ml) is added and the stoppered flask is kept at 37° for 1 h. The excess TFAA is evaporated with a gentle stream of nitrogen and the residue is distributed in 8 ml of toluene by vortex mixing. After a brief set-

tling period, a portion of the toluene is placed in red septa capped autosampler vials, and 3-μl aliquots are injected into the chromatograph at an attenuation of 512 or 1024. Fig. 1 shows a chromatogram of a urine sample containing additions of 6 μg of MOPET and 1 μg of MHPG per ml of urine.

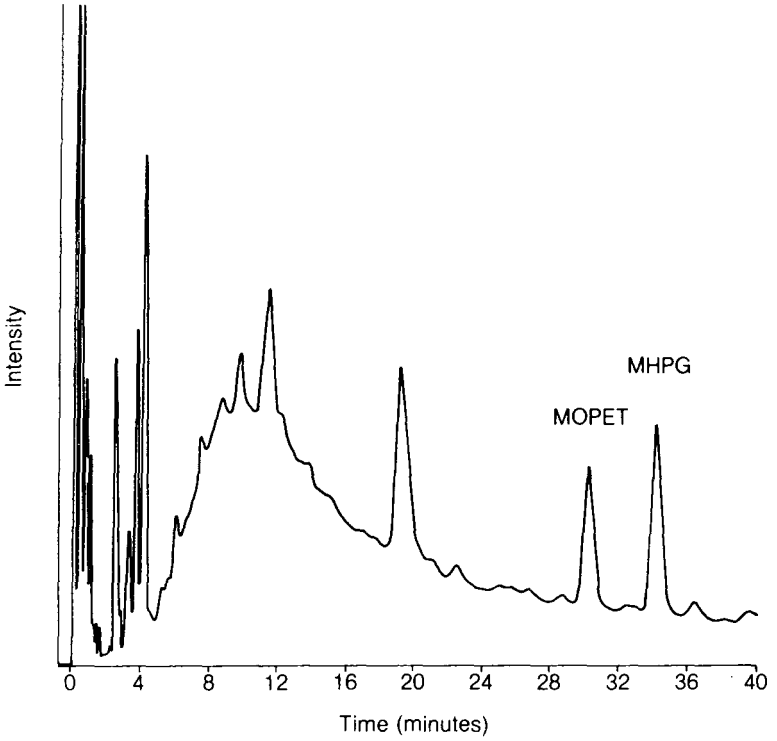

Fig. 1. An electron-capture—gas—liquid chromatogram of the TFAA derivatives of MOPET and MHPG obtained during analysis of urine with an initial 2.6 μg/ml MHPG content and additions of 6 μg/ml of MOPET and 1 μg/ml of MHPG.

A standard curve is obtained for each urine sample, using the four supplemented points. The ratio of peak heights of MHPG to MOPET is plotted against the amount of MHPG (μg) added per milliliter of urine (Fig. 2). The line is extrapolated through the Y-axis to the X-axis, which gives the micrograms of MHPG originally present in the sample per milliliter of urine. This is equivalent to projecting a horizontal line from the Y-intercept to a theoretical standard curve originating at zero, and then down to the X-axis. Fig. 2 is an analysis of a urine sample calculated by linear regression to contain 1.53 μg of MHPG per milliliter of urine.

RESULTS AND DISCUSSION

Assay procedures

There is enough MHPG present in urine to permit the use of only a portion of the organic phase after a single extraction. However, the shaking time was

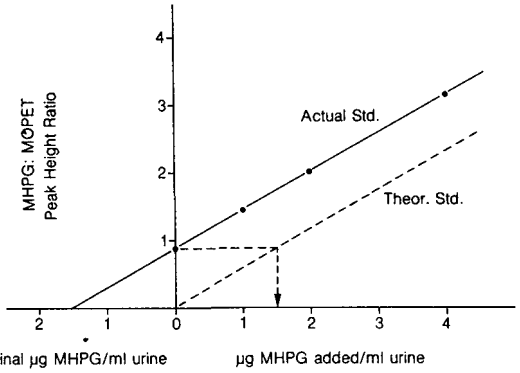

Fig. 2. Standard curve obtained for a urine sample initially containing 1.5 µg/ml of MHPG.

extended to ensure that the MHPG and MOPET had partitioned completely in the two phases. The urine samples often have different thicknesses of gel at the aqueous—organic interface, and transferring less than the total volume of ethyl acetate avoids contact with the gel. Removal of phosphates and sulfates with barium chloride [3, 4] accelerates the enzymatic hydrolysis of MHPG conjugates, but introduces additional steps that can be avoided by using enough enzyme and overnight incubation.

Dekirmenjian and Maas [2] propose a wash of the organic phase with a 1.0 *M* $KHCO_3$ solution to remove interfering acidic components. We found this step removed only chromatographic peaks that were remote from the MHPG and MOPET when using OV-225, and was deemed unnecessary.

Erratic losses in MHPG recovery were traced to the evaporative concentration step. Isoamyl alocohol in heptane was tried as an alternative solvent during the concentration step. The presence of isoamyl alcohol was found to decrease the MHPG losses during evaporation from heptane. The addition of 12% or more isoamyl alcohol to the transferred volume of ethyl acetate also decreased the MHPG losses during evaporation of the ethyl acetate.

Karoum et al. [3] preferred diethyl ether to ethyl acetate because of its better recovery of catecholamine alcoholic metabolites and easier evaporation. We avoided diethyl ether due to difficulty in its quantitative transfer and its potential danger in our hospital-based laboratory.

Direct derivatization of the vacuum dried residue eliminates re-solution and transfer steps. Evaporation of excess TFAA and resuspension in the same flask eliminates the effort of quantitative transfer and dilution to volume. Toluene used in this last dilution dissolves the MHPG—TFAA derivative, but leaves much of the more polar residue behind.

During preliminary studies, individual urine samples presented minor peaks that coincided with the MHPG and MOPET peaks. Slow peaks from previous runs also occasionally carried over into subsequent runs. The 12-ft. polar column and extended temperature programming increased the resolution and eluted most of the long retention-time peaks during each run. Karoum et al. [3] also found a long, polar column necessary (13 ft. 5% OV-210 + 1% SE-52) The temperature programming may be shortened (130°—170°, 1°/min) if overrunning peaks are not interfering.

The standard curve

We have found MOPET to be a readily available internal standard that closely follows the extraction and chromatographic resolution of MHPG. Since each urine sample contains a small but independent amount of MOPET each urine standard curve would have a slightly different MOPET divisor in its ratio calculation. This gives a different slope for each urine sample, and individual urine standard curves cannot be compared to a saline standard curve. However, supplementing the urine with MHPG makes each urine sample serve as its own standard curve and effectively determines the sample in quadruplicate. The curve is self-compensating for possible hydrolytic losses of MHPG during incubation, individual urine variations in partitioning during extraction, degradation of the derivatives, and daily variations in the response of the gas chromatograph.

These variables all change the slope but not the X-intercept. Fig. 3 demonstrates the theoretical effects on the observed slopes of a urine sample if it

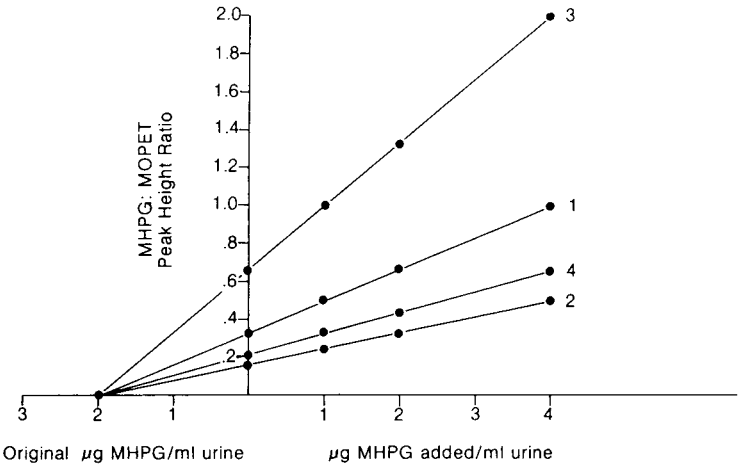

Fig. 3. Theoretical effect of consistent changes in original and added MHPG and MOPET. Chromatographic peak heights are assumed equal to the amounts of MHPG and MOPET originally present and added (MHPG, 2 μg/ml originally, 0—4 μg/ml added; MOPET, 0 or 3 μg/ml originally, 6 μg/ml added). (1) 100% MHPG:100% MOPET or 50% MHPG:50% MOPET; (2) 50% MHPG:100% MOPET; (3) 100% MHPG:50% MOPET; (4) 100% MHPG: 150% MOPET.

had systematic percentage losses in the total (endogenous and added) MHPG and/or MOPET during the analysis. The sample is assumed to contain 2 μg/ml of MHPG, 0 μg/ml of MOPET, and the peak heights are assumed proportional to the amounts added. The curves extrapolate to the same MHPG content for all the samples. The presence of 3 μg/ml of "endogenous" MOPET in a sample (100% MHPG, 150% MOPET) does not change the final MHPG value.

The effect of varying amounts of endogenous MOPET was simulated by adding small increments of MOPET (0, 0.3, 0.6, 1.2 μg/ml) to four aliquots of a urine sample. The four sets were assayed with the usual supplements of MOPET and MHPG. The increments of MOPET produced no differences (Fig. 4) in the calculated original MHPG content of the urine.

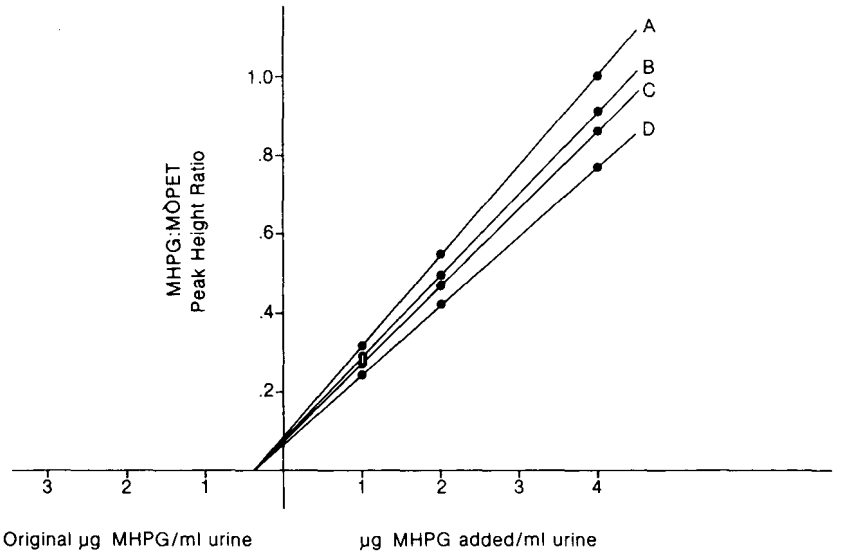

Fig. 4. Effect of increments of MOPET on determination of MHPG content of a urine sample. All the members of a urine set received either: A, 6.0; B, 6.3; C, 6.6; or D, 7.2 µg/ml of MOPET. Each set was then supplemented with 0, 1, 2, or 4 µg/ml of MHPG before assay.

The amount of MOPET present in a sample may be determined by supplementing the set with increasing amounts of MOPET, using MHPG as a constant internal standard.

The saline standard curve

The saline standard curve is not necessary for data calculation, but should be included in the assay periodically as a precautionary measure. The curve will intercept the X-axis to the right of zero if a fixed amount of MHPG is lost from each sample by evaporative losses or column degradation. The curve will intercept the X-axis to the left of zero if contaminating MHPG is brought into the assay by the reagents. The Calbiochem enzyme preparation has never introduced a visible MHPG peak at the gas chromatographic attenuations used in this assay. MHPG losses during the gas chromatographic analysis are avoided by the 4—6 injections of an MHPG-containing sample at 1-min intervals at the beginning of a run.

Precision of the assay

A urine sample was analyzed by ten sequential assays. The MHPG content and slopes were determined for each set of four points by the linear regression method. Data from these analyses revealed a coefficient of variation (C.V.) of the slope of 1.1% and of the value for MHPG of 3.1%.

Wilk et al. [1] report carrying two sets of eight identical samples through the procedure and obtaining standard deviations from the mean (C.V.) of 6.9% and 5.7%. Recalculation of the data of Table I of Karoum et al. [3] indicates that duplicate determinations of three 24-h urine samples gave a mean C.V.

of 5.5%. Sharpless [4] found the mean ± S.D. recovery of free MHPG added to urine was 78.5 ± 12.7% (n = 25), in effect a C.V. of 16.2%.

Stability of MOPET and MHPG

Dekirmenjian and Maas [2] showed that MHPG loses stability below pH 6.0. Enzymatic hydrolysis is most effective at slightly acidic pH, and introduces a possible loss of MHPG during incubation.

MHPG and MOPET stabilities under assay conditions were studied by an extended four-day incubation of nine sets of a urine sample. Each set was supplemented with 0, 1, 2, 3 or 4 μg/ml of MHPG, 6 μg/ml of MOPET and 60 μl of enzyme. The sets were then incubated for 0, 3, 6, 12, 18, 24, 48, 72, or 96 h, and each set was assayed for MHPG and MOPET. The MHPG content of the urine sample was calculated for each set by the extrapolation method, and the change in original MHPG content with time of incubation is shown in Fig. 5. The incubation period was extended to deliberately produce losses in the original and supplemented MHPG and MOPET content (see below). The extrapolation method compensated for the losses and presented a constant final value for MHPG after enzymatic hydrolysis (Fig. 5).

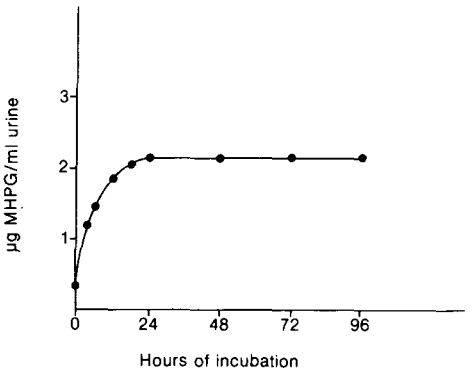

Fig. 5. The original MHPG content of a urine sample during extended incubation as determined by the extrapolation method.

The detected units of MOPET internal standards were well grouped for each time period (Table I), but showed a rapid 13% loss during the first six h of incubation. Little further change occurred during the normal incubation period. These changes do not prevent the use of MOPET as an internal standard.

Stability of MHPG was determined by replotting the curves without the internal standard. The peak heights of the 4 μg MHPG per ml urine supplemented points were taken from the curves for each supplemented set and replotted against time (Fig. 6). An analysis of the 12—96-h values by linear regression indicates that MHPG is lost from the incubation mixture at the rate of 1.0% per 24 h.

Stability of the derivatives

The appropriate amounts of MHPG and MOPET dissolved in ethyl acetate

TABLE I

STABILITY OF MOPET DURING EXTENDED INCUBATION

Incubation time (h)	MOPET units (S.D.)*	C.V.	Percentage loss
0	5.36 (0.11)	2.05	—
3	4.82 (0.10)	2.07	10.1
6	4.65 (0.20)	4.30	13.3
12	4.83 (0.11)	2.56	9.9
18	4.52 (0.04)	0.88	15.7
24	4.53 (0.17)	3.75	15.5
48	4.50 (0.13)	2.89	16.0
72	4.48 (0.07)	1.56	16.4
96	4.29 (0.09)	2.10	20.0

*cm of peak height at 1024 attenuation, 5 points averaged per time period.

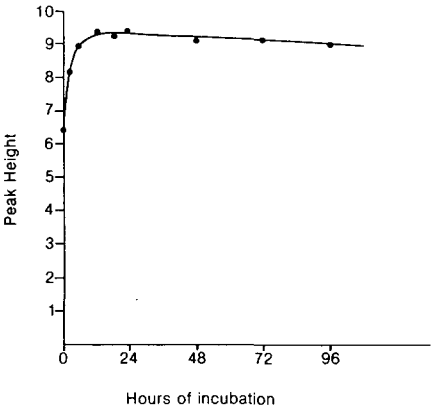

Fig. 6. The peak heights of the 4 μg MHPG per ml supplemented points during extended incubation. Peak heights are in cm at 1024 attenuation, taken from curves developed for each incubation time.

were added to round-bottom flasks to which were also transferred the ethyl acetate extracts of duplicate saline standard curves containing no standards. After normal preparation, the samples were repeatedly analyzed on the gas chromatograph over a 69-h period to determine the stability of the derivatized standards at room temperature. The slopes calculated by the MHPG:MOPET ratio remained linear, but decreased with time. The peak height of MHPG decreased continually with time, showing a 46-h half-life (calculated by the linear regression method) for the first 24 h, and a 198-h half-life thereafter. The MOPET peak height fell for the first 24 h ($t_{½}$ = 89 h) and remained steady thereafter. The ratios calculated with these varying values showed a half-life of 109 h. These losses would be theoretically compensated for during an analysis using the extrapolation method.

TABLE II

URINARY MHPG EXCRETION OF NORMAL SUBJECTS ANALYZED BY GAS CHROMATOGRAPHY

Source	Group	No. of subjects	μg MHPG/24 h (mean ± S.D.)	Range	μg MHPG/mg creatinine (mean ± S.D.)	Range
Hollister et al. [5] 1978	Men	11	2082 ± 842*	1158—4225**	1.80 ± 0.30*	0.68—1.92**
	Women	6	1634 ± 200*	1003—2018**	1.50 ± 0.31*	0.90—3.40**
	All	17	1924 ± 711*	1003—4225**	1.29 ± 0.35*	0.68—3.40**
Taube et al. [6] 1978	Women	10	1029***			
Sharpless [4] 1977	Men	6	2105	1158—2808	1.43	
	Women	5	1618	859—2016	1.32	
	All	11	1884	859—2808		
Maas [8] 1975	Men	19	1674			
	Women	21	1348			
Bond and Howlett [7] 1974	Men	7	2080		1.41	
	Women	6	1920		1.51	
Karoum et al. [3] 1973	All	9	1863	1130—2220	1.27	0.94—1.82
Dekirmenjian and Maas [2] 1970	Men	5	1600		0.95	
	Women	6	1320		1.09	
Maas et al. [9] 1968	Men	5	1600		1.15	
	Women	6	1397		1.16	
	All	11			1.16	
Wilk et al. [1] 1967	All	35			0.86	0.25—1.49

*Three consecutive 24-h urine collections averaged per subject.
**Individual sample range.
***2—4 separate 24-h MHPG values averaged per subject.

Completeness of enzymatic hydrolysis

The extent of enzymatic hydrolysis during incubation was determined by comparing the amount of MHPG detected after supplementing sets of a urine with MHPG or the equivalent amount of MHPG-SO_4. MOPET (6 μg/ml) was used as the internal standard. As the added MHPG-SO_4 is hydrolyzed, the slope of the curve increases and the X-intercept approaches that of the MHPG-supplemented sample. At complete hydrolysis the two curves are identical.

The use of 30 μl of enzyme gave incomplete (81%) hydrolysis in 24 h. Complete hydrolysis occurred when the incubation time was doubled or additional enzyme was added at 24 h and the incubation continued to 48 h.

Under the conditions of this assay, 60 μl of enzyme and 24 h incubation, the MHPG-SO_4 is completely hydrolyzed. The slopes remain superimposed after adding additional enzyme or extending the incubation time.

Application of the method

This method was used in a study of urinary excretion of MHPG in normals [5]. In Table II the results of this investigation are compared with others which measured excretion of MHPG in normal subjects. Some investigators obtain somewhat lower amounts of MHPG in their normal subjects than do others. The reasons for this difference are not entirely clear and could be many. Recent reports of MHPG excretion, both in normals as well as in depressed subjects, tend to report higher values than those originally reported. It is likely that modifications in the technique of measurement, such as those described in this paper, may have increased the extraction of MHPG and prevented its loss, resulting in the trend toward higher values.

ACKNOWLEDGEMENTS

Supported by grant MH 03030 and the Research Service, Veterans Administration Medical Center, Palo Alto, Calif., U.S.A.

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DETERMINATION OF METHENAMINE IN BIOLOGICAL SAMPLES BY GAS—LIQUID CHROMATOGRAPHY

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(Received June 11th, 1979)

SUMMARY

Methenamine (hexamethylenetetramine), a urinary disinfectant, was determined in human plasma and urine by gas—liquid chromatography with a short (10 m) open-bore glass capillary column (split ratio 1:20) and nitrogen-selective detector. An almost quantitative recovery (92.1%) was achieved by simple dilution of water-containing samples (0.5 ml) with acetone (4.5 ml). After centrifugation an aliquot (2 μl) of the supernatant was injected into the gas chromatograph. Selectivity and sensitivity of the nitrogen detector allowed the quantitation of unchanged methenamine in plasma and urine up to 24 h after a single therapeutic dose of 1 g.

Reproducibility of the method was 7.6 and 2.1% (C.V.) in serum and urine, respectively. The time required for the analysis of one sample was approx. 2 min. Due to the simple extraction and short analysis time it was possible to analyze the samples concurrently with sample taking. Absorption of standard tablets and an enterosoluble preparation of methenamine hippurate was compared.

INTRODUCTION

Methenamine (hexamethylenetetramine), whose structure is shown in Fig. 1, is a long-established urinary disinfectant which is mostly used as a salt of

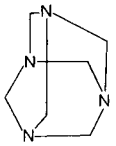

Fig. 1. The chemical structure of methenamine (hexamethylenetetramine).

*To whom correspondence should be addressed.

hippuric acid. It is especially suitable in the treatment of chronic urinary tract infections [1—4]. Its action is based on formaldehyde, which is liberated at the acidic pH of urine [5—7]. No formaldehyde has been found in tissues other than kidney and urinary tract [8, 9]. The most important advantages of methenamine salts as urinary disinfectants are (1) lack of serious side-effects, and (2) formaldehyde does not give rise to resistant bacterial strains [7, 10].

The determination of methenamine in biological samples has been performed mostly by spectrophotometry after acid hydrolysis by coupling the liberated formaldehyde with chromotropic acid [6], phenylhydrazine [11], or with 2-hydrazinobenzothiazole [12, 13]. Attempts to separate free formaldehyde from complexes with amino acids and proteins have not yielded satisfactory results [2].

In our hands the measurement of liberated formaldehyde was successful in buffered solutions but not in urine. This is possibly due to the reaction of formaldehyde with free amino groups in endogenous compounds in urine. Urinary recovery of formaldehyde was only a few per cent. The spectrophotometric methods lack sufficient sensitivity for measurement of methenamine concentrations in blood. Thus, they are not suitable in bioavailability studies. A new method was therefore developed.

EXPERIMENTAL

Subjects

Methenamine hippurate, as a 500-mg standard tablet or an enterosoluble preparation, was administered in cross-over design to six healthy volunteers (all females) mean age 35 years (range 26—48), mean weight 58 kg (range 47—70) and mean height 162 cm (range 154—170). Blood samples were collected from the cubital vein at 1-h intervals up to 10 h and at 12 and 24 h. Serum was separated as soon as possible. Urine samples, voided every 2 h, were also collected. After measurement of the volume, a 0.5-ml aliquot was separated. Extraction and analysis of the samples were performed immediately.

Apparatus

A Hewlett-Packard 5840A gas chromatograph and integrator terminal equipped with a dual flameless nitrogen-selective detector and open-bore glass capillary column (I.D. 0.3 mm, O.D. 0.9 mm) coated with a 0.23 μm thick film of OV-17, length 10 m, was used. The glass capillary column was prepared, filled and installed by Markku Reunanen and Mikko Murola in Åbo Academy, Turku, Finland. Make-up gas (nitrogen) with a flow-rate of 30 ml/min was passed to the detector. Hydrogen and air flow-rates were 3.0 ml/min and 50 ml/min, respectively, The split ratio for carrier gas (nitrogen) was 1:20. Carrier gas flow-rate through the capillary column was 1.0—1.2 ml/min. All gases were of standard purity and were purchased from AGA, Helsinki, Finland. Injector, oven and detector temperatures were 240°, 150° and 250°, respectively.

Quantitation of the integrated peaks was performed by internal standardization.

Reagents

Methenamine hippurate (synthesized by Leiras, Turku, Finland) and *p*-acetylbenzonitrile, used as internal standard (Aldrich-Europe, Janssen Pharmaceutica, Beerse, Belgium) were used without further purification. $NaHCO_3$ and acetone, both analytical grade, were obtained from Merck, Darmstadt, G.F.R.

Preparation of standard solutions

Standard solutions of methenamine were prepared by dissolving 100 mg of methenamine hippurate in acetone (100 ml). This solution was diluted with acetone to correspond to 0, 0.6, 2, 8 and 20 μg of methenamine hippurate per ml for serum samples, and 50, 100, 200 and 400 μg of methenamine hippurate per ml for urine samples. Internal standard was dissolved in acetone for use as extraction solutions at final concentrations of 5 and 100 μg/ml for serum and urine samples, respectively.

Analytical method

Serum, 0.5 ml, was shaken for 1 min with 4.5 ml of the extraction solution. After centrifugation about 500 μl were transferred to a clean test tube from which a 2-μl sample was injected into the gas chromatograph. Urine samples, 0.5 ml, were treated similarly except that the sample was made slightly alkaline by adding about 0.5 g of solid $NaHCO_3$ before extraction (to avoid hydrolysis of methenamine to formaldehyde at the acidic pH of the urine) and the extraction solution was used with the higher concentration of internal standard.

RESULTS

Precision of the analysis was studied by injecting identical samples fourteen times repeatedly into the gas chromatograph. The concentration of methenamine corresponded to 5 μg/ml (serum) and 250 μg/ml (urine). The concentrations of the internal standard were 2.5 and 500 μg/ml, respectively. The ratio of peak areas of sample to internal standard was calculated. The results, expressed as coefficient of variation (standard deviation/mean) × 100, are presented in Table I.

TABLE I

PRECISION, REPRODUCIBILITY AND RECOVERY OF METHENAMINE DETERMINATION IN SERUM AND URINE

For further details see text.

	Precision		Reproducibility		Recovery		
	C.V.	*n*	C.V.	*n*	$\bar{x}$ (%)	S.D.	*n*
Serum	4.2	14	7.6	9	93.1	5.3	12
Urine	1.1	14	2.1	9	91.2	1.8	12

Reproducibility was estimated by preparing and extracting nine identical samples from serum and urine. The concentrations were similar to the precision test. The results are presented in Table I. No day-to-day variation was calculated because the samples were analyzed immediately.

Recovery of the method was tested at concentrations of 10, 20 and 30 μg/ml in serum and at 100, 200 and 500 μg/ml in urine. The amounts of internal standard were 5 μg/ml (serum) and 250 μg/ml (urine). Samples without extraction (acetone solutions) were compared with extracted ones. Recovery per cent (mean 92.1) was similar in serum and urine, as shown in Table I, and was not dependent on methenamine concentration.

Chromatograms for serum and urine samples appear in Fig. 2A and B. Retention times of methenamine and internal standard were about 0.7 and 1.3 min, respectively. The standard curve was linear over the concentration range required in this study. Absorption curves of methenamine in one volunteer after a standard and an enterosoluble tablet following a single dose of 1 g are presented in Fig. 3. The urinary excretion of methenamine in the same volunteer after the same dose is illustrated in Table II.

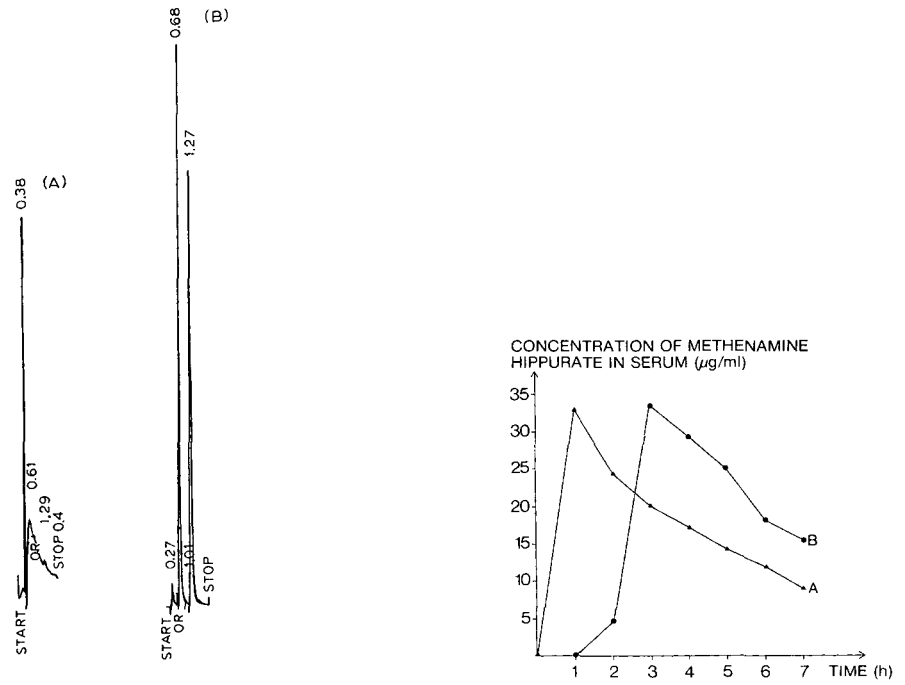

Fig. 2. Typical chromatograms of serum without methenamine and internal standard (A), and chromatogram of urine sample (B). Retention times of methenamine and internal standard were about 0.7 and 1.3 min, respectively.

Fig. 3. Typical absorption curve of methenamine for standard (A) and enterosoluble (B) tablets following a single dose of 1 g.

TABLE II

CUMULATIVE URINARY EXCRETION OF METHENAMINE IN ONE VOLUNTEER AFTER A SINGLE ORAL DOSE OF 1 g

Time of urine collection (h)	Amount excreted (mg)	
	Standard	Enterosoluble
0	0	0
0—2	289.0	65.0
2—4	477.5	266.0
4—6	594.8	546.7
6—8	654.7	706.5
8—10	707.2	841.1
10—12	740.8	892.2
12—14	767.7	945.2

DISCUSSION

Determination of methenamine in human samples has been a difficult problem. So far no reports of its concentrations in human serum have been reported. Even in the urine, where the concentrations are high, unchanged methenamine has not been measured. It has been cleaved by acid hydrolysis to formaldehyde, which has been quantitated by spectrophotometry after coupling with coloured complexes [6, 11—14]. The special difficulties of methenamine determination are (a) its lability in both acidic and alkaline conditions, (b) its hydrophilic nature, which seems to make its extraction from water-containing biological samples to water-insoluble organic solvents impossible, (c) rapid cleavage of the ring system if evaporation (concentration) is attempted, (d) its instability if an attempt is made to remove water from the samples (drying), and (e) the samples cannot be stored. For these reasons the extraction of methenamine cannot be made by the conventional method, that is extracting with organic solvent at a suitable pH, concentrating the sample by evaporation, drying (if necessary) and dissolving the sample in a small volume of solvent which is then analyzed by gas—liquid chromatography (GLC).

We failed in our attempts to concentrate the samples or to remove the traces of water by inorganic salts. The sample was therefore mixed with acetone and analyzed directly by GLC. The diluted sample contains 10% of water. This did not seem to disturb the function of either the glass capillary column or detector -- possibly due to very small amounts of water entering the glass capillary column after the 1:20 split.

Methenamine is thermally stable in GLC analysis — at least it gives a single symmetrical peak in an OV-17 column as shown by Strom and Jun [15]. The method with a packed column and flame ionization detection was, however, used only for pharmaceutical purposes. Methenamine with four nitrogen atoms in the molecule (—NH groups) gives an intense response in the nitrogen-selective detector. It was therefore reasonable to use this detector in analyzing methenamine. The sensitivity of the detector proved to be sufficient for diluted serum samples stored for up to 24 h.

Most determinations of methenamine in human samples have been performed as urinary formaldehyde by spectrophotometry [6, 11—14]. Polar-

ography [16], bioautographic visualization [17], refraction—extraction [18] and volumetric methods [19] have also been used. These methods are not suitable for the determination of methenamine in serum samples, because formaldehyde is liberated only in the urinary tract and not in serum.

The present method was applied to a bioavailability study of methenamine hippurate from various preparations marketed in Finland. A single dose of 1000 mg of methenamine hippurate was administered. Absorption curves of standard and enterosoluble tablets show that C_{max} is achieved later following administration of the enterosoluble preparation. The bioavailability seems to be the same for both preparations.

Our method does not measure the biologically active form of methenamine (= formaldehyde). Analysis of free formaldehyde is, however, as a whole a different problem and is not discussed in this paper. Measurement of total urinary formaldehyde after acid hydrolysis offers no advantages over the present method; on the contrary, in our hands the spectrophotometric methods suffered from poor sensitivity and poor recovery (10—20%), possibly due to reactions of formaldehyde with free amino groups in endogenous urinary compounds.

Details of the pharmacokinetics of methenamine in human serum and urine will be published elsewhere.

CONCLUSION

The present method allows the rapid and reliable measurement of methenamine in human serum and urine. It is very suitable in pharmacokinetic studies of methenamine.

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Journal of Chromatography, 181 (1980) 17—24
Biomedical Applications

CHROMBIO. 437

ELECTRON-CAPTURE GAS CHROMATOGRAPHY OF PLASMA SULPHONYLUREAS AFTER EXTRACTIVE METHYLATION

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(First received May 25th, 1979; revised manuscript received September 13th, 1979)

SUMMARY

Conditions for the extractive alkylation of eight sulphonylurea hypoglycemic drugs have been evaluated. Extractive methylation of the compounds was achieved within 90 min using tetrabutylammonium as counter-ion (0.1 *M* at pH = 6.9) with 5% methyl iodide in dichloromethane as organic phase. Mass spectral analysis showed derivatives methylated at the sulphonamide nitrogen. A higher pH or use of tetrapentylammonium as counter-ion caused hydrolysis of the sulphonylureas.

The derivatives showed a high electron-capture response with minimum concentrations detectable in the range $1—4 \times 10^{-16}$ moles sec^{-1}.

Therapeutic plasma concentrations of glipizide and tolbutamide were determined by direct extractive methylation of the compounds from the plasma sample. The glipizide derivative was determined by electron-capture gas chromatography down to about 20 ng/ml in a 0.5-ml plasma sample. The relative standard deviation at the 0.2 μg/ml level of glipizide was 6% (n = 6). The corresponding figure in the determination of tolbutamide at the 10 μg/ml level was 3% (n = 10).

INTRODUCTION

Oral hypoglycemic drugs of the sulphonylurea type have been in clinical use for about twenty years in the treatment of diabetes. Several analytical techniques have been employed in the analysis of the compounds from biological samples, the most frequently used being gas chromatography. Sulphonylureas have been determined by gas chromatography with flame ionization detection after methylation with dimethylsulphate [1—3] or diazomethane [4, 5]. After methylation, derivatives with good chromatographic properties and enhanced thermal stability were obtained. As well as a methyl derivative, some methyl enol ether was found [4, 6]. A low injection port temperature was claimed to be essential to minimize pyrolysis of the derivative.

Sulphonamide drugs have a high detectability in the electron-capture

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detector and tolbutamide and chlorpropamide have been determined by electron-capture gas chromatography after methylation with diazomethane [7]. Methylation followed by trifluoroacetylation has also been reported [8, 9]. In recent years, the low-dose hypoglycemic agents glibenclamide and glipizide have been introduced. Determination of therapeutic plasma concentrations of these drugs has been performed with radiolabelled material [10, 11] or by radioimmunological assays [12, 13]. The selectivity of these methods for metabolites has not been verified.

Extractive alkylation has found a widespread use in the analysis of acidic drugs and applications to the determination of sulphonamides in biological samples have appeared [14, 15]. The present paper discusses conditions for the extractive methylation of sulphonylurea drugs. The direct analysis of the compounds from plasma is demonstrated with tolbutamide and glipizide.

EXPERIMENTAL

Apparatus

A Pye GCV gas chromatograph was used and equipped with a flame ionization and an electron-capture detector operated in the constant-current mode. The glass column (210 cm × 0.2 cm) was filled with 3% OV-17 on Gas-Chrom Q (100—120 mesh) and operated at 300° for the analysis of the glipizide derivative and at 220° for the tolbutamide derivative. The flow-rate of nitrogen carrier gas was 40 ml/min. The injector and detector temperatures were 330°.

The derivatives were identified by mass spectral analysis in an LKB 2091 gas chromatograph—mass spectrometer. The glass column (90 cm × 0.2 cm) contained 3% OV-17 on Gas-Chrom Q (100—120 mesh). The ionization energy was 70 eV.

Reagents and chemicals

Tetrabutylammonium, 0.1 *M*, was prepared by neutralization of the hydrogen sulphate salt (Labkemi, Stockholm, Sweden) and diluted to volume with buffer. Tetrapentylammonium ion solution, 0.2 *M*, was made from the iodide salt (Eastman—Kodak, Rochester, N.Y., U.S.A.) by shaking overnight with an equivalent amount of silver oxide in water and, after filtration, diluting to volume with water.

Methyl iodide, dichloromethane and toluene were supplied by Merck (Darmstadt, G.F.R.). Toluene was distilled before use. Silver sulphate was prepared as a saturated solution in water.

A stock solution containing 100 μg of tolbutamide per ml was made up in water. Aliquots of 0.05, 0.10, 0.20 and 0.40 ml of this solution were diluted to 1.0 ml with plasma. Chlorpropamide, 10 μg/ml in water, was used as internal standard.

In the determination of glipizide, a stock solution was prepared containing 1.0 μg of the drug per ml. Aliquots of 0.05, 0.10, 0.20 and 0.40 ml of this solution were diluted to 1 ml with plasma. Glibenclamide dissolved and diluted to 5 μg/ml was used as internal standard.

Identity of methylated sulphonylureas

The following prominent peaks were seen in the mass spectra from the sulphonylureas after extractive methylation.

Methyl acetohexamide: *m/e* (percentage relative abundance) = 91 (28), 98 (80), 119 (71), 155 (43), 183 (100), 198 (95), 338 (M^+; *m/e* 8).

Methyl carbutamide: *m/e* = 92 (43), 109 (100), 156 (62), 285 (M^+; *m/e* 1).

Methyl chlorpropamide: *m/e* = 92 (41), 109 (100), 156 (62), 304 (M^+; *m/e* 2).

Methyl glibenclamide: *m/e* = 82 (38), 97 (37), 169 (100), 171 (33), 198 (16), 381 (2).

Methyl glipizide: *m/e* = 93 (60), 98 (25), 111 (64), 150 (100), 459 (M^+; *m/e* = 28).

Methyl tolbutamide: *m/e* = 91 (100), 113 (74), 121 (38), 155 (75), 284 (M^+, *m/e* approx. 0).

N-methyl-(*p*-methylbenzene)sulphonamide from glibornuride and tolazamide: *m/e* = 91 (100), 155 (21), 185 (M^+, *m/e* 24).

Methods

Evaluation of methylation conditions. The sulphonylurea (10^{-3} *M*) was dissolved together with internal standard (hexadocosane or hexatriacontane, 0.2 mg/ml) in dichloromethane. To a 1-ml aliquot of this solution, 2.0 ml of tetrabutylammonium ion solution in 0.1 *M* buffer solution were added or 1 ml of 0.2 *M* tetrapentylammonium ion solution and 1 ml of 0.4 *M* buffer solution. Methyl iodide, 50 μl, was added and the mixture shaken at room temperature for the time given. The reaction was quenched and some microlitres were taken for analysis by gas chromatography with flame ionization detection. The ratio of the peak height of the methyl derivative to that of the internal standard was calculated.

Minimum detectable concentration. Methyl derivatives of the sulphonylureas were prepared in the mg/ml range. Dilutions in toluene were analyzed by electron-capture gas chromatography and the minimum detectable concentration was calculated from the amount giving a signal three times the background noise level.

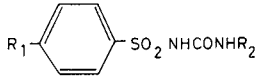
Determination of glipizide in plasma. To a plasma sample of glipizide (0.5 ml), 0.1 ml of internal standard solution and 2.0 ml of 0.1 *M* tetrabutylammonium ion solution in buffer (pH 6.9) were added. This solution was shaken for 1 h with 3 ml of dichloromethane containing 5% of methyl iodide. After centrifugation for 15 min at 500 *g* as much as possible of the organic phase was transferred to another tube and evaporated in a stream of nitrogen. A 0.5-ml volume of toluene was added and this solution was washed with saturated silver sulphate solution. A few microlitres of the organic phase were taken for analysis by electron-capture gas chromatography.

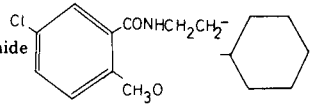
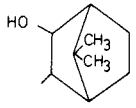
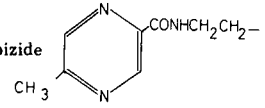
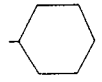
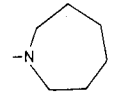
A standard curve was constructed by treating 0.5 ml of the standard samples according to the procedure above.

Determination of tolbutamide in plasma. A plasma sample of tolbutamide (0.1 ml) was mixed with 0.1 ml of internal standard solution and 2.0 ml of 0.1 *M* tetrabutylammonium ion solution in buffer (pH 6.9). This solution was shaken for 1 h with 1 ml of dichloromethane with 5% methyl iodide. The rest of the procedure was then essentially the same as for glipizide above.

TABLE I

EXTRACTIVE ALKYLATION OF SULPHONYLUREAS



No.	Generic name	Structure		Reaction time (min) using 0.1 *M* tetrabutylammonium at pH 6.9 and 5% methyl iodide in dichloromethane	Relative retention of monomethyl derivative compared to hexadocosane	Minimum detectable conc. ($\times 10^{-16}$ moles/sec)
		R_1	R_2			
1	Acetohexamide	CH_3CO-		30	3.3	2.8
2	Carbutamide	H_2N-	-$(CH_2)_3$-CH_3	90	3.5	4.2
3	Chlorpropamide	Cl-	-$(CH_2)_3$-CH_3	40	0.45	1.2
4	Glibenclamide			40	4.0*	4.2
5	Glibornuride	CH_3-		—	(0.17)**	—
6	Glipizide			40	1.8*	3.1
7	Tolazamide	CH_3-		—	(0.17)**	—
8	Tolbutamide	CH_3-	-$(CH_2)_3$-CH_3	20	0.50	1.8

*Relative retention compared to hexatriacontane.

**Formation of N-methylbenzenesulphonamide.

RESULTS AND DISCUSSION

Extractive methylation of sulphonylureas

The extractive alkylation of sulphonylurea drugs could be controlled to give either methylation of the sulphonamide nitrogen or hydrolysis to a sulphonamide and an amide. At a low pH in the aqueous phase methylation of the sulphonamide nitrogen took place, while complete hydrolysis was achieved using tetrapentylammonium as counter-ion at a pH > 10.

For selectivity towards metabolites, the derivatization was focused on the methylation of the sulphonamide nitrogen. The time for complete methylation using tetrabutylammonium ion as counter-ion at pH 6.9 and with 5% methyl iodide in dichloromethane is given in Table I. In all cases, except for tolazamide and glibornuride, the desired derivative was formed, as identified by mass spectrometry (see Experimental section).

Tolbutamide. Sulphonylureas are extremely sensitive to hydrolysis. Therefore conditions that yield a high concentration of hydroxide ion in the organic phase must be avoided. Use of tetrapentylammonium ion as counter-ion or a high pH in the aqueous phase, gave a low yield of derivative as can be seen from Table II. Tetrabutylammonium ion was therefore used as counter-ion. With this counter-ion a reaction time of 90 min was required for complete reaction at pH 5.3. In buffers with a pH exceeding 7.0 a maximum formation rate was seen with complete reaction within 20 min. In buffer pH 10, a 15% degradation of the derivative was observed after 4 h. The derivative, on the other hand, was stable for more than 3 h in the reaction mixture using buffer of pH 6.9.

TABLE II

METHYLATION TIMES OF TOLBUTAMIDE

Organic phase: dichloromethane with 5% methyl iodide, 1 ml.

Aqueous phase (2 ml) counter-ion	pH	Time for complete reaction (min)
Tetrabutylammonium (0.05 *M*)	5.3	90
Tetrabutylammonium (0.05 *M*)	6.3	45
Tetrabutylammonium (0.05 *M*)	6.9	20
Tetrabutylammonium (0.05 *M*)	10.0	20
Tetrapentylammonium (0.05 *M*)	6.9	(20)*
Tetrapentylammonium (0.05 *M*)	10.0	(30)**

*Maximum yield: 30%.
**Maximum yield: 15%.

Glipizide. Three products could be detected after extractive methylation of glipizide. The formation and degradation of the products with time using 0.05 *M* tetrabutylammonium ion as counter-ion in 0.1 *M* sodium hydroxide is shown in Fig. 1. Peak I corresponds to the methylated derivative of glipizide and was rapidly degraded. Peak II was an unidentified product and peak III was the sulphonamide hydrolysis product. The reaction conditions were chosen to give the methylated product and a low pH in the aqueous phase was used. A complete derivatization was achieved in 40 min using buffer of pH 6.9.

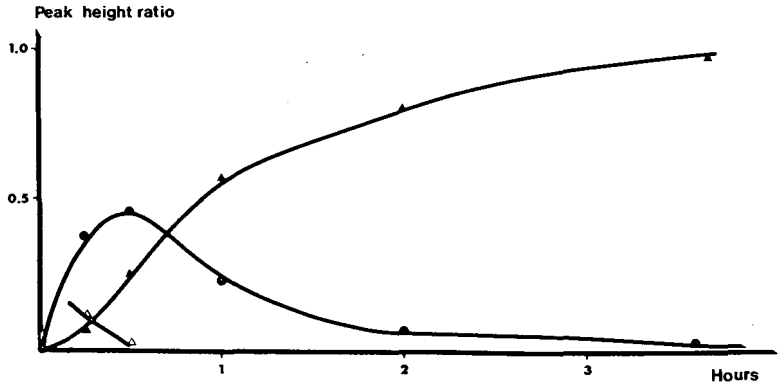


Fig. 1. Time course of the extractive methylation of glipizide. Conditions: 0.05 *M* tetrabutylammonium ion solution in 0.1 *M* sodium hydroxide (2 ml) and 5% methyl iodide in dichloromethane (1 ml). (△—△) product I = methylated derivative: (●—●) product II = unidentified product; (▲—▲) product III = hydrolysis product.

Acetohexamide. The derivative from acetohexamide was very sensitive to hydrolysis and it was necessary to perform the reaction at pH 6.0 with 0.05 *M* tetrabutylammonium ion as counter-ion.

Carbutamide. Only the sulphonamide nitrogen was methylated in the case of carbutamide, which means that the aniline moiety remains intact. For complete derivatization 90 min were required.

Chlorpropamide and glibenclamide. With the reaction conditions used, 40 min were required for complete methylation of the sulphonamide nitrogen of chlorpropamide and glibenclamide. Hydrolysis was pronounced at pH >10.

Tolazamide and glibornuride. Apart from the hydrolysis product, N-methyl-(*p*-methylbenzene)sulphonamide, no other derivatives could be detected from either tolazamide or glibornuride even in buffers of low pH.

Gas chromatography and electron-capture detection of methylated sulphonylureas

The gas chromatographic stability of methylated sulphonylureas has been reported to be poor [2, 4]. Therefore, small amounts (0.8 ng) of the tolbutamide derivative were repeatedly chromatographed on the stationary phase OV-17 together with an inert internal standard using injector temperatures in the range 200—350° and detector temperatures in the range 250—350°. In all cases, the relative standard deviation was in the range 1.4—3.0% (n = 10), indicating that stability problems were of small importance.

For some derivatives (for example, glibenclamide and glipizide) a slight tailing of the peaks was observed, most probably caused by remaining acidic hydrogens in the molecules. The relative retentions of the derivatives are given in Table I and two gas chromatograms are shown in Fig. 2.

The sulphonamide grouping has a high electron-capture response after methylation [14—16]. The response was particularly good with the sulphonamide moiety in close conjugation with an aromatic ring. Conjugation of the sulphonamide grouping to a carbonyl group as in the sulphonylureas would

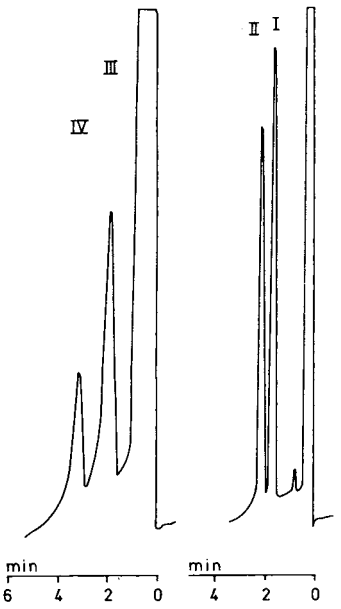


Fig. 2. Gas chromatograms after extractive alkylation of plasma sulphonylureas. Left panel: analysis of glipizide (III), 0.4 μg/ml. Internal standard: glibenclamide (IV), 0.5 μg added. Right panel: analysis of tolbutamide (II), 10 μg/ml. Internal standard: chlorpropamide (I), 1.0 μg added. For gas chromatographic conditions see Experimental section.

further increase the response. Minimum detectable concentrations of the methylated sulphonylureas were in the range $1-4 \times 10^{-16}$ moles/sec (see Table I). The electron-capture response of N-methyltolbutamide was found to be independent of the detector temperature in the range 200—350°.

Determination of sulphonylureas from plasma samples

Antidiabetic sulphonylurea drugs are extensively metabolized in man. For some sulphonylureas, the metabolites are also reported to exhibit pharmacological activity [11, 17]. The possibility of co-determination of metabolites could not be neglected after hydrolysis of the sulphonylureas and methylation of the sulphonamide nitrogen was carried out.

The plasma concentrations after therapeutic doses of sulphonylurea drugs are usually in the lower μg/ml range. Direct extractive methylation from a 0.1-ml plasma sample of the drugs was possible, as has recently been demonstrated for other sulphonamide drugs [14, 15]. A chromatogram from an analysis of plasma tolbutamide can be seen in Fig. 2. The relative standard deviation in the analysis of 10 μg of tolbutamide per ml of plasma was 3.0% (n = 10).

Although the plasma concentrations of glipizide or glibenclamide are in the submicrogram/ml range, direct extractive methylation of the plasma sample could also be used in these cases owing to the high detection selectivity of the method. Use of tetrabutylammonium ion as counter-ion at pH 6.9 gave no interfering components from the biological sample and glipizide could be detected down to 20 ng/ml in a 0.5-ml plasma sample. Before gas chromato-

graphic analysis, methyl iodide was removed by evaporation, and tetrabutylammonium iodide formed in the reaction by washing with saturated silver sulphate solution. Rectilinear standard curves through the origin were obtained in the range 50—400 ng of glipizide per ml. The relative standard deviation in the determination of glipizide at the 200 ng/ml level was 6.0% (n = 6). A typical gas chromatogram is seen in Fig. 2. The procedure for glipizide is at present in use for pharmacokinetic studies of the drug in man.

ACKNOWLEDGEMENT

Dr. Ulf Bondesson, Department of Drugs, National Board of Health and Welfare, Uppsala, Sweden, is gratefully acknowledged for the mass spectral analyses.

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SIMPLIFIED METHOD FOR DETERMINATION OF THE TETRACYCLIC ANTIDEPRESSANT MIANSERIN IN HUMAN PLASMA USING GAS CHROMATOGRAPHY WITH NITROGEN DETECTION

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(Received July 2nd, 1979)

SUMMARY

A simplified gas chromatographic method for determination of the antidepressant drug mianserin in human plasma is described. Application of a nitrogen-sensitive detector reduces the assay procedure to extraction, concentration and gas chromatographic determination. The method is suitable to determine mianserin in human plasma at the 1 ng/ml level on a routine basis. At the 20 ng/ml level the deviation of the mean from the true value and the relative standard deviation amount to 1.0% and 6.8%, respectively.

INTRODUCTION

Until now, quantitation of mianserin (Fig. 1) in biological samples for clinical studies and animal experiments has been performed using mass fragmentography at our laboratories. Although the assay for mianserin [1, 2] has been gradually improved over the past five years, and the analyses have become more and more automated to handle a large number of samples per year [3], the use of mass fragmentography itself hampers the general applicability of the assay method. Gas chromatography (GC) using a specific nitrogen-sensitive detector is a possible alternative for mass fragmentography. However, the use of alkali flame ionization detectors (AFID) for gas chromatographic detection of nitrogen-containing compounds has never become wide-spread because of the signal instability observed during daily routine operation [4].

The introduction of a new generation of nitrogen/phosphorus-sensitive detectors (NPD) has dramatically changed the situation [5—7]. With these types of detector, also referred to as thermionic ionization detectors, signals

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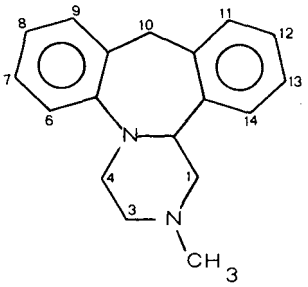

Fig. 1. Mianserin (1,2,3,4,10,14b-hexahydro-2-methyldibenzo[*c*,*f*]-pyrazino[1,2-*a*]-azepine). Also the lab code Org GB 94 is in use denoting the salt mianserin monohydrochloride.

of high stability during long-term operation are obtained. Although the detector design differs from manufacturer to manufacturer [5—7] and the principle of operation is still rather obscure, all these nitrogen-sensitive detectors have an independent electrically-heated bead as alkali source, which is operated in an air-hydrogen environment. The supply of hydrogen is not enough to provide a self-sustaining hydrogen—air flame, thereby generating a flame zone around the glowing alkali bead only. In the low-temperature plasma surrounding the bead, organic molecules are pyrolyzed; the highly reactive radicals formed are involved in reactions with alkali atoms, while the ions formed are responsible for the detector signals observed [5—12]. Regardless of the mystic surrounding the reaction mechanisms, the NPD has already found many applications in the field of drug research [13—25].

The sensitivity and specificity of the nitrogen-sensitive detector and the general accessibility of gas chromatography in other laboratories, forced us to investigate in detail the merits of the GC assay with nitrogen detection for determination of mianserin in plasma. In this paper, the assay method is described in detail. A comparative study of the present assay method and the previously used method employing mass fragmentography [1, 2] will be published elsewhere [26].

MATERIALS AND METHODS

Solvents

All solvents were purchased from Merck (Darmstadt, G.F.R.). Ammonia, *n*-hexane and methanol were of Suprapur, Uvasol and analytical-reagent grade quality, respectively.

Glassware

The glassware was cleaned using normal laboratory washers (Miele type G19) and standard washing agents (Neodisher F, N and S, Chemische Fabrik Dr. Weigert, Hamburg, G.F.R.). After this washing procedure, the glassware was cleaned ultrasonically in a hydrochloric acidified water bath, rinsed with distilled water and dried. Before use, the glassware was rinsed thoroughly with the organic solvent to be used in the assay procedure.

Internal standard

For the GC assay method, 7-methylmianserin was chosen as internal standard. Because of their structural relationship, mianserin and 7-methylmianserin behave chemically quite similarly during the assay procedure.

Gas chromatography with nitrogen detection

A Hewlett-Packard gas chromatograph Model 5710A, equipped with a 3 m × 2 mm I.D. glass column packed with 1% OV-25 on Gas-Chrom Q (80—100 mesh) was used. The Hewlett-Packard N/P detector Model 18789A was operated at a voltage of 15—18 V for heating the bead. The temperatures of injector, column and detector oven were 300°, 240° and 300°, respectively. Nitrogen was used as a carrier gas at a flow-rate of 20 ml/min. The gas flow-rate for the N/P detector was 3 ml/min for hydrogen and 50 ml/min for air.

Assay procedure

The internal standard, dissolved in water (40 ng/ml), was added to the plasma sample in approximately the same amount as the expected mianserin concentration, together with 100 μl of concentrated ammonia to obtain pH > 11. After equilibration for at least 3 h, the plasma was extracted with 5 ml of *n*-hexane by thoroughly mixing and centrifuging for 5 min at 1500 *g*. The *n*-hexane extract was evaporated to dryness in a 10-ml conical tube at 45° under a gentle stream of nitrogen. The wall of the conical tube was rinsed thoroughly with 500 μl of methanol, the methanolic solution was again evaporated to dryness at 45° under nitrogen, and the residue finally dissolved in 8—20 μl of methanol. To avoid detector overloading and cross-over of previous injections, only an aliquot of 1—7 μl from the methanolic solution was injected into the gas chromatograph for quantitation. The volume of sample injected was dependent on the expected drug plasma level; below 10 ng/ml the maximum solvent volume (7 μl out of 8 μl) was injected; for plasma levels within the range of 10—200 ng/ml 30% or less of the 20 μl methanolic solution was used for injection.

Standardization and quality control on the results of analysis

Standard solutions required for establishment of the calibration curve for the GC—NPD method contained mianserin and 7-methylmianserin in the concentration ratios 1:4; 1:2; 1:1; 2:1; and 4:1. The standard solutions were added to human plasma free of drug (blank) and analyzed by GC—NPD after sample processing as described previously in order to correct for the minor difference in yield of extraction from plasma between mianserin and its 7-methyl derivative.

On each day of measurement, plasma samples containing unknown concentrations of the drug, samples for standardization, blank human plasma samples and blank human plasma samples spiked with mianserin were analyzed randomly. The blank human plasma samples with and without mianserin added, served as controls for the overall analytical procedure.

Data processing

For calculation of the calibration functions and mianserin levels, the measured peak heights of mianserin and internal standard together with required data on calibration mixtures, original sample volume and amount of internal standard added, were fed into a computer via a visual display terminal with keyboard connected to the DEC PDP-11 RSTS computer facilities of Akzo Pharma, Oss, The Netherlands.

RESULTS AND DISCUSSION

Calibration curve

The calibration curve for the GC—NPD method was calculated using polynomial regression analysis. A typical example of the calibration curve (peak height ratio y versus concentration ratio x) is represented by $y = -0.007x^2 + 1.400x - 0.025$ where each concentration ratio was measured in triplicate.

Detection limit

Picogram amounts of pure mianserin can be detected with the N/P detector. However, the lower limit of detection for reliable mianserin determination in human plasma is not governed by the sensitivity for mianserin of the N/P detection alone. Also the work-up procedure and the endogenous components from the biological matrix could possibly contribute to the detector output signal. In these cases, the mianserin levels are influenced by the ratio of background signal to native response.

During the assay procedure, the total amount of mianserin injected into the GC—NPD system is determined by the yield of extraction (approx. 90%) and the solvent volume used for injection (maximum 7 μl out of 8 μl). Therefore, a maximum of approximately 80% of the amount of mianserin originally present in the plasma sample can be subjected to GC—NPD. The simplicity of the assay procedure, the high yield of extraction of mianserin from plasma and the possibility of efficient solvent transfer ensure that the loss of mianserin during the assay procedure is low. However, upon determination of mianserin levels below 1 ng/ml problems are encountered because at these levels the GC peak of mianserin is superimposed on the tails of the solvent and plasma impurity peaks. Consequently, the described assay method is sensitive enough to determine, on a routine basis, mianserin plasma levels above the 1 ng/ml level.

Specificity

Gas chromatograms, which were obtained after injection of an extract from blank human plasma, blank human plasma spiked with a known amount of mianserin, and human plasma from a patient undergoing chronic mianserin treatment are shown in Fig. 2. No interference from endogenous plasma constituents eluting at the retention times of mianserin and the internal standard was found. Possible interference by the metabolites of mianserin was also investigated. The retention times of the major metabolites [27] relative to that of mianserin are presented in Table I. Only the mianserin N-oxide could possibly interfere with mianserin in the GC—NPD assay owing to its thermal

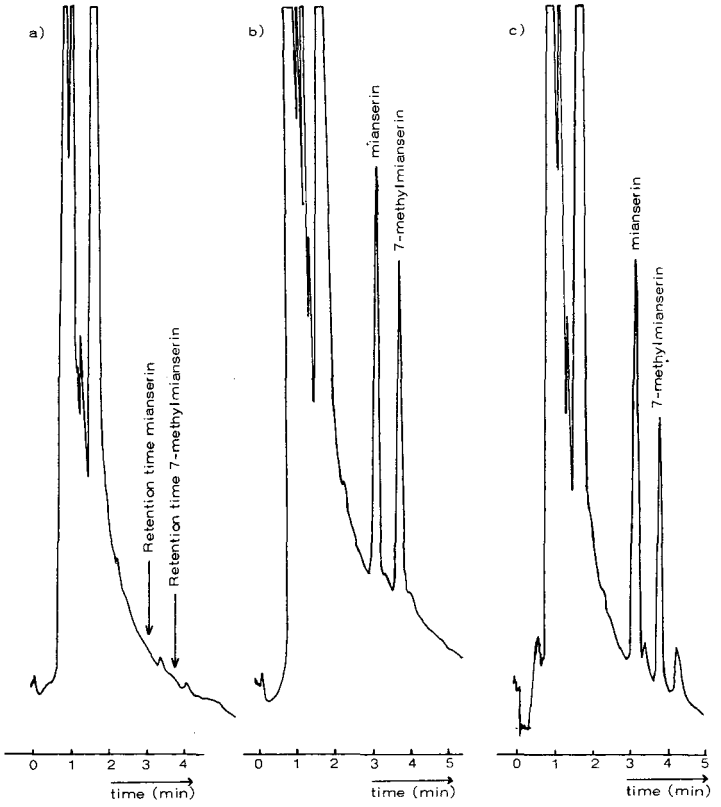

Fig. 2. Gas chromatograms after injection of a *n*-hexane extract of 1 ml human plasma samples: (a) blank; (b) blank, spiked with 10 ng of mianserin and 10 ng of the added internal standard, and (c) plasma from patients receiving mianserin treatment; to the sample 10 ng of the internal standard were added, the mianserin content was calculated to be 11.5 ng.

TABLE I

RETENTION TIMES OF THE MAJOR MIANSERIN METABOLITES RELATIVE TO MIANSERIN

Compound	Relative retention time
Mianserin	1.00
8-Hydroxymianserin	3.27
Desmethylmianserin	1.36
Mianserin N-oxide	1.00*
Internal standard (7-methylmianserin)	1.24

*Decomposes thermally into mianserin as confirmed by GC—MS.

decomposition into mianserin. However, the N-oxide cannot be extracted from plasma under the assay conditions employed.

Other drugs concomitantly in use during mianserin therapy could possibly interfere with mianserin or the internal standard 7-methyl-mianserin. The as-

say procedure does, however, ensure a reasonable specificity; only drugs which can be extracted from basic plasma can appear in the *n*-hexane extract, most basic drugs can be separated from each other by GC [25] and only nitrogen- (or phosphorus)-containing compounds will be detected by the specific N/P detector.

Accuracy and precision

The deviation of the mean from the true value and the relative standard deviation as a measure for, respectively, the accuracy and precision of the GC—NPD assay method, were determined by analyzing blank plasma samples spiked with a known amount of mianserin. These spiked samples were analyzed intermittently over a period of three months during analyses of blank plasma samples and clinical samples. The accuracy and precision at the 20 ng/ml plasma level are shown in Table II.

TABLE II

GC—NPD ASSAY CHARACTERISTICS OF REFERENCES SAMPLES CONTAINING 20.0 NG MIANSERIN PER MILLILITRE OF PLASMA

Mean	(ng/ml)	19.80
Standard deviation	(ng/ml)	1.34
Deviation of the mean from the true value	(%)	− 1.0
Relative standard deviation	(%)	6.8
Number of data	(*n*)	41

CONCLUSIONS

The novel GC—NPD assay method for the determination of mianserin in human plasma offers ample sensitivity and specificity to determine mianserin levels as low as 1 ng per ml plasma during long-term daily operations. Compared with the previously described gas chromatographic—mass spectrometric (GC—MS) assay method [1, 2], the GC—NPD method involves only a simple extraction and concentration step, while the GC—MS method requires intermediate sample clean-up before introduction into the GC—MS system to enable daily routine analysis [2]. Furthermore, the GC—NPD assay method uses medium-priced, general accessible and reliable analytical instruments. Application of the GC—MS assay method requires the presence of sophisticated, costly analytical instruments together with highly-skilled personnel to operate them, while the equipment itself is subject to frequent breakdowns. Using packed GC columns, the specificity of the GC—NPD method can, however, not be compared with the specificity of a mass spectral method. Application of capillary GC columns can increase the specificity of the GC—NPD method, whenever problems are encountered owing to a lack of specificity. It has been demonstrated that GC—MS assay methods can be automated to a great extent [3, 28]. Using an autosampler for sample injection and a computer system for data acquisition, collection, processing and final drug level calculation, com-

plete automation will be easier with the GC—NPD method than with a GC—MS method.

The GC—NPD as well as the GC—MS assay method have been applied in our laboratory to plasma samples from depressed patients chronically treated with mianserin [29]. The results of the statistical evaluation of both assay methods with respect to intra-laboratory variation and (possible) systematic deviation between the results of analyses, are published separately [26].

ACKNOWLEDGEMENTS

The authors would like to thank Dr. J.J. de Ridder for initiating the use of a nitrogen-sensitive detector for mianserin determinations. Thanks are due to Mrs. J.P.A. van de Logt-Kaal for performing the mianserin plasma analyses.

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SENSITIVE GAS CHROMATOGRAPHIC ASSAY FOR THE QUANTITATION OF BRETYLIUM IN PLASMA, URINE AND MYOCARDIAL TISSUE

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(First received April 17th, 1979; revised manuscript received August 6th, 1979)

SUMMARY

A sensitive analytical method has been developed for the quantitation of bretylium in plasma, urine and myocardial tissue. Bretylium and the internal standard, UM-360 (*o*-iodobenzyltrimethylammonium), are extracted and isolated as the iodide salts. Sodium benzenethiolate is added and the mixture heated to 100° for one hour. This results in the formation of 2-bromobenzyl phenyl thioether and 2-iodobenzyl phenyl thioether, which can be separated and quantitated by gas chromatography. Good reliability and reproducibility can be obtained using electron-capture detection with quantities of bretylium as small as 1 ng.

INTRODUCTION

Bretylium tosylate (bretylol, *o*-bromobenzylethyldimethylammonium tosylate) is a unique antiarrhythmic agent possessing antifibrillatory actions [1,2]. The pharmacokinetic properties of bretylium are poorly described due to the lack of an analytical method with both specificity and sensitivity for the quaternary. An earlier method used to measure bretylium in urine [3] did not possess the sensitivity needed to measure bretylium levels in plasma. The previous method was based upon the ability of the quaternary to bind methyl orange, and lacked specificity for bretylium. The method used in the present study for quantitation of bretylium is based upon a procedure used for quantitation of acetylcholine [4] as modified for use in quantitating bretylium by Kuntzman et al. [5]. The procedure involves the removal of the *o*-bromobenzylgroup from bretylium by sodium benzenethiolate and the formation of an *o*-bromobenzyl phenyl thioether which can be measured with excellent

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sensitivity by electron-capture detection after separation by gas chromatography.

METHODS

General principle

Bretylium and UM-360 (*o*-iodobenzyltrimethylammonium) are extracted and isolated from samples as the iodide salt. Addition of sodium benzenethiolate results in the formation of *o*-bromobenzyl phenyl thioether and *o*-iodobenzyl phenyl thioether derivatives which can be separated and quantitated by gas chromatography.

Apparatus

A gas chromatograph (Hewlett-Packard Model 7610A) equipped with a ^{63}Ni electron-capture detector was used. Glass columns (1.83 m × 4 mm I.D.) with the following packings were employed: 4.3% OV-101 on Gas-Chrom Q (100—120 mesh), 5.0% OV-1 on Gas-Chrom Q (80—100 mesh), 3% OV-17 on Gas-Chrom Q (100—120 mesh), and 4% OV-225 on Gas-Chrom Q (100—120 mesh) (Applied Science Labs., State College, Pa., U.S.A.). Samples (0.1—2 μl) were injected manually using standard commercial microsyringes (Hamilton, Reno, Nev., U.S.A.). The following temperatures were used: injection port, 250°; column oven, 190°; and electron-capture detector, 250°. The carrier gas, argon—methane (19:1), flow-rate was 50 ml/min.

A Finnigan gas chromatograph—mass spectrometer (electron impact mode) was used for identification of the major chromatographic peaks. Helium was used as the carrier gas. A 3% OV-17 on Gas-Chrom Q (100—120 mesh) (1.7 m × 2 mm I.D.) column was used. Injection port, column, and detector temperatures were as described above.

Extraction

Bretylium and UM-360 were extracted from biological fluids as the iodide salts using the method of Vidic et al. [6] as modified by Pohlmann and Cohen [7]. UM-360 chloride, 50 μl of a 5.5 μg/ml solution, and 1 ml distilled water were added to: 1 ml plasma or serum, 1 ml dissolved tissue sample, or 100 μl urine. Chloroform (3 ml) was added and the tubes were vortexed for 10 sec followed by centrifugation at 1500 *g* for 5 min. The chloroform layer was removed and discarded. Potassium triiodide [iodine—potassium iodide—water (1:2:10 w/w/v)], 200 μl, and chloroform, 3 ml, were added. The samples were shaken gently on a reciprocating flat bed shaker (100 rpm) for one hour followed by centrifugation at 1500 *g* for 5 min. The chloroform layer was transferred to a conical glass vial (Reactivial, Pierce, Rockford, Ill., U.S.A.) and evaporated to a dry residue under a stream of dry nitrogen. The presence of elemental iodine in the dry residue does not interfere with subsequent derivatization and quantitation although it can be removed by the addition of 50 μl of 10% ascorbic acid in methanol followed by vortexing and evaporation of the methanol.

Derivatization

Sodium benzenethiolate (3 mg/ml in ethyl acetate), 200 μl, was added and

the vial capped securely. The samples were refluxed in a sand bath heated to 100—120° for one hour and allowed to cool at room temperature. The ethyl acetate was removed under a stream of dry nitrogen and the residue dissolved in 100 μl hexane or cyclohexane. Injection of the ethyl acetate after derivatization and cooling to room temperature reveals the presence of late peaks which interfere with subsequent injections. These peaks were removed by evaporation of the ethyl acetate and dissolution of the residue in cyclohexane or hexane.

Preparation of samples for analysis

Male mongrel dogs weighing 14.0—16.3 kg were anesthetized with intravenous sodium pentobarbital, 30 mg/kg. A left external jugular vein cannula was inserted for drug administration and a second cannula was inserted into the inferior vena cava through the right femoral vein for withdrawal of blood samples for bretylium analysis. Blood was withdrawn into commercially prepared heparinized containers (Vacutainer, Becton-Dickinson, Toronto, Canada). A bolus of bretylium tosylate (6 mg/kg) was administered and plasma samples obtained at appropriate intervals. The animals (n = 3) were sacrificed 12 h after the administration of bretylium and 300—350-mg sections of atrial, left ventricular, and right ventricular myocardium were removed for analysis of bretylium concentration. A 12-h urine sample was removed from the bladder. The myocardial samples were dissolved in 500 μl of 12 N sodium hydroxide with 800 μl of 7 N hydrochloric acid added after tissue dissolution. The above experiments were designed to demonstrate the ability of the assay to detect bretylium in biological fluids and tissue.

RESULTS

Quantitation of bretylium and extraction efficiency

Electron-capture detection was used for the measurement of the halogenated thioethers. Typical chromatograms obtained from injected standards and plasma are shown in Fig. 1. Peak height ratios (*o*-bromobenzyl phenyl thioether:*o*-iodobenzyl phenyl thioether) were used for quantitation. Standard curves were prepared for plasma, urine and tissue by the addition of known quantities of bretylium to the samples. A linear correlation exists for samples representing bretylium plasma concentrations of 25—8000 ng/ml; r = 0.998. Linearity also exists for standards representing as little as 1 ng/ml bretylium or as much as 20 μg/ml bretylium; 1—20 ng/ml (r = 0.992) and 2—20 μg/ml (r = 0.995). The quantity of UM-360 added to the last two standards was modified to facilitate measurement of peak heights. The injected sample volume was varied so the detector response ranged from $0.1—2.0 \cdot 10^{-10}$ A and retained a linear response ratio. Standard curves for urine, 10—200 μg/ml (r = 0.999) and myocardial tissue, 1—20 μg/ml (r = 0.990) also were linear.

Bretylium, 25—8000 ng, and UM-360, 250 ng, dissolved in methanol, were added to conical glass vials and the methanol evaporated under a stream of dry nitrogen. These samples were run in parallel with plasma bretylium standards representing 25—8000 ng/ml bretylium. UM-360, 250 ng, dissolved in methanol, was added to the dry chloroform extract of plasma and the methanol evaporated under dry nitrogen. Recovery of bretylium from plasma using

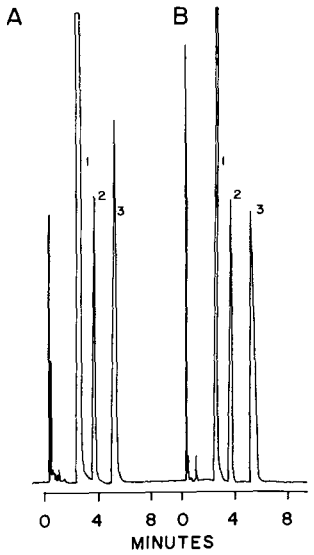

Fig. 1. Gas chromatograms from standards and plasma. Sample A was prepared from stock solutions of bretylium and UM-360. Peaks: 1 (retention time 2.9 min) = benzenethiol disulfide, a contaminant formed from sodium benzenethiolate derivatization; 2 (retention time 3.6 min) = *o*-bromobenzyl phenyl thioether, a derivative of bretylium; 3 (retention time 5.3 min) = *o*-iodobenzyl phenyl thioether, a derivative formed from UM-360.

potassium triiodide extraction as described previously was 95.9 ± 3.0%.

Mass spectroscopy and identification of major peaks

A total ion chromatogram and mass spectra of the three major peaks are given in Fig. 2. Peak 1 of the total ion chromatogram, with a base peak (*m/e* = 109) corresponding to cleavage of the disulfide bond and a second peak (*m/e* = 218) representing the molecular species was shown to be benzenethiol disulfide. Peak 2, with a base peak (*m/e* = 171) corresponding to fragmentation of the thioether with formation of the resonance stabilized bromotropylium ion and a second peak representing the molecular species (*m/e* = 280) was shown to be *o*-bromobenzyl phenyl thioether. Peak 3, with a base peak (*m/e* 217) corresponding to formation of an iodotropylium ion by fragmentation of the thioether, was shown to be *o*-iodobenzyl phenyl thioether.

Canine plasma, urine and myocardial tissue samples

Plasma levels of bretylium taken at appropriate intervals over a period of 12 h after a bolus intravenous injection of bretylium tosylate, 6 mg/kg, are given in Fig. 3. Plasma bretylium levels varied from a maximum of nearly 20 μg/ml just after bretylium administration to a minimum of just over 2 μg/ml at 12 h.

Myocardial tissue levels of bretylium were measured at the time of sacrifice, 12 h after the administration of bretylium (Fig. 4). Atrial tissue levels of bretylium were lower than those seen in right and left ventricular myocardium. A seven-fold ratio of ventricular myocardial tissue to plasma, bretylium concentration (μg/g myocardial tissue:μg/ml plasma) was seen.

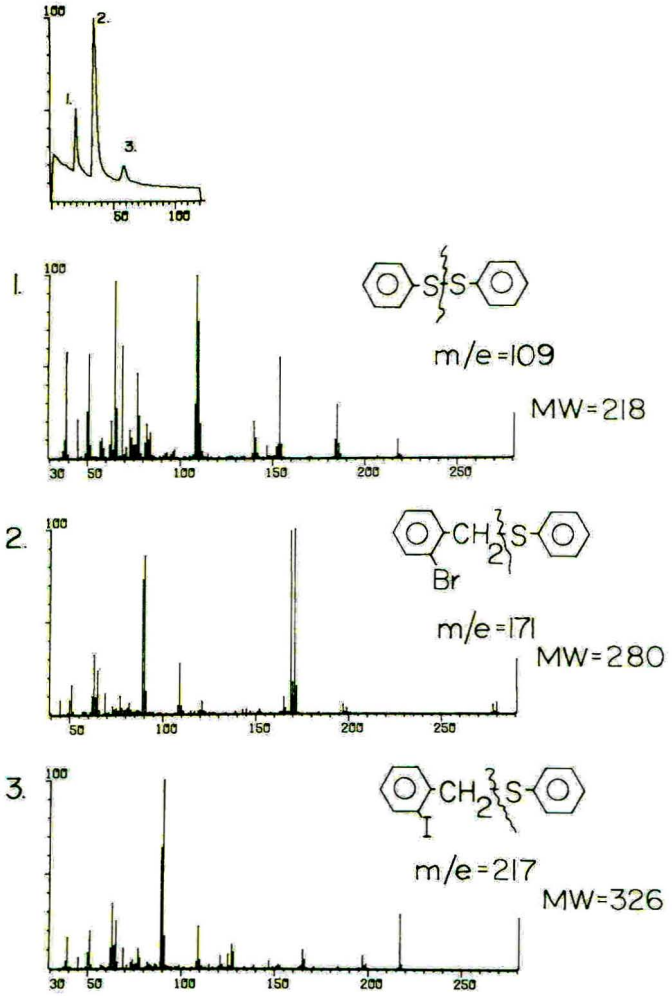

Fig. 2. Mass spectroscopic analysis. The three major peaks measured by electron impact mass spectroscopy corresponded to the three peaks measured by electron capture in Fig. 1. 1, (*m/e*=109, *m/e*=218), benzenethiol disulfide; 2, (*m/e*=171, *m/e*=109, *m/e*=280), *o*-bromobenzyl phenyl thioether; 3, (*m/e*=217, *m/e*=109) *o*-iodobenzyl phenyl thio ether.

A 12-h urine sample was collected and the excreted bretylium quantitated. The total 12-h excretion of bretylium was 23.4 ± 3.9 mg (50.6 ± 8.3% of the administered dose).

All samples were performed in duplicate with a mean difference of 3.8 ± 1.8% between samples.

DISCUSSION

The method of Kuntzman et al. [5] has a number of problems which prevent its general use for pharmacokinetic studies. (1) A large number of sample transfers and extractions are necessary for the isolation of bretylium and the elimination of substances from plasma and urine with similar retention

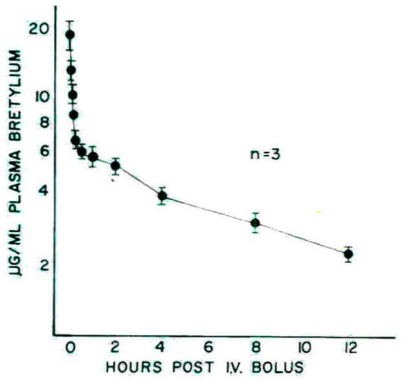

Fig. 3. Plasma bretylium concentrations. Plasma bretylium concentrations in an anesthetized dog after an intravenous (i.v.) bolus injection (6 mg/kg) of bretylium tosylate (mean ± standard error of the mean).

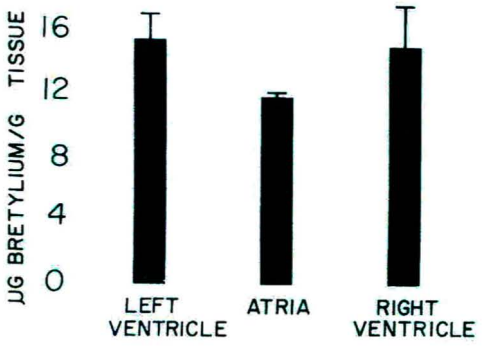

Fig. 4. Myocardial tissue concentrations of bretylium. Bretylium concentrations in canine myocardial tissue, 12 h after a single intravenous bolus injection of 6 mg/kg bretylium tosylate (mean ± standard error).

times to those of the derivatized product and external standard. (2) Quantitation of the *o*-bromobenzyl phenyl thioether is possible only by the addition of an external standard, occurring after the extraction and isolation of bretylium. (3) The smallest detectable quantity of bretylium, 70 ng, is greater than the plasma levels necessary for observation of linear first-order pharmacokinetics in man. (4) The poor chromatographic separation of *o*-bromobenzyl phenyl thioether from benzenethiol required the use of conditions which resulted in long retention times for the *o*-bromobenzyl phenyl thioether (14 min) and the external standard (18 min).

The assay procedure for bretylium as given in this paper overcomes many of the problems occurring with the previous method. A single sample wash, followed by chloroform extraction of bretylium as the triiodide salt, is the only procedure necessary for the isolation of bretylium in a relatively pure form. Derivatization followed by evaporation and dissolution of the halogenated thioethers yields a clean chromatogram with no interfering peaks. This is in contrast to the multi-step procedures previously used to isolate bretylium in a relatively pure form [3] and to obtain a derivatized product with sufficient purity to achieve a clean chromatogram [5].

The use of an internal standard undergoing identical isolation and derivatization procedures as the measured product, bretylium, eliminates the problems which can occur due to inaccurate volume transfers occurring prior to the addition of an external standard. Also, the addition of an internal standard serves as a control for both the extraction procedure and derivatization procedure. The failure to detect bretylium in a sample, therefore, can be attributed to the absence of bretylium in a specimen, thus eliminating the possibilities of faulty extraction or derivatization.

The assay is both specific and sensitive for bretylium. Quantities of bretylium in excess of 1 ng are measured easily. The bretylium plasma levels in the

anesthetized dog were considerably higher than are seen in humans at similar dosages of bretylium [5]. However, we still have shown the ability to measure bretylium in quantities as small as one nanogram from plasma standards.

Benzenethiol and *o*-bromobenzyl phenyl thioether were easily separated by a number of column packings (see Apparatus) with retention times for the *o*-bromobenzyl phenyl thioether of 3.2—5.0 min and for *o*-iodobenzyl phenyl thioether of 4.2—6.2 min. No peaks with higher retention times were seen. This allows relatively rapid quantitation of injected samples.

The above assay possesses the required specificity, sensitivity and ease of sample preparation needed for pharmacokinetic studies of bretylium in man and animals.

ACKNOWLEDGEMENT

This study was supported by a grant from the United States Public Health Service HLBI-HL-05806-19.

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PAIRED-ION CHROMATOGRAPHIC ANALYSIS OF TAMOXIFEN AND TWO MAJOR METABOLITES IN PLASMA

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(First received May 8th, 1979; revised manuscript received August 21st, 1979)

SUMMARY

A method is described for the clinical analysis of the non-steroidal anti-estrogenic, anti-neoplastic agent, tamoxifen and its 4-hydroxy and N-desmethyl metabolites in human plasma. The analytes are extracted from biological fluid with diethyl ether and subsequently converted to fluorescent phenanthrene derivatives by irradiation with UV light. The fluorophores are separated by paired-ion chromatography on a reversed-phase (C_{18}) column. Spectrofluorometric monitoring of the column eluent allows quantitation of analytes as their phenanthrene derivatives to levels of 100 pg/ml of plasma.

INTRODUCTION

Tamoxifen (I; ICI 46,474 or Nolvadex, Imperial Chemical Industries, Macclesfield, Great Britain) is a non-steroidal anti-estrogenic compound being used successfully in the treatment of metastatic breast cancer [1—5]. We recently described [6] an analytical method for monitoring tamoxifen and its 4-hydroxy derivative (II) in plasma. After extraction from biological fluid, the analytes are photochemically converted to fluorescent phenanthrene derivatives which are then separated by liquid chromatography on a μBondapak CN column. Spectrofluorometric monitoring of the column eluent allows the detection of approximately 2 ng of analyte per ml of biological fluid.

This method has now required modification in light of recent findings by Adam et al. [7] indicating that a major route of detoxication of tamoxifen (in addition to aromatic hydroxylation [8]) involves hepatic N-demethylation producing desmethyltamoxifen (III) and that this metabolite may in itself possess

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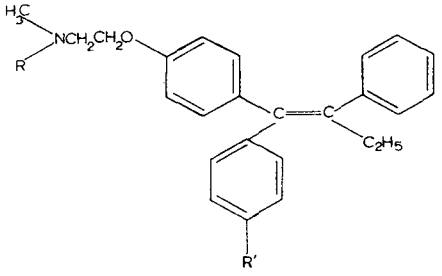
anti-estrogenic activity. Furthermore, the fluorescent products formed from I and II were somewhat unstable in the photolysis media, requiring that reaction times be strictly controlled (± 2 min) to avoid loss in fluorescence during reaction.



I : R = CH_3, R' = H
II : R = CH_3, R' = HO
III : R = H, R' = H

MATERIALS AND METHODS

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A solvent delivery system, Model U6K injector, Model 440 dual-channel absorbance detector, operated at 254 nm, and a Schoeffel FS 970 fluorescence LC detector (Schoeffel, Westwood, N.J., U.S.A.). The excitation monochromator of this detector was fixed at 256 nm and emitted radiation was passed through a filter with 340-nm cut-off. A 30 cm × 4.6 mm I.D. μBondapak C_{18} 10-μm particle size column (Waters Assoc.) was used for all separations.

Materials

Tamoxifen, 4-hydroxytamoxifen (II) and desmethyltamoxifen (III) were provided by Imperial Chemical Industries and used without further purification. Mobile phase for high-performance liquid chromatography (HPLC) was prepared with Fisher HPLC grade methanol (Fairlawn, N.J., U.S.A.). Sodium pentanesulfonate was purchased from Eastman (Rochester, N.Y., U.S.A.) and used as obtained.

Procedure

Samples (5 ml) of fresh plasma containing known amounts of I, II and III (prepared by the addition of 25—100-μl aliquots of methanolic stock solutions of I, II or III to 5 ml of plasma) were transferred to 45-ml, heavy-duty screw cap (with PTFE liner) centrifuge tubes and extracted once with 25 ml of diethyl ether (AR) for 20 min. The phases were subsequently separated by centrifugation of the mixture (1300 *g* for 15 min) and the aqueous layer was frozen in a dry-ice—acetone bath. An aliquot of the diethyl ether layer (20 ml) was then removed and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with vortex mixing in 1 ml of HPLC mobile phase [methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* sodium pentanesulfonate].

Photochemical activation

This solution was then transferred to 1-cm^2 quartz cuvettes and irradiated for 20 min with a 15-W Hg vapor lamp (maximum output at 253.7 nm; General Electric No. G15T8) placed 10 cm from the quartz reaction vessel. Optimum reaction conditions were determined by irradiating solutions of tamoxifen, II and III, in various solvent mixtures, varying the distance between the lamp and the cell, and monitoring fluorescent intensity as a function of time.

Chromatographic analysis

A 50—200-μl aliquot of the reaction mixture was then chromatographed on a μBondapak C_{18} column. Components were eluted isocratically with a mobile phase consisting of methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* sodium pentanesulfonate.

Components were quantitated as the photolyzed product by measuring peak area and comparing it with a standard curve constructed after analysis of plasma samples containing known amounts of I, II or III over the concentration range of 0.1—500 ng/ml of plasma (i.e. 0.1, 0.5, 1.0, 5.0, 10, 50, 100, 250, 500 ng/ml). Calibration curves used to validate the assay were thus prepared from samples containing nine different concentrations of analyte (and a blank). Four samples were prepared at each concentration and each sample was analyzed in triplicate. For routine assays only five concentrations were used (0.1, 1.0, 5.0, 50, 250 ng/ml) to prepare calibration curves and samples were prepared and analyzed in duplicate.

RESULTS

Extraction procedures

Tamoxifen (I) and its 4-hydroxy (II) and desmethyl (III) metabolites were removed from plasma by a single extraction with 4 volumes of diethyl ether. This extraction afforded an average recovery of 89 ± 4% for tamoxifen, 97 ± 4% for II and 83 ± 4% for III over the concentration range stated. Recoveries at specific concentrations are provided in Table I. During extraction, I and III are stable; however, II is converted to the corresponding phenanthrene derivative to an extent of ca. 50% over the 20-min extraction period. This decomposition does not affect the overall analysis, since II is ultimately quantitated as the corresponding phenanthrene. Chloroform, methylene chloride and ethyl acetate were poorer extractants for these analytes, having less favorable partition characteristics and being less selective (i.e. resulting in co-extraction of additional contaminants). Less polar solvents proved unsuitable since they resulted in reduction in extraction efficiency of I, II and III, apparently due to adsorption of analytes to container surfaces in these more lipophilic solvents, as has been reported by Thakker et al. [9] for other hydrophobic drugs. Ion-pair extraction [6] of I, II and III (in plasma adjusted to pH 1 with sulfuric acid) with diethyl ether and 0.1 *M* trichloroacetate offered no advantage over direct extraction and in fact was more time consuming and produced a more cluttered chromatogram. Addition of ion-pairing agents did improve the extractability of I, II and III into non-polar solvents (as previously seen by Thakker et al. [9], but not to efficiencies obtained with diethyl ether as extractant.

TABLE I

RECOVERY OF TAMOXIFEN AND METABOLITES FROM PLASMA

Concentration* (ng/ml) in plasma	Recovery (%)**,***		
	Compound I	Compound II	Compound III
0.10 §	86 ± 6	91 ± 5	87 ± 5
0.50	91 ± 5	96 ± 3	88 ± 4
1.0	87 ± 1	101 ± 5	86 ± 2
5.0	96 ± 2	94 ± 2	79 ± 5
10.0	86 ± 5	95 ± 4	83 ± 2
50	92 ± 3	98 ± 3	85 ± 1
100	84 ± 4	100 ± 2	78 ± 5
250	90 ± 3	96 ± 2	79 ± 2
500	88 ± 1	104 ± 5	80 ± 3

* Known amount of analyte added to known volume of human plasma, as described in Materials and methods.

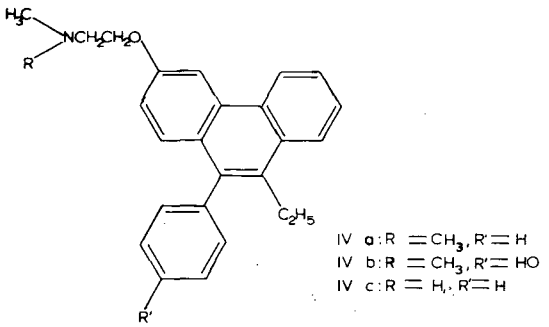
** Determined as the ratio of the area of the analyte peak obtained after carrying out the assay on a plasma sample (5 ml) spiked with a known amount of I, II or III; to the area of the analyte peak resulting after carrying out the assay on the same amount of I, II or III added to the mobile phase (5 ml).

*** Represents the average recovery ± S.D. for four plasma samples containing the same known concentration of analyte. Each sample is analyzed chromatographically in triplicate.

§ Represents the detection limit, defined as the minimum detectable fluorescent intensity which is three times the baseline level.

Photochemical conversion of analytes to fluorescent products

Tamoxifen, II and III exhibit no native fluorescence. We have previously demonstrated [6] that irradiation of solutions of I or II with UV light converts these materials to the corresponding phenanthrenes (IVa and b) which are strongly fluorescent (λ_{ex} = 256 nm; λ_{em} = 320 nm). These products (IVa and b) degrade in the photolysis media resulting in a loss of fluorescence with time [6]. Photochemical activation of III appeared to proceed through the identical reaction sequence, producing the corresponding fluorescent phenanthrene (IVc), which then decomposed. The addition of a small amount of acid to the photolysis media significantly inhibited the degradation of IVa—c to non-fluorescent products. The stabilizing influence of acid was observed in various photolysis media [dioxane—heptane (70:30); methanol; HPLC mobile phase]



IV a: R = CH_3, R' = H
IV b: R = CH_3, R' = HO
IV c: R = H, R' = H

and held for IVa—c. HPLC mobile phase was selected as the reaction medium since it offered maximum yield and stability of IVa—c and subsequently gave rise to the best chromatographic properties. Irradiation time and distance of the reaction vessel from the lamp were adjusted to maximize the rate of formation of fluorophores while minimizing degradation of the fluorophores in a manner analogous to that previously described [6]. An irradiation distance of 10 cm was chosen and photolysis carried out for 20 min. Conversion to phenanthrene was quantitative under these conditions. The formation of IV followed first-order kinetics (Fig. 1). The rate constants for formation of IV from I, II, and III are given in Table II.

TABLE II

RATE OF FORMATION OF PHENANTHRENES (IV) FROM PHENYLSTILBENES

Irradiation carried out in methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* pentanesulfonic acid. Distance between lamp and sample cuvette was 10 cm. Reaction carried out as described in experimental sections.

Reaction*	k** (min^{-1})	$t_{1/2}$ (min)
I → IVa	0.15	4.6
II → IVb	0.29	2.4
III → IVc	0.11	6.5

* I, II, III present initially at concentration of 50 ng/ml.
** Reaction followed first-order kinetics.

Reaction was thus complete in 30 min. Degradation of IV ($\leqslant$ 10%) was observed only after photolysis had proceeded for 40 min; therefore, photolysis conditions need not be adhered to as strictly as those previously described [6]. Fig. 1 also shows that the fluorescence intensity of IVa is approximately 1.2 times that of IVc and 0.8 times that of IVb. The reason for this difference in relative intensities has not been determined but apparently resides with differences in either the molar absorptivity of the stilbenes or phenanthrenes or quantum yield of the latter.

Chromatographic analysis

Separation of components (I, II, III, IVa—c) could not be accomplished by reversed-phase or normal-phase chromatography, because of the inability to resolve compound IVb and IVc. However, using paired-ion chromatography with a reversed-phase (C_{18}) column, separation of tamoxifen species and corresponding phenanthrenes in biological samples could be accomplished. Using methanol—water mixtures and alkylsulfonate ion-pairing agents, the capacity factors (k') for IVa—c were most sensitive to change over the range 70—80% methanol, varying from ca. $k' = 1.9$ to 5 for tamoxifen and over a similar range for IVb and IVc. The system was much less sensitive to the nature of the ion-pairing agent. Using a mobile phase of methanol—water (80:20) k' for tamoxifen was 1.9 when 1-pentanesulfonate (2.5 m*M*) was used as counter-ion and 2.3 when 1-decanesulfonate (2.5 m*M*) was chosen as ion-pairing agent.

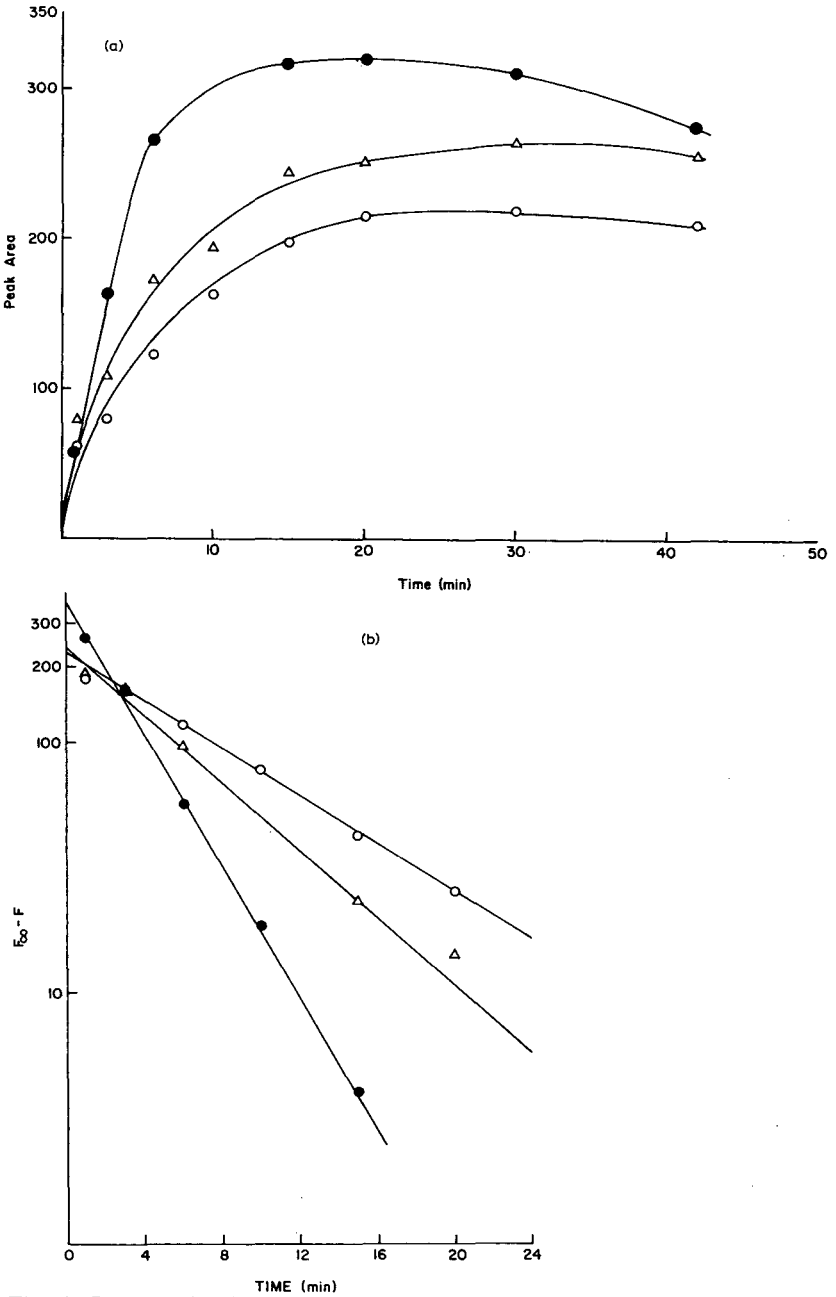


Fig. 1. Increase in fluorescence occurring when tamoxifen (△), N-desmethyltamoxifen (○) and 4-hydroxytamoxifen (●), extracted from plasma, are irradiated with UV light (distance between light and reaction vessel = 10 cm). Reaction carried out as described in the text. Data presented (a) on cartesian coordinates and (b) as a semi-logarithmic plot.

Separation of analytes with maximum resolution from each other and from co-extracted contaminants was obtained using a mobile phase of methanol—water (73:27) containing 0.5% acetic acid and sodium pentanesulfonate (2.5

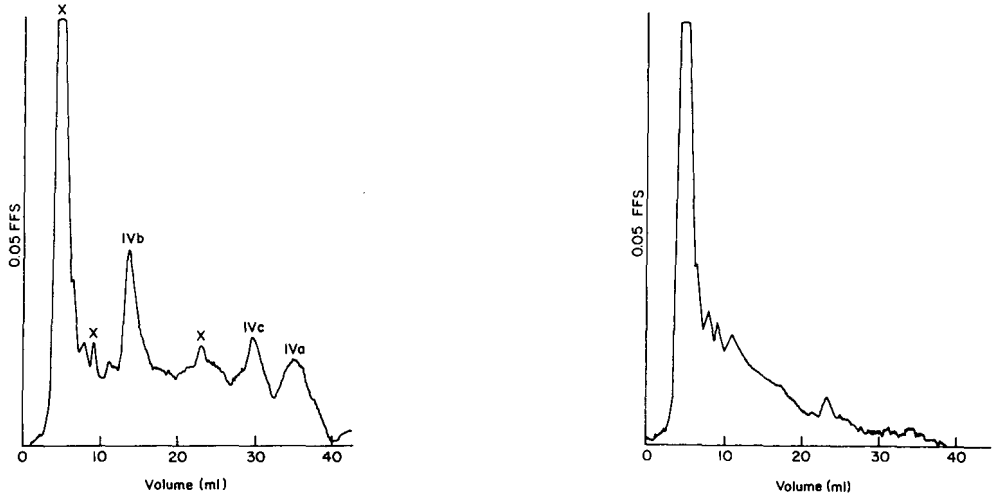


Fig. 2. Chromatography of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen as their phenanthrene derivatives (IVa—c) obtained by extraction of I, II and III from plasma (each component present at a concentration of 1 ng/ml) and irradiation with UV light (as described in the text). Components were separated by paired-ion reversed-phase (C_{18}) partition chromatography using methanol—water (73:27) containing 0.5% acetic acid and 2.5 m*M* sodium pentanesulfonate as mobile phase. Flow-rate = 2 ml/min. Peaks at V_R = 7 and 22 ml (×) represent co-extracted contaminants also found in plasma blanks.

Fig. 3. A plasma blank containing none of the drug species, but carried through the analytical sequence.

m*M*) (Fig. 2). The retention volumes (V_r) for the analytes are as follows: I, 26 ml; II, 12 ml; III, 24 ml; IVa, 34 ml; IVb, 13.6 ml; IVc, 27.2 ml. Under these conditions no interference was observed from extraneous peaks seen in plasma blanks (Fig. 3).

Components were quantitated as IV by measuring peak area, which was linearly related to concentration of I, II or III in the range 0.1—500 ng/ml of plasma. A standard curve constructed after analysis of plasma samples containing known amounts of I, II and III over this concentration range resulted in the line $y = 0.17x + 3.44$ (correlation coefficient: 0.999) for tamoxifen (monitored as IVa) when subjected to linear regression analysis, the line $y = 0.16x + 1.44$ (correlation coefficient: 0.999) for 4-hydroxytamoxifen (as IVb) and the line $y = 0.21x + 4.99$ (correlation coefficient: 0.998) for N-desmethyltamoxifen (as IVc). Less than 4% variation in these curves was observed as day-to-day variation in the response factor. Over this concentration range, when samples were subjected to the analysis scheme described here, overall analytical recovery of tamoxifen from plasma was 89 ± 4% (n = 9), recovery of II was 97 ± 4% (n = 9) and recovery of III was 83 ± 4% (n = 9). The detection limits for I, II and III are 0.1 ng/ml of plasma at the 99% confidence level as measured as their fluorescent derivatives and as determined by direct analysis of drug-supplemented plasma samples at 100 pg/ml vs. plasma blanks.

This method provides suitable sensitivity to monitor therapeutic levels of

tamoxifen [6, 10] for at least 24 h after oral administration of the drug (dose: 20 mg, daily). 4-Hydroxytamoxifen is a minor metabolite [7] and appears at levels $\leqslant$ 20 ng/ml. The major metabolite, III, was detected ($<$ 10 ng/ml) 1 h after the initial dose and reached steady-state levels of 250—350 ng/ml after approximately 40 doses (3 weeks). The sensitivity of the method permits the monitoring of the drug and two of its metabolites for long periods of time and offers an opportunity for also following subtherapeutic amounts of drug.

DISCUSSION

Tamoxifen and its metabolites II and III can be conveniently and efficiently removed from biological fluid by a single extraction with diethyl ether. The difficulty suggested by other groups [7] in detecting appreciable levels of II is apparently due to its facile conversion to IVb during the extraction and, therefore, would only be observed when the methodology dictates first monitoring II as the phenanthrene, IVb, and secondly, forming this derivative prior to the chromatographic step.

The increased stability of IVa—c observed in acid solution may be caused by protonation of the amine function, eliminating the nucleophilic character of the nitrogen. We previously showed [6] that decomposition of IV to less fluorescent products involves loss of the aminoalkanol side chain, which could be initiated by intramolecular nucleophilic attack by the amine nitrogen. Depression of nucleophilicity would thus increase stability.

Chromatographic separation of IVa—c could not be accomplished by either reversed-phase or normal-phase chromatography because resolution of peaks arising from IVb and IVc could not be achieved. Paired ion chromatography with a reversed-phase (C_{18}) column provided resolution of all peaks of interest. The small effect observed in k' in varying the length of the alkyl chain on the hetaeron from C_5 to C_{10} is most likely due to the inherent hydrophobicity of IVa—c and, therefore, partitioning of the ion-pair is only minimally affected by the chain length of the alkylsulfonate.

This tamoxifen assay has the following advantages over the previously reported procedure [6]: (1) it allows simultaneous and selective monitoring of I, II and III; (2) the photolysis products (IVa—c) are more stable in the reaction media; (3) a cleaner chromatogram is produced because photolysis is carried out in the mobile phase and (4) sensitivity is improved by a factor of ca. 20. The method is currently being used to monitor tamoxifen and its 4-hydroxy and desmethyl metabolites in the plasma of women who have been administered the drug in the management of breast cancer.

ACKNOWLEDGEMENTS

This work was supported in part by training grant CA-09242 awarded by the National Cancer Institute, National Institutes of Health (PHS, DHEW) and a grant from Imperial Chemical Industries, Americas.

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Journal of Chromatography, 181 (1980) 51—57
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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATIONS OF AMINOPYRINE AND ITS METABOLITES IN MAN

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(First received June 1st, 1979; revised manuscript received August 6th, 1979)

SUMMARY

A quantitative high-performance liquid chromatographic method, using a polystyrene—divinyl benzene (Hitachi No. 3010 gel) column and aqueous methanol as the mobile phase, was employed for the determination of aminopyrine and its related compounds, 4-acetylaminoantipyrine, 4-aminoantipyrine and 4-monomethylaminoantipyrine. Baseline separation could be achieved within 25 min. The method was applied to the recovery of these materials from control urine and human urine. Before separation human urine was adjusted to pH 9 and extracted with ethyl acetate, chloroform and diethyl ether.

INTRODUCTION

Aminopyrine (AM) has widely been used as an analgesic and antipyretic drug. The following compounds are known as the main metabolites of aminopyrine in human urine: 4-acetylaminoantipyrine (4-AcAA), 4-aminoantipyrine (4-AA) and 4-monomethylaminoantipyrine (4-MAA).

Common methods for the analysis of aminopyrine and its metabolites are based on spectrophotometric [1—4], gas chromatographic [5] and mass fragmentographic [6] determinations. High-performance liquid chromatography (HPLC) has been applied to the determination of antipyrine in biological fluids [7].

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In the present paper, rapid HPLC was introduced for the determination of AM metabolites without any derivatization using a porous polymer gel column. We report the separation and individual quantitation of these metabolites in less than 25 min and the application of the method to human urine and the recovery test. The technique can also provide a simple and inexpensive method for the HPLC analysis of aminopyrine and its metabolites in biological fluids.

MATERIALS AND METHOD

Apparatus

An Hitachi Model 635 high-pressure liquid chromatograph equipped with an Hitachi spectrophotometer Model 200-10, and Model 834 chromatoprocessor was used for the analyses. The detector wavelength was 254 nm. Two grams of Hitachi gel No. 3010 porous liquid chromatographic packing, particle size 15—20 μm, were suspended in 10 ml of methanol. After brief vigorous shaking of the suspension, the packing was swollen by allowing to stand for 1 h. Two-thirds of the supernatant was discarded, then the remaining suspension was shaken vigorously and introduced into the stainless-steel column (2.1 mm × 500 mm) through the packing apparatus. Samples (1—2 μl) were injected through a septum using a Hamilton HP 305 syringe. The mobile phase was methanol—water (1:1) and was degassed by sonicating for 10 min before use. The column was maintained at 30° by a regulated water jacket, and the flow-rate was 1.2 ml/min.

Reagents

JP* grade aminopyrine was recrystallized from ligroin (b.p. 77—80° fractions). 4-Aminoantipyrine and 4-acetylaminoantipyrine were obtained from Wako Pure Chemical (Tokyo, Japan) and purified by recrystallization with chloroform for 4-AA and with diluted ethanol for 4-AcAA. 4-Monomethylaminoantipyrine was prepared by Morita's method [8].

Pyrrole 2-carboxylic acid was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and recrystallized from diluted ethanol before use.

TEK-CHEK® No.1 (control urine for routine analysis) was purchased from Ames (Elkhart, Ind., U.S.A.), and reconstituted by the addition of 15 ml of water before use.

Procedure for recovery test

To 10 ml of human fresh urine excreted from healthy adults, 312.5 μg each of authentic 4-AcAA, 4-AA, 4-MAA and AM were added, and the urine sample was brought to pH 9 with 1 *N* NaOH. The urine was then concentrated to a small volume, and extracted successively with 30 ml each of ethyl acetate, chloroform, and ethyl ether. The combined extracts were dehydrated over anhydrous sodium sulfate.

To the dried extract, 250 μg of pyrrole 2-carboxylic acid as an ethanolic solution were added as an internal standard. The organic phase was evaporated to dryness in vacuo. A stream of nitrogen gas was passed through the residue, and 50 μl of methanol were added to dissolve AM and its metabolites. A 1—2-μl aliquot of this solution was injected into the chromatograph which was

*JP = Pharmacopeia of Japan.

equipped with an ultraviolet (UV) spectrophotometer (254 nm).

AM and its metabolites were eluted with methanol—water (1:1). The quantities of these materials were determined by comparing the peak areas with that of pyrrole 2-carboxylic acid.

RESULTS AND DISCUSSION

The structures of aminopyrine and its major metabolites are shown in the metabolic pathway in Fig. 1. As can be seen from Fig. 2, a typical HPLC elution pattern was observed after the injection of a mixture of 4-acetylaminoantipyrine, 4-aminoantipyrine, 4-monomethylaminoantipyrine, aminopyrine and pyrrole 2-carboxylic acid as internal standard. An almost complete separation of these compounds was accomplished in less than 25 min, the retention times (min) being 4-AcAA 3.4, 4-AA 7.3, 4-MAA 12.1, AM 19.8. Therefore, the baseline separation obtained using Hitachi gel No. 3010 with aqueous methanol as the mobile phase allows for the individual quantitation of these compounds.

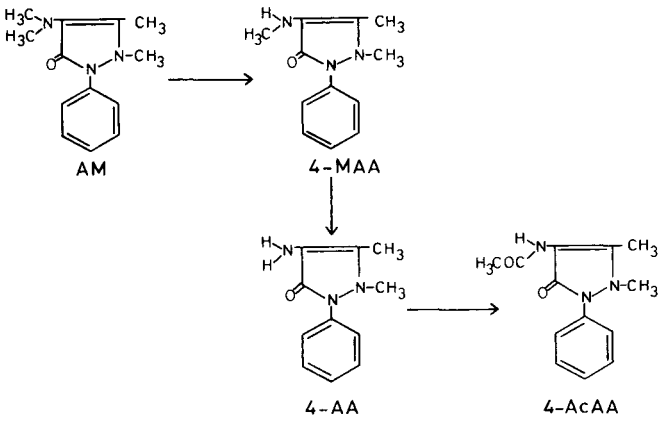

Fig. 1. Structures of aminopyrine and related compounds.

The linear relationship between the peak area ratio for aminopyrine, its metabolites and pyrrole 2-carboxylic acid and the amount of these compounds present per injection is shown in Fig. 3. The areas of the chromatographic peaks were calculated by chromatoprocessor.

The accuracy of determining AM and its metabolites by this method was found to be better than ± 2.5%, with correlation coefficients between 0.997 and 0.999.

Table I shows the results of analysing known amounts of AM and its related compounds by HPLC. The results show that these compounds are well determined in the range 0.44—20 μg for 4-AcAA, 4-AA and 4-MAA, and 5.0—20 μg for AM. The coefficients of variation for the method are below 5% (n=5) for 4-AcAA and 4-AA at 0.45 μg, for 4-MAA at 0.56 μg, and for AM at 5.0 μg. The detection limits were 25, 50, 70 and 120 ng for 4-AcAA, 4-AA, 4-MAA and AM, respectively, with a signal-to-noise ratio of 2:1.

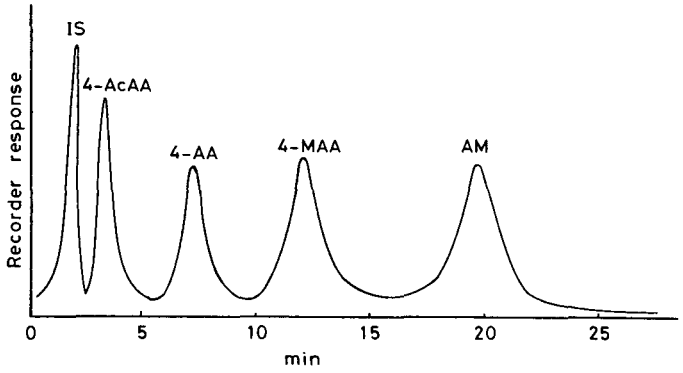

Fig. 2. Typical HPLC elution pattern for a standard mixture of 4-acetylaminoantipyrine (4-AcAA, 5.0 μg), 4-aminoantipyrine (4-AA, 5.0 μg), 4-monomethylaminoantipyrine (4-MAA, 5.0 μg) and aminopyrine (AM, 25.0 μg), with pyrrole 2-carboxylic acid (IS, 2.5 μg) as an internal standard.

The method was examined for the possible interference of urinary constituents. Human urine has many constituents such as uric acid, urea, creatine, hippuric acid, indican, hormones and enzymes. It is crucial that such compounds are not extracted from the urine by the organic solvents. Urine was therefore adjusted to various pH values in the range 2—9, and the solvent extraction for AM and its metabolites carried out. Fig. 4 shows chromatograms of AM and its metabolites extracted from human urine at various pH values. The extracts at pH 2 or 3 contained significant amounts of urine constituents, and each peak overlapped with the peak of the internal standard and 4-AcAA.

With the absorption measured at 254 nm, and the pH of the urine gradually raised to 4, 5 and 6, significant amounts of urine constituents were similarly ex-

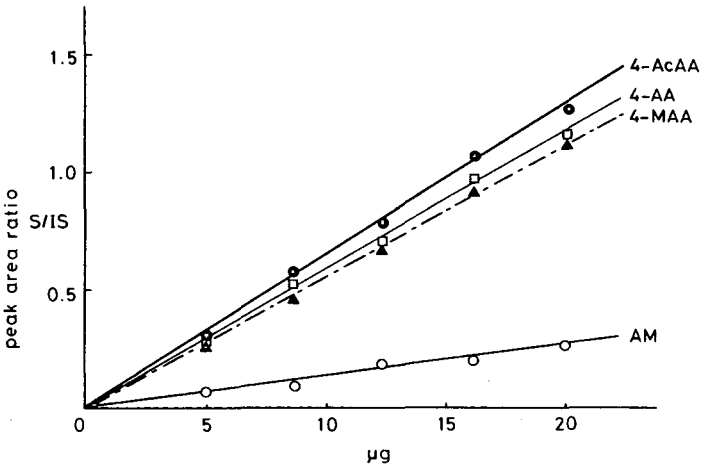

Fig. 3. Calibration curves for aminopyrine and its metabolites with pyrrole 2-carboxylic acid as an internal standard. Each point represents the average of at least five determinations.

TABLE I

DETERMINATION OF AMINOPYRINE AND ITS RELATED COMPOUNDS

$n = 5$.

Amount taken (μg)	4-AcAA		4-AA		4-MAA		AM	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
20.00	98.9	1.0	99.7	2.4	96.9	2.6	100.9	2.2
16.25	100.4	1.0	103.2	2.0	98.9	4.3	101.0	1.5
12.50	95.2	0.7	99.8	2.3	99.6	3.3	100.0	1.5
8.75	101.4	0.6	95.4	1.1	95.5	2.6	99.8	2.2
5.00	95.6	2.5	92.4	2.5	99.2	1.9	109.4	2.3
1.11	97.3	3.7	100.0	2.7	96.8	3.4	96.4	5.6
0.56	98.2	3.6	99.6	2.9	98.0	4.6		
0.444	97.7	4.7	96.4	2.7	95.9	5.6		
0.088	94.3	6.0	90.9	8.0	90.9	8.8		

tracted by the solvent. The extracts at pH 8 also contained a small amount of the urine constituents, but the extracts at pH 9 contained almost none.

On the basis of the analytical results and the properties of the AM metabolites, the following method was used in extraction procedures from urine. The pH of human urine was adjusted to 9, then the urine was concentrated to approx. 1 ml for the solvent extraction, because 4-AcAA is highly soluble in water [9].

In the light of the amount of aminopyrine given as a single dose from a Pharmacopeia of Japan, approximately 300 μg each of aminopyrine and its metabolites were added to 10 ml of human urine from healthy volunteers. After ad-

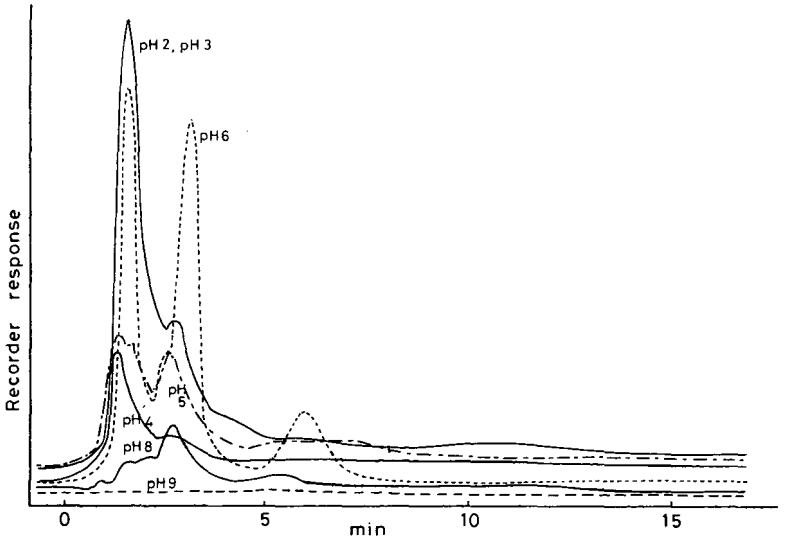

Fig. 4. HPLC of the extracts obtained by solvent extraction of freshly voided human urine at several pH values without the addition of any materials to the urine.

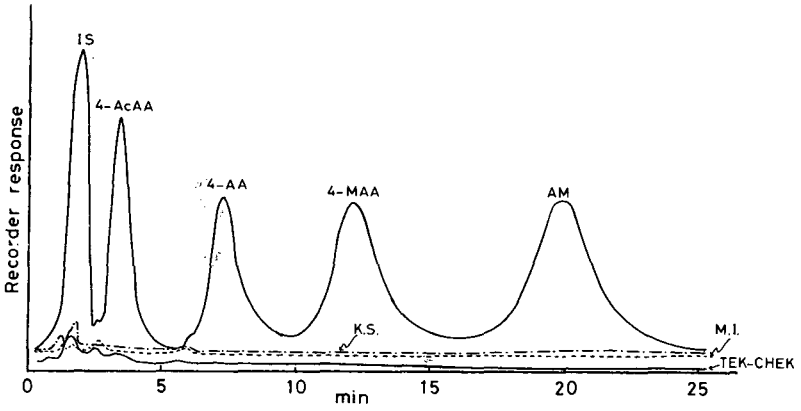

Fig. 5. HPLC of aminopyrine and its metabolites after they had been added to human urine and extracted with the organic solvents at pH 9.

justing the pH of the urine to 9, ethyl acetate, chloroform and ethyl ether extractions were carried out by mechanical shaking for 10 min with 30 ml of each solvent. The HPLC chromatograms of the combined extracts are shown in Fig. 5. The two chromatograms drawn with dotted and broken lines represent those of the blank test, in which AM and its metabolites had not been added to the urine. Furthermore, as the control experiment, the chromatogram of the "control" urine, which is usually utilized as a sensitivity control of the urinary test, is shown by a solid line. The chromatogram thus obtained showed contamination by a small amount of endogenous urinary constituents, which appeared as small peaks and shoulders on the chromatogram. The values for determination were not corrected in this case.

Table II shows the results of the recovery test for AM and its metabolites added to human urine.

TABLE II

RECOVERY OF AM AND ITS METABOLITES ADDED TO HUMAN URINE

Values are given as mean ± S.D. (n=5).

Urine sample	4-AcAA (31.25 μg)	4-AA (31.25 μg)	4-MAA (31.25 μg)	AM (31.25 μg)
TEK-CHEK®	29.87 ± 0.81 (95.5 ± 2.62%)	27.13 ± 1.32 (86.8 ± 4.23%)	23.71 ± 0.70 (75.9 ± 2.25%)	27.53 ± 5.77 (88.1 ± 18.5%)
K.S.	30.00 ± 0.24 (96.1 ± 0.78%)	26.88 ± 1.76 (86.0 ± 5.64%)	28.95 ± 1.44 (92.6 ± 4.60%)	30.11 ± 8.19 (96.3 ± 26.2%)
M.I.	22.40 ± 1.46 (73.1 ± 5.0%)	24.25 ± 2.97 (77.6 ± 9.5%)	25.64 ± 1.41 (82.0 ± 3.8%)	26.43 ± 7.98 (84.6 ± 25.5%)
T.N.	30.80 ± 1.45 (98.5 ± 4.65%)	30.97 ± 1.42 (99.1 ± 4.55%)	27.78 ± 2.81 (88.9 ± 9.00%)	31.52 ± 4.29 (100.9 ± 13.7%)

The idiosyncrasies of the volunteers seem to be revealed as differences in recoveries. The percentage recovery of all AM related compounds is found to be 89% on the average, when the recovery data are included for TEK-CHEK®.

The determination described here for AM and its metabolites has advantages that include complete baseline separation and specific identification of AM metabolites but do not involve special and time-consuming procedures. In addition, the method can also be applied to the determination of aminopyrine and its metabolites in urine without further purification and derivatization.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T. Nagatomo and Mr. M. Ikeda for helpful discussions and supplying human urine samples.

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Journal of Chromatography, 181 (1980) 59—65
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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CARBAMAZEPINE METABOLITES EXCRETED IN RAT URINE

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(Received July 3rd, 1979)

SUMMARY

A procedure for the separation and isolation of the urinary metabolites of carbamazepine by reversed-phase high-performance liquid chromatography is described. After extraction from urine, the metabolites were separated on either an analytical or semi-preparative C_{18} μBondapak column by gradient elution with methanol—water—acetic acid. Following derivatization the metabolites isolated by the use of the semi-preparative column were analyzed by gas chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

Carbamazepine (5*H*-dibenzo-[*b*,*f*]azepine-5-carboxamide) is an effective agent for the control of epileptic seizures particularly those of psychomotor epilepsy. Although the drug was introduced in the early 1960's, 25—50% of the metabolites excreted in urine have not been identified. In the initial studies [1] two possible metabolites were detected in cerebrospinal fluid by thin-layer chromatography (TLC). Subsequently seven metabolites were detected by TLC in the urine of patients receiving carbamazepine (CBZ) chronically [2]. Carbamazepine 10,11-epoxide and 10,11-dihydroxy-10,11-dihydrocarbamazepine were identified by gas chromatography—mass spectrometry (GC—MS) in 1972—1973 [3, 4]. In subsequent studies, iminostilbene [5—8], *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine [9] and 1-hydroxy-, 2-hydroxy- and 3-hydroxycarbamazepine and 9-hydroxymethyl-10-carbamoylacridan [8] were identified as urinary metabolites by MS. The glucuronide metabolites of carbamazepine have also been investigated by MS. In 1976, Bauer et al. [10] isolated an N-glucuronide of carbamazepine from rat liver perfusate and more recently eight new glucuronide conjugates were identified as urinary metabolites in man [11].

The identification of metabolites of carbamazepine by GC and GC—MS is difficult because the metabolites are not resolved on packed columns and extensive rearrangement to iminostilbene and acridine derivatives occurs during GC analysis [5]. Because of the mild conditions employed, high-performance liquid chromatography (HPLC) provides another approach to this analytical problem. Methods based on HPLC have been employed for the quantification of CBZ and CBZ epoxide in plasma [12] and in saliva [13]. In this paper we describe an HPLC method for the separation and isolation of the metabolites of carbamazepine excreted in rat urine.

EXPERIMENTAL

Reagents

All reagents were analytical grade. Glass distilled methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). The HPLC grade acetic acid was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.). The 3% OV-17 column packing and bis-trimethylsilylacetamide (BSA) were purchased from Applied Science Labs. (State College, Pa., U.S.A.). Glusulase was obtained from Endo Labs. (Garden City, N.Y., U.S.A.).

Carbamazepine and carbamazepine epoxide were obtained from Ciba-Geigy (Ardsley, N.Y., U.S.A.). Iminostilbene, acridine and 10,11-dihydrocarbamazepine were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). The *cis*-10,11-dihydrodiol of carbamazepine and 10-hydroxy-10,11-dihydrocarbamazepine were a gift from Prof. A. Frigerio.

Instrumentation

High-performance liquid chromatography. C_{18} μBondapak analytical columns (3.9 mm × 30 cm) and semi-preparative columns (7.8 mm × 30 cm) were obtained from Waters Assoc. (Milford, Mass., U.S.A.). Reversed-phase HPLC analyses were carried out by gradient elution utilizing a dual solvent delivery system (Waters Assoc., Model 6000A), a solvent programmer (Waters Assoc., Model 660) and a UV-III detector (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) set at 254 nm. OmniScribe recorders (Houston Instruments, Austin, Texas, U.S.A.) were employed.

Gas chromatography and gas chromatography—mass spectrometry. GC separations were carried out on 3.7 m × 2 mm glass W columns packed with 3% OV-17. The analyses were temperature programmed from 150° at 2°/min. The MS analyses were carried out on a LKB 9000-PDP/12 analytical system using 1.85 m × 2 mm glass coil columns packed with 3% OV-17. Separations were programmed from 170° at 5°/min.

Animal procedure

Male Sprague-Dawley rats (200 g) received 20 mg of carbamazepine daily for 8 days in special rat biscuits. The biscuits were prepared by adding 22 ml of a warm 18% solution of gelatin to 9 g of ground Purina rat chow in a 3 in. square plastic weighing boat. After mixing thoroughly, 20 mg of carbamazepine was stirred into the paste which solidified on cooling. The biscuits were made daily and kept in the refrigerator until used. The rats were housed individually in

metabolism cages and 24-h urine samples were collected daily. The urine samples were stored at −20°.

Sample preparation

After enzymatic hydrolysis of the urine with glusulase at pH 4.5—4.8 for 17 h at 37°, the carbamazepine metabolites were extracted by the ammonium carbonate—ethyl acetate procedure [14]. For profiling metabolites on an analytical column, an aliquot (usually 1/10) of a diluted 24-h urine sample was used. The urine extraction was carried out in a centrifuge tube fitted with a PTFE-lined screw cap. For collection of metabolites from a semi-preparative column, an ammonium carbonate—ethyl acetate extract of a complete 24-h urine sample was used; the extraction was carried out in a separatory funnel. The extracts were taken to dryness, redissolved in methanol and transferred to Reacti-vials. The final volume of the analytical sample was 20—50 μl and the final volume of sample for analysis on the semi-preparative column was 150 μl. After centrifugation of the sample, an aliquot was injected onto the HPLC column; 1—2 μl were used with the analytical column and 20—50 μl were used with the semi-preparative column.

High-performance liquid chromatographic analysis

When the separations were carried out with an analytical column, a 40-min gradient system (gradient 6) was used with a column pressure of 1100—1200 p.s.i. and a flow-rate of 1.2 ml/min. The solvent system consisted of solvent A: methanol—water—acetic acid (20:80:0.1) and solvent B: methanol—water—acetic acid (50:50:0.1). The percentage of B varied from 10 to 90. A modified solvent system was used with a semi-preparative column and consisted of solvent A: methanol—water—acetic acid (33:67:0.1) and solvent B: methanol—water—acetic acid (50:50:0.1). A 20-min gradient system with gradient 6 was used. The flow-rate was 1.2 ml/min and the column pressure was 800 p.s.i. The time for a single gradient analysis was 50—60 min on a semi-preparative column and 60 min on an analytical column.

Gas chromatographic and gas chromatographic—mass spectrometric analysis

The individual fractions collected from a semi-preparative column (2—5 injections) were pooled and most of the solvent was removed (Rotovap). After lyophilization of the seventeen pooled fractions, the residues were transferred with methanol to Reacti-vials. An aliquot of each residue (usually 1/3) was taken to dryness under a nitrogen stream and redissolved in 10 μl of pyridine and silylated with 10 μl of BSA. After heating at 60° for 1 h, the fractions were analyzed by GC and GC—MS. Several of the fractions contained more than one metabolite and some overlap of metabolites between adjacent fractions was observed.

RESULTS

Fig. 1a shows the separation of urinary metabolites on a semi-preparative column and Fig. 1b shows the separation of reference standards on the same column. The dotted lines in Fig. 1a show where the 17 fractions were collected.

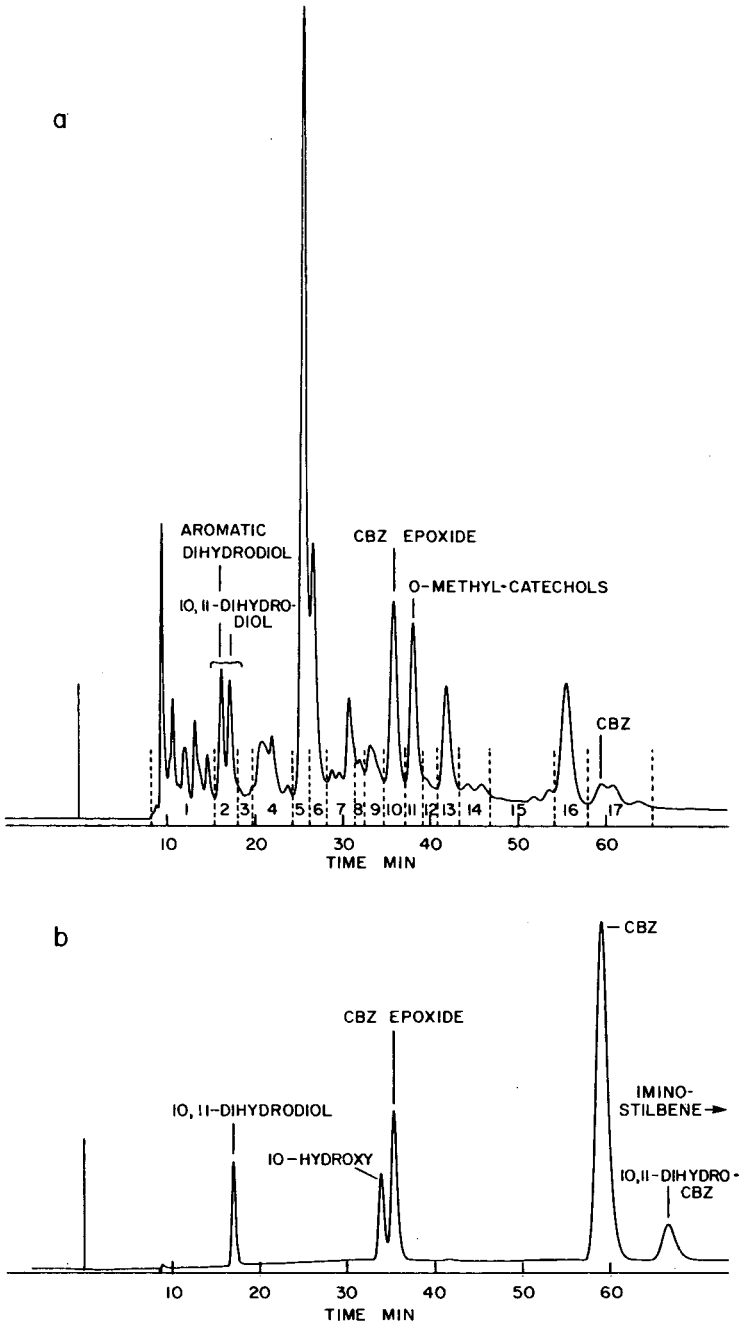

Fig. 1. HPLC separation of carbamazepine metabolites on a C_{18} μBondapak semi-preparative column by gradient elution. (a) Separation of urinary metabolites excreted by a rat after being fed carbamazepine for eight days; (b) separation of reference standards on the same column.

GC analyses of fractions 2 and 11 are shown in Fig. 2a and 2b, respectively. The major metabolite in fraction 2 was identified as a dihydrodiol of carbamazepine. This dihydrodiol had different GC and GC—MS properties from synthetic *cis*-10,11-dihydroxy-10,11-dihydrocarbamazepine [4]. The base peak in the mass spectrum of this new metabolite (TMS derivative) was observed at *m/z*

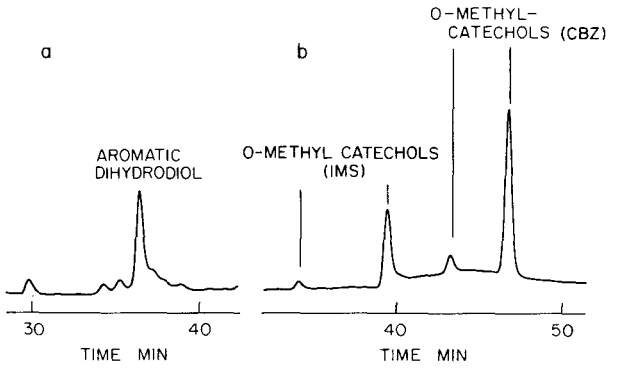

Fig. 2. GC analysis with a 3% OV-17 column of fraction 2 (a) and fraction 11 (b) collected from a semi-preparative HPLC column. The structures were determined by GC—MS analysis.

191 (Fig. 3) indicating that the dihydrodiol had been formed on one of the aromatic rings; the methylene unit (MU) of the TMS derivative was 28.0. The base peak in the mass spectrum of the *cis*-10,11-dihydrodiol of carbamazepine (TMS derivative) was observed at *m/z* 282 and the MU was 26.8. The metabolites in fraction 11 were identified by GC—MS as hydroxymethoxy derivatives of carbamazepine. The mass spectrum of one of the isomeric hydroxymethoxycarbamazepines is shown in Fig. 4. It was not possible to assign the positions of the O-methyl and hydroxyl groups from the mass spectrum, but the fragmentation indicated that the metabolite was an O-methylcatechol derivative of carbamazepine [15].

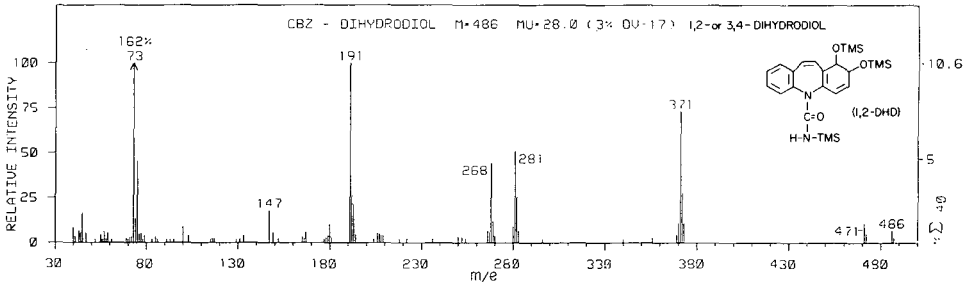

Fig. 3. Electron impact mass spectrum of the 1,2- or 3,4-dihydrodiol of carbamazepine.

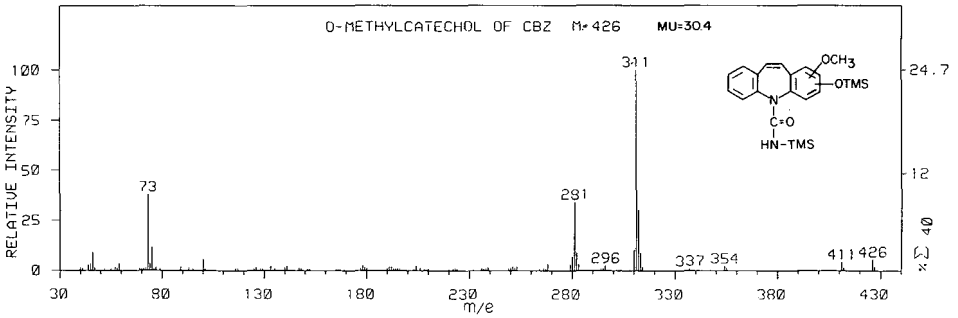

Fig. 4. Electron impact mass spectrum of one of the O-methylcatechol metabolites of carbamazepine.

DISCUSSION

The separation of metabolites of carbamazine by GC has been difficult because the metabolites are not resolved on either packed or capillary columns. However, the metabolites have been separated by TLC and the eluted metabolites analyzed by direct inlet MS [5, and references cited therein]. The structures of the metabolites have also been investigated after permethylation which permitted the GC and GC—MS analyses of the intact glucosiduronic acid derivatives as well as the unconjugated metabolites of carbamazepine [11]. In this study, we have been able to separate the metabolites by reversed-phase HPLC. The separation of the metabolites by gradient elution into seventeen or more fractions greatly facilitated the identification of the metabolites by GC and GC—MS. In addition to the dihydrodiol and hydroxymethoxy derivatives of carbamazepine already described, mono-, di-, tri- and tetrahydroxy derivatives of carbamazepine have been isolated by HPLC. The GC and GC—MS properties of these metabolites will be reported separately.

There was no evidence of degradation or rearrangement of carbamazepine or the other reference compounds during HPLC analysis; a single peak was always observed for each standard. To check that degradation had not occurred during lyophilization, *cis*-10,11-dihydroxy-10,11-dihydrocarbamazepine was collected from the semi-preparative HPLC column using the solvent system described. After removal of most of the methanol (Rotovap), the solution of the *cis*-dihydrodiol was lyophilized and the residue redissolved in methanol. When the lyophilized sample was analyzed by HPLC, a single peak with the same retention time as the *cis*-dihydrodiol was observed. No iminostilbene derivatives were formed during HPLC analysis or during the work-up of the eluted *cis*-dihydrodiol. A comparable experiment was carried out with carbamazepine epoxide. Only a single peak due to the epoxide was observed when the lyophilized sample was analyzed by HPLC.

In our laboratory iminostilbene and iminostilbene derivatives have been observed as rearrangement products during GC analysis of the free and derivatized *cis*-dihydrodiol. The rearrangement of carbamazepine metabolites to iminostilbene derivatives during GC analyses is illustrated in Fig. 2b. Since iminostilbene and its derivatives were separated from the carbamazepine analogs on the HPLC

column, it was assumed that the O-methylcatecholiminostilbenes in Fig. 2b were formed during GC analysis. This conclusion was supported by the observation that the size of the iminostilbene peaks varied during repetitive analyses of the same sample.

The HPLC procedure described was not satisfactory for quantitative analysis because some of the peaks were not symmetrical (Fig. 1a) and analysis of the individual fractions by GC—MS indicated that several of the apparently symmetrical peaks contained more than one metabolite (Fig. 2a and 2b). The separations on semi-preparative columns and on analytical columns having 10,000—12,000 theoretical plates were comparable. It is possible that a 5-μm C_{18} μBondapak column would provide the resolution necessary for quantitative analysis. The procedure, however, is very useful for isolation and identification and for comparison of urinary profiles because of the stability of the metabolites under the mild conditions of HPLC analysis.

ACKNOWLEDGEMENT

This work was supported by Grant GM-24092 of the National Institute of General Medical Sciences.

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DETERMINATION OF PLASMA MEPINDOLOL LEVELS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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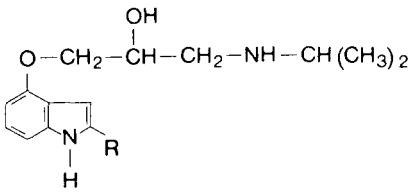
(Received June 21st, 1979)

SUMMARY

A method for determining the plasma concentration of the β-receptor blocking agent mepindolol by high-performance liquid column chromatography (paired-ion chromatography) and electrochemical detection is described. Pindolol is used as an internal standard and the detection limit after extraction of 1 ml of plasma is less than 1 ng of mepindolol. Reproducible results can be obtained with relative standard deviations from replicate analyses of 5 ng/ml plasma samples within ± 4.7%. The method is also suitable for quantification of plasma pindolol levels with mepindolol as an internal standard.

INTRODUCTION

Mepindolol sulphate (bis-[1-(isopropylamino)-3-(2-methylindol-4-yloxy)-2-propanol] sulphate) is a new, non-selective β-receptor blocking agent with slight intrinsic sympathomimetic activity. It is reported to be two to three times as potent as pindolol [1]. Its pharmacokinetic properties have been evaluated by a fluorimetric method [2] and by ^{14}C-labelled drug analysis [3]. Comparison



R = H : pindolol
R = CH_3 : mepindolol

of the two assays shows that, by spectrofluorimetry, (1) the plasma level of mepindolol is higher, (2) the elimination half-life in the plasma is longer, and (3) the amount of unchanged drug excreted with the urine is larger. The reason for this may be that metabolites similar to the drug molecule might have been assayed fluorimetrically together with mepindolol, thus showing higher and longer lasting drug concentrations in plasma and urine.

We therefore decided to develop a new method of detection which should be at least as sensitive as the fluorimetric assay mentioned above but without the use of radioactively labelled substances and without the need of derivatization.

EXPERIMENTAL

Subjects and medication

Four healthy female volunteers (age range 23—46 years, mean value 36 ± 11, and 54—66 kg in weight) were each given 20 mg of mepindolol sulphate orally as 10-mg tablets after a standardized breakfast. Blood samples were taken at 0, 1, 2, 3, 4, 5, 7, 9, 12 and 24 h after the drug administration. They were immediately centrifuged and the plasma stored frozen until analysis.

Reagents

Mepindolol and mepindolol sulphate were obtained from Schering, Berlin, G.F.R., and pindolol was a gift from Sandoz, Basle, Switzerland. All solvents (benzene, isoamyl alcohol, methanol, ethyl acetate and diethyl ether) were of analytical-reagent grade and were used without further purification. Sodium hydroxide, 0.1 *N* solution, and 0.1 *N* acetic acid were each prepared by dissolving one ampoule of "fixanal" (Riedel-de Haen, Hannover, G.F.R.) in 1 l of distilled water.

Standard solutions

Just before use, solutions of 10 μg of mepindolol and 100 μg of pindolol, each in 100 ml of methanol, were prepared.

Glassware

All glassware used in the extraction procedure was cleaned with chromic acid, washed with distilled water and methanol and dried at 150° before use.

Extraction procedure

One millilitre of plasma was pipetted into an 8-ml stoppered test tube and 5 ng of pindolol, 200 μl of 0.1 *N* sodium hydroxide solution and 5 ml of benzene—isoamyl alcohol (20:1, v/v) were added. After thorough mixing on a Vortex mixer for 1 min and centrifugation at 1200 *g* for 5 min, the organic phase was transferred to another test tube and re-extracted into 200 μl of 0.1 *N* acetic acid by mixing for 1 min and centrifugation for 5 min at 1200 *g*. The organic phase was discarded and the acetic acid extract washed twice with 500 μl of diethyl ether; 150 μl of the aqueous phase were used for analysis.

Standard samples prepared from blank plasma spiked with 5, 10 and 20 ng of mepindolol were analyzed along with the unknown samples.

The extraction efficiency was determined with 1-ml plasma samples containing 8 or 16 ng of ^{14}C-labelled mepindolol (n = 5 for each concentration), the ^{14}C-activity being measured in the aqueous phase of the extract.

Chromatographic system

The high-performance liquid chromatography (HPLC) system consisted of a solvent delivery pump (Knauer, Berlin, G.F.R. type 52.00), a LiChrosorb RP-18 chromatographic column (10 μm particle size, 250 mm × 4.6 mm; Knauer) and an electrochemical detector (E 611, cell EA 1096/2; Metrohm, Filderstadt, G.F.R.) using a glassy carbon working electrode and an Ag/AgCl/KCl reference electrode. Injection was accomplished with a Rheodyne RH 7120 system. The mobile phase consisted of methanol—water (65:35, v/v) with 0.01 *M* sodium dodecylsulphate and 2 ml of acetic acid per litre. The eluent was degassed under reduced pressure before use. The chromatographic system was operated at ambient temperature, with an eluent flow-rate of 2.0 ml/min. The electrochemical potential of the working electrode was set at +1.4 V against the reference electrode. The current range used was 5—20 nA according to the concentration of the drug. The detector signal was converted to a chromatographic trace by a W+W recorder at an input voltage of 1 V.

Calibration curve

The standard curve was constructed with 1-ml blank plasma samples containing 0, 1, 2.5, 5, 7.5, 10, 15, 20, 30 and 50 ng of mepindolol and 5 ng of pindolol. These samples were extracted by the method described above. Peak heights of internal standard and drug were measured and the calibration curve (peak height ratio of mepindolol:pindolol versus the concentration of mepindolol) was constructed.

Unknown plasma samples were processed together with three calibration points (5, 10, and 20 ng of mepindolol) which were used to correct for inter-assay variability.

The overall accuracy of the assay was calculated from two series of experiments: determination of five samples of 1 ml of plasma with 20 ng and with 5 ng of mepindolol.

Pharmacokinetic evaluation

To determine the absorption and elimination half-lives time courses of the plasma levels of each test subject were plotted separately on a semi-logarithmic scale. The elimination rate constant (k_e) was obtained from the slope of the terminal straight line. The absorption rate constant (k_a) was constructed by the "feathering" method [4]. t_{max} was calculated from

$$t_{max} = \frac{\ln (k_a/k_e)}{k_a - k_e}$$

RESULTS AND DISCUSSION

Assay

This report describes a highly sensitive and selective method for the deter-

mination of mepindolol in plasma utilizing HPLC with electrochemical detection. Extraction is performed in two steps by first using an organic solvent mixture (benzene–isoamyl alcohol) and then re-extracting into 0.1 *N* acetic acid and washing the aqueous phase with diethyl ether. The overall recovery of this procedure was found to be 69.9 ± 4.4% and 64.7 ± 6.2% for 8 and 16 ng of mepindolol, respectively, as determined by radioactivity measurements of the extract after spiking plasma samples with ^{14}C-labelled drug (see Table I). The mean value of recovery for the two concentrations was 67.3 ± 5.8%.

TABLE I

OVERALL EXTRACTION RECOVERIES OF MEPINDOLOL

Recoveries were determined by radioacitivity measurements after spiking 1 ml plasma samples with ^{14}C-labelled drug.

Mepindolol (ng)	
Added to plasma	Recovered
8.00	5.66
	5.36
	5.45
	6.18
	5.32
16.00	11.33
	10.96
	9.74
	10.84
	8.93

Plasma constituents and possible co-extracted metabolites of mepindolol are then separated from the drug by HPLC using a reversed-phase system with ion-pair formation (see Fig. 1).

Detection is performed by means of an electrochemical detector in the oxidative mode. This is possible because the indole moiety can easily be oxidized or react with electrophiles. The main site of oxidation will always be the 3-position of the molecule [5]. The introduction of an additonal methyl group in position 2 as with mepindolol should enhance nucleophilicity at position 3 by an inductive effect thus making mepindolol even more susceptible to oxidation than pindolol. As can be seen from Fig. 2 (electrochemical response at varying potential settings) pindolol starts to be oxidized at somewhat higher voltages. The potential setting of +1.4 V used in the detection system described above is considerably higher than that applied in the determination of catecholamines and related compounds (+0.50 to +0.85 V) as described in the literature [6–11] and it may not be increased further because of electrolysis of the eluent.

Plasma concentrations of mepindolol were determined by comparing the peak heights of the drug and the internal standard added to the plasma specimens before extraction. A typical calibration curve of the assay is

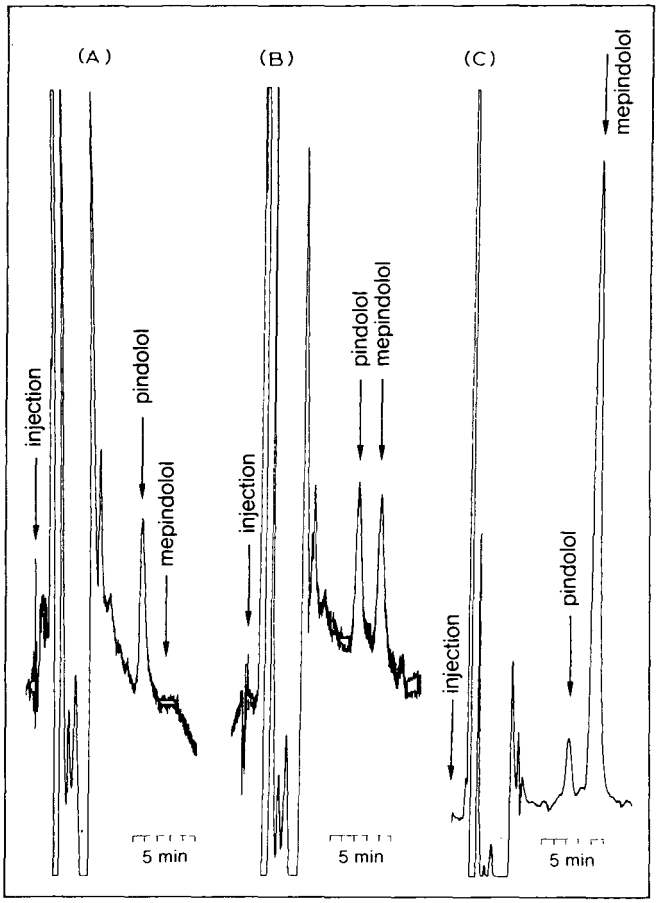


Fig. 1. HPLC chromatograms of (A) blank plasma samples spiked with 5 ng of pindolol, (B) 5 ng of pindolol + 7.5 ng of mepindolol, and (C) of a plasma sample obtained from test subject No. 4 2 h after oral administration of 20 mg of mepindolol sulphate.

illustrated in Fig. 3. The equation

$$\frac{\text{peak height of mepindolol}}{\text{peak height of pindolol}} = (0.133 \times \text{concentration of mepindolol}) - 0.061$$

was obtained, with the y-intercept of -0.061 being almost zero. The correlation coefficient was 0.998 and demonstrated the linearity of the data. This calibration curve was used for the analysis of unknown plasma samples. Concentrations of more than 50 ng/ml thus have to be regarded as estimates.

The overall accuracy of the assay expressed as standard deviation of five consecutive determinations of 5 and 20 ng of mepindolol per ml was 4.7 and 4.2%, respectively (see Table II). When assayed on four different days peak height ratios for 20 ng of drug per ml were obtained as 2.67, 2.90, 2.60 and 2.77, yielding a standard deviation of the inter-assay accuracy of 6.2%. Although this value was quite tolerable, standard samples prepared from blank plasma spiked with 5, 10 and 20 ng of mepindolol per ml were analyzed

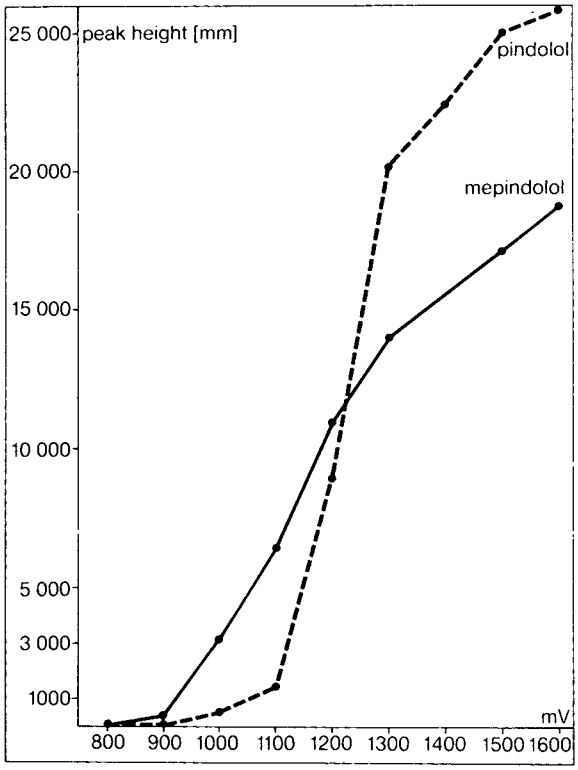


Fig. 2. Electrochemical response (peak height) of 2.5 μg each of pindolol and mepindolol at various potential settings.

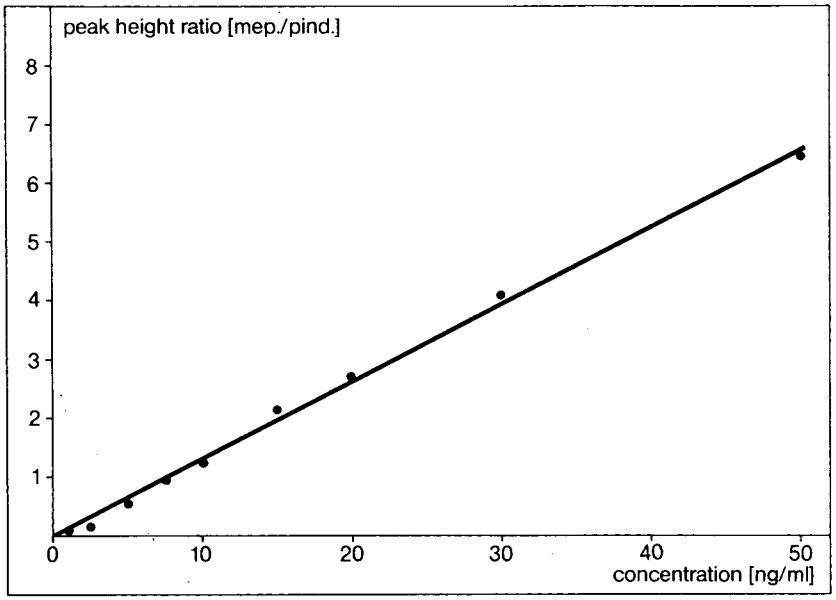


Fig. 3. Calibration curve for the determination of unknown mepindolol concentrations in 1 ml of plasma, obtained by spiking with 5 ng of pindolol and various amounts of mepindolol.

TABLE II

PRECISION OF THE ASSAY

Five consecutive determinations of 5 ng of mepindolol and 20 ng of mepindolol per ml of plasma were made.

Mepindolol (ng/ml)	Peak height (mm)			
	Pindolol	Mepindolol	Mep./Pind.	Mean ± S.D.
20	35	108	3.09	
	33	97	2.94	
	38	108	2.84	2.90 ± 0.12
	37	105.5	2.85	
	39	108	2.77	
5	66.5	29	0.44	
	63	30	0.48	
	80.5	40	0.50	0.47 ± 0.02
	58	27.5	0.47	
	71	34	0.48	

TABLE III

INDIVIDUAL PHARMACOKINETIC PARAMETERS OF FOUR TEST SUBJECTS AFTER ORAL ADMINISTRATION OF 20 mg OF MEPINDOLOL SULPHATE

Test subject	Absorption $t_{1/2}$ (h)	Concentration maximum		Elimination $t_{1/2}$ (h)
		(h)	(ng/ml)	
1	0.7	3	36.3	3.8
2	1.2	3	57.8	4.3
3	—	1	69.0	4.1
4	0.4	2	74.2	3.5
Mean ± S.D.	—	2.3 ± 1.0	59.3 ± 16.8	3.9 ± 0.4
Mean value *	0.4	1.5	47.3 ± 18.8	4.0

*Calculated from the mean values' curve of the four volunteers.

along with the unknown samples on different days to correct for the inter-assay variance.

The detection limit after extraction of 1 ml of plasma was less than 1 ng of mepindolol.

Study of plasma levels

Mepindolol was absorbed with a half-life of 0.4 h (calculated from a curve constructed with the mean values of the four test subjects) and reached its maximum at about 2 h (calculated: 1.5 h) at a level of 47.4 ± 18.8 ng/ml. The individual pharmacokinetic values of the four volunteers are listed in Table III (see also Fig. 4). As can be seen from Table IV, there is no difference in the results of the ^{14}C-assay and the method described above, especially when the data are corrected for the body weight of the test subjects. In that case — when taking the body weight of the male subjects (^{14}C-assay) as a standard — the mean value of the maximum concentration of the HPLC assay becomes 41.1 ± 16.3 ng/ml.

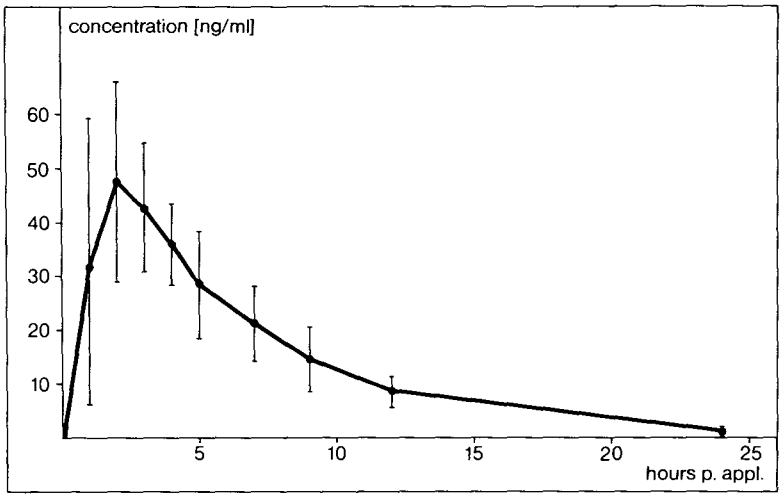


Fig. 4. Plasma level of mepindolol (mean ± S. D.) after oral administration of 20 mg of mepindolol sulphate to four healthy female volunteers.

TABLE IV

COMPARISON OF RESULTS OBTAINED BY THREE DIFFERENT METHODS AFTER ORAL ADMINISTRATION OF MEPINDOLOL SULPHATE TO HEALTHY VOLUNTEERS

Assay	Fluorimetry	^{14}C-method	HPLC
No. test subjects	5	5	4
Sex	Male + female	Male	Female
Dose (mg)	10	20	20
Absorption $t_{1/2}$ (h)	1.1	0.4	0.4
Concentration maximum			
(h)	2.8	1.6	1.5
(ng/ml)	41.4 ± 8.1	37.2 ± 20.7	47.3 ± 18.8
range (ng/ml)	33.4—54.3	21.9—71.6	36.3—74.2
Elimination $t_{1/2}$ (h)	4.6 ± 1.6	4.2 ± 1.3	3.9 ± 0.4

In the fluorimetric assay, however, an identical value is obtained with half the dose administered. The reason for this may be that metabolites chemically similar to the drug molecule might have been fluorimetrically assayed together with mepindolol, thus showing higher and longer lasting plasma concentrations.

The same holds for urinary excretion. In the report by Gugler et al. [2], 24 h after oral administration of 10 mg of mepindolol sulphate 17.4% of the dose was recovered in the urine in the unchanged form. ^{14}C-Labelled drug analysis [3], however, has shown that after double the dose less than 1% was to be found in the urine.

Thus, the HPLC assay of mepindolol described above differs from the fluorimetric method known in the literature by

(1) use of an internal standard for controlling the extraction procedure,
(2) separation of plasma constituents and mepindolol metabolites from the unchanged drug by HPLC (metabolic patterns will be reported elsewhere [12]),
(3) high sensitivity without the need of derivatization, and
(4) rapid sample preparation, thus permitting a higher efficiency in routine work.

The method will be used in further clinical pharmacokinetic studies in our laboratory and may also be applied to the determination of pindolol.

ACKNOWLEDGEMENT

The author thanks Prof. Dr. W. Schwartzkopff, Fett- und Stoffwechselambulanz der Freien Universität Berlin, for skillfully conducting the human study.

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Journal of Chromatography, 181 (1980) 77—84
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CHROMBIO. 441

CHROMATOGRAPHIC ANALYSIS OF GRISEOFULVIN AND METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A simple and accurate assay for the determination of griseofulvin and its metabolites in biological fluids using high-performance liquid chromatography is described. Using a reversed phase column and a mobile phase solvent of 45% acetonitrile in 0.1 *M* acetic acid, baseline separation of griseofulvin and several analogues was obtained. The described method allows one to quantitatively determine griseofulvin, 6-demethylgriseofulvin, and griseofulvic acid, a newly identified metabolite in man, in urine and plasma samples. Treatment of plasma samples prior to the analysis is simply made by deproteinizing the samples with an equal volume of acetonitrile. For urine samples, the procedure involves diethyl ether extraction with subsequent evaporation to dryness and reconstitution with the mobile phase solvent.

INTRODUCTION

Griseofulvin is an orally effective antifungal agent for the treatment of dermatophylic infections. Studies [1, 2] have shown that griseofulvin undergoes metabolic O-demethylation in man and animals to form several metabolites. To date, only two of the metabolites are positively identified as 6-demethylgriseofulvin and 4-demethylgriseofulvin [3]. The unidentified metabolites may include griseofulvic acid, which has been found in incubated fungi solutions of griseofulvin [4], and some dihydroxy-griseofulvin derivatives. These compounds, such as griseofulvic acid which is capable of undergoing methanolysis under mild conditions to form isogriseofulvin [5, 6], may be in part responsible for the toxicity and irregular responses of the

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drug found in clinical studies [7, 8]. Thus, it seems to be desirable to have an accurate method for monitoring concentrations of griseofulvin and its metabolites in biological fluids.

Several methods for the determination of griseofulvin and 6-demethylgriseofulvin in urine and plasma have been reported. These include the spectrofluorometric [9, 10], gas—liquid chromatographic [10—12] and liquid column chromatographic [13, 14] techniques. The spectrofluorometric method is generally considered to be time consuming and non-specific due to its extraction procedures and the inability of separating griseofulvin from its metabolites in fluorescence measurements. The gas—liquid chromatographic method is reported to be sensitive and specific, but the assay procedure involves tedious derivatization of the drug and often gives inconsistent results when using an electron-capture detector. The use of liquid column chromatography in the analysis of griseofulvin in plasma and 6-demethylgriseofulvin in urine has been reported by Nation et al. [13] and by Papp et al. [14], respectively. Improvement of these methods can be made by changing the chromatographic conditions to achieve better separations, so that a simple procedure may be obtained for simultaneous measurement of griseofulvin and its metabolites. Under this guideline, a method for the determination of griseofulvin, 6-demethylgriseofulvin, and griseofulvic acid in urine and in plasma has been developed in this laboratory. Based on this method, a previous study [15] has shown that griseofulvic acid is also a urine metabolite of griseofulvin in man.

EXPERIMENTAL

Materials

Griseofulvin (I) (Imperial Chemical Industries, Macclesfield, Great Britain) and boron tribromide (Alfa Division, Ventron, Danvers, Mass., U.S.A.) were used as obtained. 6-Demethylgriseofulvin (II), 7-chloro-6-hydroxy-4,2′-dimethoxy-6′-methylgris-3,4 -dione, was isolated from dog urine as a griseofulvin metabolite according to the method of Harris and Riegelman [16]. Griseofulvic acid (III), 7-chloro-4,6-dimethoxy-6′-methylgrisan-3,2′,4′-trione, was synthesized by selective boron tribromide ether cleavage of griseofulvin at —60°. 4-Demethylgriseofulvin (IV), 7-chloro-4-hydroxy-6,2′-dimethoxy-6′-methylgris-3,4′-dione, was prepared according to the method of Arkley et al. [5]. 7-Chloro-4-hydroxy-6,4′-dimethoxy-6′-methylgris-3′-ene-3,2′-dione (V) was prepared with selective boron tribromide ether cleavage of griseofulvin at room temperature followed by quenching the reaction mixture with methanol. Compounds II—V were positively identified as named by melting point, nuclear magnetic resonance and mass spectroscopic measurements. These data and the synthetic procedures are published elsewhere [6].

The solvents used in this study were chromatographic grade. All other chemicals used were reagent grade.

Equipment

A high-performance liquid chromatographic (HPLC) system including a Model 6000A pump and a U6K injector (Waters Assoc., Milford, Mass., U.S.A.) equipped with a Model SF-770 UV and a Model SF-970 fluorescence detector

(both from Schoeffel Instruments, Westwood, N.J., U.S.A.) was used. The outputs of the detectors were displaced on a recorder (Omniscribe, Houston Instruments, Austin, Texas, U.S.A.) having a full scale range of 10 mV. The HPLC analysis was made with a reversed-phase μBondapak C_{18} column (30 cm × 4 mm I.D., particle size 10 μm) (Waters Assoc.).

Fluorometric studies were made with a spectrophotofluorometer (Aminco-Bowman, American Instruments, Silver Spring, Md., U.S.A.) equipped with a 150 W xenon lamp and a 1P21 photomultiplier tube.

Chromatographic conditions

The mobile phase was prepared by degassing a mixture of 450 ml acetonitrile with 550 ml of 0.1 *M* acetic acid. Chromatographic analysis using the above solvent was carried out at a flow-rate of 1.0 ml/min. The column temperature was ambient. The sample injection size was 5 μl for urine samples and 10 μl for plasma samples. The column eluate was monitored by a UV detector at 290 nm and by a fluorescence detector with excitation at 280 nm and emission above 389 nm. The fluorescence wavelength of griseofulvin and derivatives was found to be 430 nm when excited at 310 nm using a spectrophotofluorometer. The UV detector was operated at 0.01 a.u.f.s., the fluorescence detector was operated at 0.2 μA range with an applied potential of 520 mV.

Sample preparations

Stock solutions of 1 mg/ml of compounds I—V were prepared in methanol or acetonitrile. For chromatographic analysis, standard solutions in the concentration range of 0.5—6 μg/ml of these compounds were prepared by transferring desired amounts of the stock solutions, with micro syringes, into test tubes containing 2 ml of the mobile phase solvent.

The analysis of commercial griseofulvin tablets [Schering (microsized), Kenilworth, N.J., U.S.A., Ayerst Laboratories (ultra-micronized), New York, N.Y., U.S.A., McNeil Laboratories (microsized), Fort Washington, Pa., U.S.A.] was carried out as follows: 10 tablets were weighed and reduced to a fine powder, 5—10 mg of the tablet powder were weighed and dissolved in 10 ml methanol. The solutions were filtered and further diluted with the mobile phase solvent for chromatographic analysis. The analyses were performed in triplicate.

Treatment of urine samples prior to the analysis was performed as follows: 4 ml of a urine sample were acidified to pH 3 and extracted with 8 ml of anhydrous diethyl ether; after centrifugation, 6 ml of the diethyl ether layer were transferred into a centrifuge tube containing 6 ml of 0.1 *M* acetic acid, the sample was shaken and centrifuged to remove any acid—water soluble substances from the diethyl ether layer. For chromatographic analysis, 4 ml of the diethyl ether layer were evaporated to dryness and reconstituted with 2 ml of the mobile phase solvent.

For plasma samples, deproteination of the samples using an equal volume of acetonitrile was carried out prior to the analysis. The acetonitrile treated sample was simply vortexed and centrifuged (at 480 *g* for 1 min) to obtain the clear supernate for HPLC injections.

RESULTS AND DISCUSSION

An HPLC assay for the determination of plasma levels of griseofulvin, using a reversed-phase column and a mobile phase of water—acetonitrile (1:1) mixture, has been reported by Nation et al. [13]. This method is found to be adequate for the determination of griseofulvin, but it does not allow one to measure the griseofulvin metabolites. Since the metabolites are the phenolic or hydroxy derivatives of griseofulvin, the pH of the mobile phase plays an important role in the separation of these compounds using reversed-phase chromatography. When a neutral pH solution is used as the mobile phase, the griseofulvin metabolites are essentially not retained by the column due to the fact that these compounds are ionized or partially ionized in the mobile phase solvent. Under acidic conditions, however, the metabolites become protonated, and they can be successfully separated for quantitative analysis. An example of the separation of I (peak d), II (peak a), III (peak b) and V (peak c) is shown in Fig. 1, where the chromatograms from UV (bottom) and fluorescence (top) detections were obtained using a mobile phase of 45% acetonitrile at pH 3.5.

Compound IV, 4-demethylgriseofulvin, which is not shown in Fig. 1, has a retention time of 7.0 min under the same chromatographic conditions. This compound was not included in the chromatogram because it was not found in the biological samples due to its very low abundance [3].

It is interesting to point out that the fluorescence yields of the griseofulvin derivatives depends largely on the substitutions at the 4- and 6-positions of their aromatic ring. Spectrofluorometric studies showed that the replacement of the 6-methoxy group of griseofulvin by a hydroxy group (such as in II), reduces more than half of the griseofulvin fluorescence. When the 4-methoxy group is replaced by a hydroxy group, such as in IV and V, the compound becomes nearly non-fluorescent. Griseofulvin derivatives possessing both 4- and 6-methoxy groups, such as in III, showed approximately equal fluorescence yield with griseofulvin. The same results were also found in the HPLC studies. As it can be seen in Fig. 1, compounds I and III are strongly fluorescent when compared with their UV absorptions, compound II, 6-demethylgriseofulvin, is moderately fluorescent, whereas compound V is nearly non-fluorescent. Compound IV is also non-fluorescent.

The advantage of using both fluorescence and UV detectors is that the identity and purity of a separated component in a chromatogram may be further evaluated with the peak response ratio of the two detections. That is, if the peak response ratio of a component is different from that of the known standard, then either the peak represents a different species, or some impurities may be eluted with the same retention time. In the present study, baseline separations of compounds III and IV were not obtained under the chromatographic conditions described. However, careful studies of the biological samples revealed that these samples do not contain measurable amounts of compound IV based on the fluorescence—UV peak response ratio. Therefore, no attempt was made to separate compounds III and IV at this time.

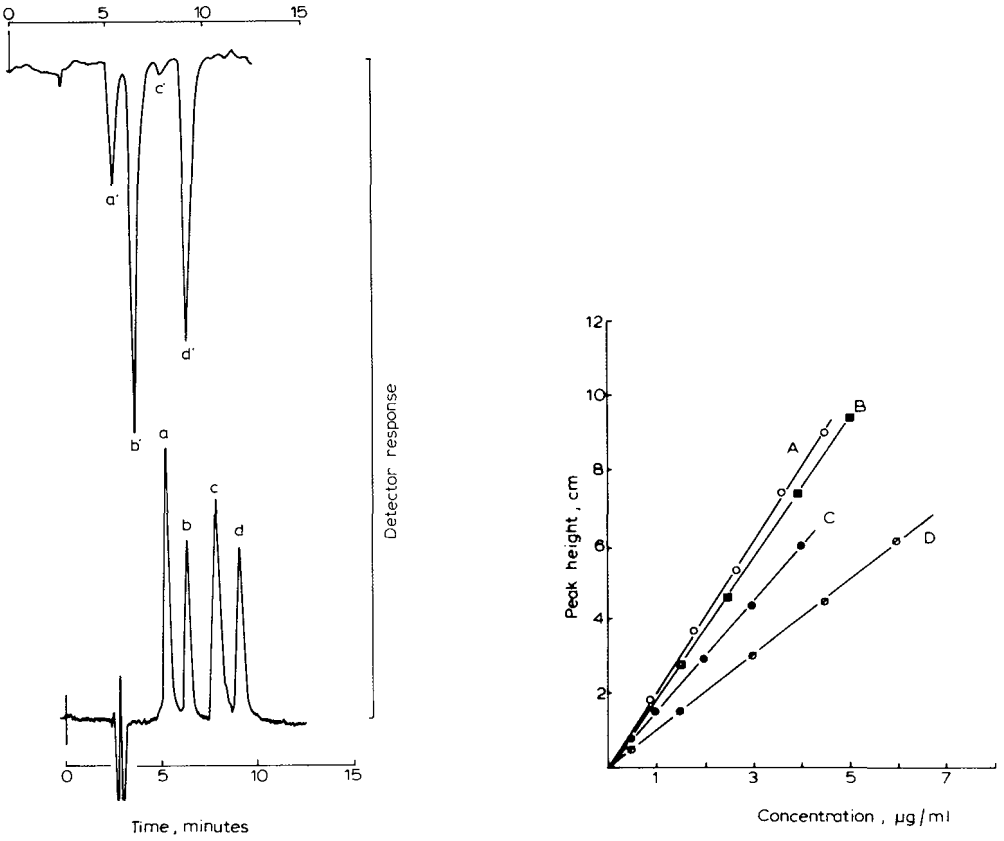

Fig. 1. Chromatograms of griseofulvin (peaks d and d', t_R = 9.0 min, 3 µg/ml); 6-demethylgriseofulvin (peaks a and a', t_R = 5.2 min, 3.6 µg/ml); griseofulvic acid (peaks b and b', t_R = 6.3 min, 2.5 µg/ml) and 7-chloro-4-hydroxy-6,4'-dimethoxy-6'-methylgris-3'-ene-3,2'-dione (peaks c and c', t_R = 7.8 min, 6 µg/ml) obtained from fluorescence (top) and UV (bottom) detections.

Fig. 2. Calibration curves for compounds I (C), II (A), III (B) and V (D) from UV detection.

Fig. 2 shows the calibration curves of compounds I, II, III, and V, where the peak heights obtained from UV detection are plotted as a function of their concentrations. Using the procedures given in the Experimental section, solutions containing as low as 0.05 µg/ml of these compounds can be detected. The fluorescence detection gives even better sensitivity for the determination of I and III. Compound V was not observed in biological samples. Therefore, it was used in the present study as an internal standard.

Fig. 3 shows the chromatograms of a urine sample from a normal subject who was given an oral dose of 250 mg griseofulvin (McNeil Laboratories, microsize). The sample was collected 4 h after the oral dose and extracted according to the procedures given in the Experimental section. Three components were found in the chromatograms, they are identified as I (peak d), II (peak a), and III (peak b), respectively. This sample contains only a trace amount of

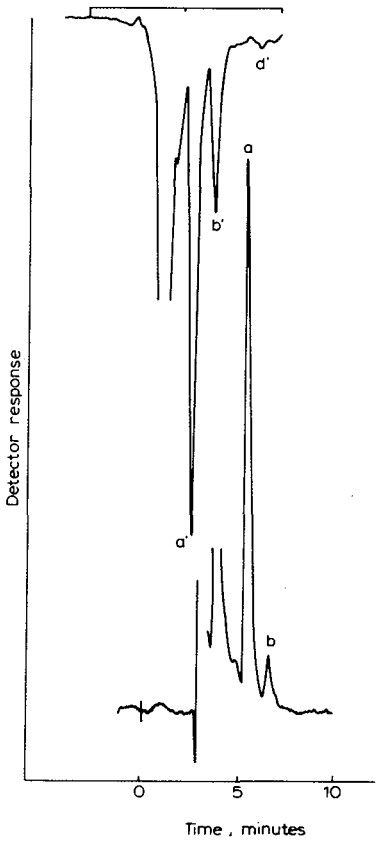

Fig. 3. Chromatogram of a urine sample from a normal subject. Peaks a and a', 6-demethylgriseofulvin; b and b', griseofulvic acid; d', griseofulvin.

griseofulvin which agrees with the literature report that less than 1% of griseofulvin is excreted in the urine [7]. The major metabolite is 6-demethylgriseofulvin with the presence of a significant quantity of griseofulvic acid. 4-Demethylgriseofulvin, which has been reported to be present in the urine in about 1% of the total dose, was not found in this or other samples. The finding of griseofulvic acid (III) in the urine has been reported in a previous communication [15]. In the present study, urine samples from four subjects who were given different brands of griseofulvin tablets were collected and analyzed. All samples showed the presence of griseofulvic acid. The tablets were analyzed prior to the administration and were found to contain griseofulvin in the indicated strengths with the absence of any degradation products or impurities.

The presence of griseofulvic acid (III) in the urine brings an interesting point into the metabolism of griseofulvin. The formation of III is postulated to occur at the 2'-position of griseofulvin via microsomal demethylation and subsequent tautomerization of the 2'-enol to the 2',4'-dione. From the chemical point of view, the formation of III as a metabolite is really not surpising since the vinyl methyl ether at the 2'-position is more readily cleaved than the aromatic ethers at the 4-and 6-positions. Yet both 4- and 6-demethylgriseo-

fulvin, but not griseofulvic acid, have been found to be the metabolites of griseofulvin in man [3]. The fact that griseofulvic acid has been found to be present in incubated agar solutions containing fungi and griseofulvin [4] also supports the finding that griseofulvic acid is a human metabolite of griseofulvin. Griseofulvic acid is chemically reactive, it readily forms isogriseofulvin via methanolysis under mild conditions [5, 6]. Preliminary studies carried out in this laboratory showed that griseofulvic acid is strongly bound to plasma proteins. Thus, this compound may be, at least in part, responsible for the toxicity of the drug. Detailed studies of the protein binding of grisofulvin metabolites and their possible toxicological effects are currently being investigated in this laboratory.

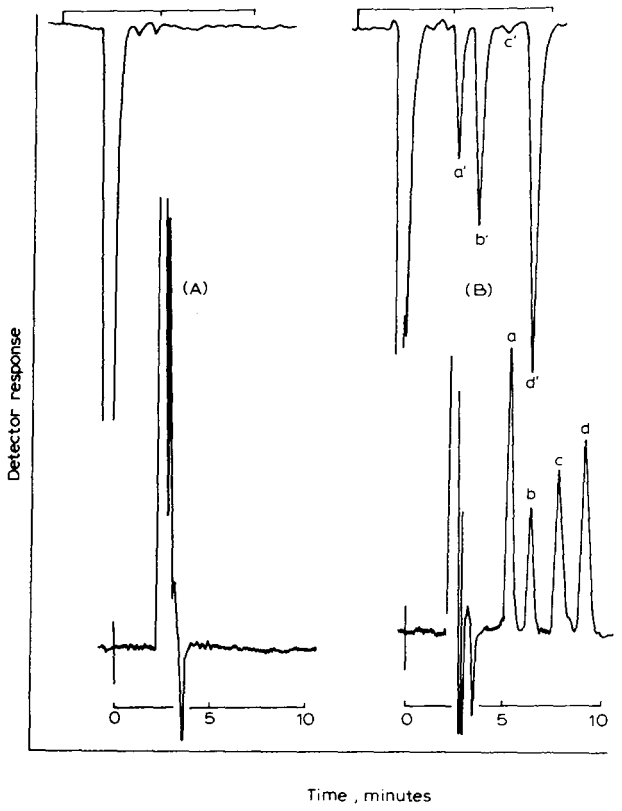

Fig. 4. Chromatograms of a 10-μl injection: (A), a blank plasma sample and (B), a plasma sample containing 2.9 μl/ml of 6-demethylgriseofulvin (peaks a and a'); 2.0 μg/ml of griseofulvic acid (peaks b and b'); 4.4 μg/ml of compound V (peaks c and c', used as an internal standard) and 3.4 μg/ml of grisofulvin (peaks d and d').

Fig. 4 shows the chromatograms of (A) a blank plasma sample, and (B) a plasma sample spiked with griseofulvin and its metabolites. The samples were treated according to the procedure given in the Experimental section. As it can be seen, there is no interference from the plasma ingredients.

In order to evaluate the sample preparation procedures for quantitative

TABLE I

ANALYSIS OF GRISEOFULVIN (I), 6-DEMETHYLGRISEOFULVIN (II), AND GRISEOFULVIC ACID (III) IN IN VITRO URINE SAMPLES

Concentration added (μg/ml)	Recovered*					
	I		II		III	
	μg/ml	(%)	μg/ml	(%)	μg/ml	(%)
0.5	0.50 ± 0.05	100.0	0.52 ± 0.03	104.0	0.48 ± 0.02	96.0
1.0	0.95 ± 0.04	95.0	0.96 ± 0.03	96.0	1.00 ± 0.04	100.0
2.0	1.90 ± 0.08	95.0	2.05 ± 0.07	102.5	1.95 ± 0.05	97.5
3.0	3.10 ± 0.10	103.3	2.86 ± 0.12	95.3	2.85 ± 0.10	95.0
4.0	3.90 ± 0.12	97.5	4.10 ± 0.15	102.5	3.88 ± 0.10	97.0

*Average of 3 determinations.

determination of compounds I, II and III, plasma and urine samples with known concentrations of these compounds in the range of 0.5—4 μg/ml were prepared for HPLC analysis. The results, obtained in triplicate, showed complete recovery of the added compounds in urine samples with an average standard error of ± 5% (see Table I). For the plasma samples, the standard errors were found to be less than ± 5%. Since these samples were simply treated with an equal volume of acetonitrile, no loss of the added compounds was anticipated. The error, however, could come from the non-additive volume changes when the plasma is mixed with acetonitrile. Based on these results, it is seen that this method provides an accurate and simple procedure for the determination of urine and plasma levels of griseofulvin, 6-demethylgriseofulvin and griseofulvic acid.

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Journal of Chromatography, 181 (1980) 85—89
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Note

Capillary column gas—liquid chromatographic—mass spectrometric assay for 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid, the major human urinary metabolite of prostaglandins E_1 and E_2 ★

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(Received July 10th, 1979)

Prostaglandins (PG's) and their involvement in disease states have been the subject of much study during the past decade. Unraveling their role in inflammation is a particularly active area of research, especially as anti-inflammatory drugs have been shown to be prostaglandin synthetase inhibitors [1]. Significant progress in such areas of biology is often highly dependent upon the development of methods for assay of the involved biochemical agents. Hamberg and Samuelsson [2] have shown that the major human urinary metabolite of PGE_1 and PGE_2 is 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid (PGM), and Hamberg [1] and Seyberth et al. [3] have demonstrated that administration to humans of therapeutic doses of drugs such as indomethacin and aspirin significantly reduces the urinary levels of PGM (presumably reflecting inhibition of PGE_1 and PGE_2 biosynthesis). These authors employed the dimethyl ester-di-([$^2H_3$]methyloxime) of tritium-containing PGM as the internal standard in a selected ion monitoring (SIM) gas—liquid chromatographic—mass spectrometric (GLC—MS) assay. This deuterated derivatized species is introduced into the assay procedure after endogenous PGM has been partially purified and derivatized to its dimethyl ester—dimethyloxime. If possible, an internal standard should be added to a biological specimen at the beginning of an assay procedure. ^2H- and ^3H-labeled PGM has now been prepared by Rosegay and Taub [4]. We wish to report on the use of this doubly-labeled compound as the internal standard in the determination of human urinary PGM levels in patients with rheumatoid arthritis treated

★Presented in part at the 26th Annual Conference on Mass Spectrometry and Allied Topics, St. Louis, Mo., May 28—June 2, 1978.

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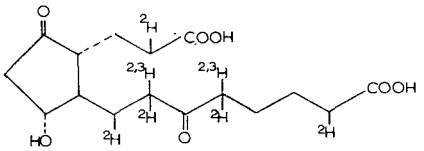
with indomethacin [5]. The procedure is based on those reported earlier [1, 3] with several exceptions, viz. (1) the internal standard is added to the urine prior to the initiation of the assay, and (2) capillary column GLC with splitless injection is employed with electron impact MS. Chemical ionization MS detection in combination with selective stationary phase packed column GLC is also utilized.

EXPERIMENTAL

Isolation procedure

Except for those aspects that involve the internal standard, the isolation procedure is essentially that used by Seyberth et al. [3]. The internal standard was labeled with tritium ($4.0 \cdot 10^5$ dpm/μg) to facilitate the isolation of the metabolite and deuterium (up to 10 ^{2}H atoms per mole).



The electron impact mass spectrum of this compound (as its dimethyl ester—dimethyloxime—TMS ether) shows the M—(90 + 31) fragment ion as base peak; the major species in this characteristic cluster is $^2H_7$, with no $^2H_0$ or $^2H_1$ ions present [6]. The urine sample (20 ml), after addition of 1 μg internal standard and adjustment to pH 4, is charged to an XAD-2 column (15 ml of resin), washed with an excess of water and eluted with ca 50 ml ethanol. After evaporation the residue is derivatized first with diazomethane (0.5 ml ethereal diazomethane) and secondly, with methoxylamine·HCl (15 mg per ml pyridine, overnight at room temperature). The residue after flash evaporation is subjected to reversed-phase partition chromatography on a column prepared by coating 4.5 g of silanized Hyflo-Supercel with 4 ml of stationary phase (lower phase of the equilibrated system 720 ml methanol, 480 ml water, 150 ml chloroform and 50 ml heptane). Radioactive monitoring of the eluate (5-ml cuts; mobile phase is the upper phase from the above-mentioned equilibrated system) is carried out and the 3 or 4 fractions of highest radioactivity content pooled. The residue after evaporation is subjected to thin-layer chromatography (silica gel G) with a developing solvent prepared by equilibrating ethyl acetate, trimethylpentane and water (2:1:2, upper phase). The zone of interest ($R_F \approx 0.50$), located by radioscanning, is eluted with diethyl ether; the diethyl ether solution is reduced to dryness and the residue derivatized with 20 μl of bis-trimethylsilyltrifluoroacetamide—pyridine (2:1) for 0.5 h at room temperature.

Instrumentation

A Finnigan Model 3200 GC—MS instrument is used for the measurements on the derivatized isolate (dimethyl ester—dimethyloxime—TMS ether; endogenous metabolite and internal standard). A 10 m × 0.25 mm SE-30 glass capillary column with splitless injection is used for the electron impact ionization

work; column temperature, 230°; carrier gas (helium) flow-rate, ca. 2 ml/min. Mass spectrometer operating conditions: ionizing potential 70 eV; emission current 0.8 mA; electron multiplier 1800 V. The chemical ionization data are obtained using a 5 ft. 3% OV-17 packed column at 260°; injection port temperature 270°; carrier gas flow-rate 15 ml/min and reagent gas methane (source pressure 1 Torr). Mass spectrometer operating conditions same as above except an ionizing potential of 150 eV. SIM is effected through use of the Finnigan 6110 data system (electron impact) and the Programmable Multiple Ion Monitor (PROMIM) accessory (chemical ionization) monitoring the M—(90 + 31) ions (electron impact) and MH^+ ions (base peak, chemical ionization) arising from the two species of interest. The resulting intensity ratio is then employed to calculate the urinary output of the metabolite per 24 h.

Calculation

$$\text{Amount of PGM per 24 h} = K \frac{I_{365}}{I_{372}} \times \frac{\text{Total 24-h urine volume (ml)}}{20 \text{ ml}}$$

or

$$= K \frac{I_{487}}{I_{494}} \times \frac{\text{Total urine volume (ml)}}{20 \text{ ml}}$$

where K = 0.25 (the fraction of the M—(90 + 31) or MH^+ ion cluster arising from the $^2H_7$ species).

A detection limit of 0.5 ng/ml urine (with a 20-ml urine aliquot) was indicated. Multiple (six) assays of a pooled 24-h urine (11.5 μg per 24 h) gave a coefficient of variation of 10.3%.

RESULTS AND DISCUSSION

Fig. 1 shows the electron impact ion response plots obtained with isolates (equivalent urine volumes) from a patient before administration of indomethacin (left panel) and while on the drug (right panel). With the capillary column at 230°, PGM was found to elute at scan No. 220 (4 min). The *m/e* 365 ion response at scan No. 210 does not interfere with the analysis because of the chromatographic resolution provided by the capillary column. It can be clearly seen that the *m/e* 365 ion response is markedly reduced following the administration of the drug. Glass capillary columns have been used by other workers for improved chromatographic resolution in the measurement of prostaglandins [7, 8] and thromboxane B_2 [9] (see also ref. 11).

To ascertain the effect of indomethacin on the excretion of PGM, three 24-urine collections were made on each patient: during the last days of the "washout period" (control), the "oral indomethacin period", and the "oral and suppository indomethacin period". Aliquots (20 ml) from each of these three one-day collections were added to vials containing 1 μg internal standard and stored at −17° prior to assay. The urine content (μg per 24 h) of PGM in a number of patients treated with indomethacin is shown in Table I. A wide range of control PGM levels was found among the patients, but in each case

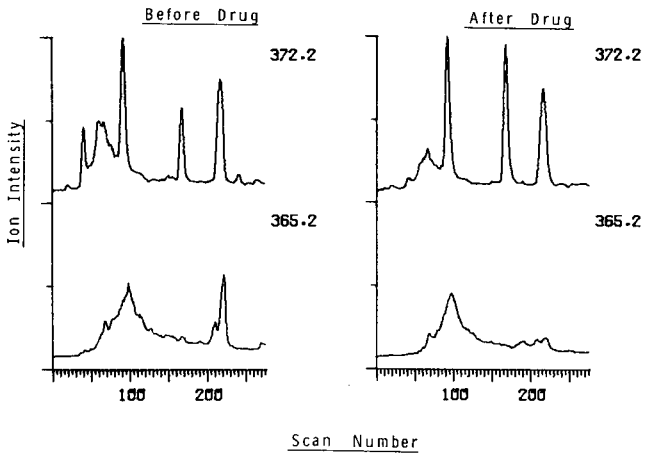


Fig. 1. Electron impact *m/e* 365 and 372 ion response plots obtained with isolates from a patient before administration of indomethacin (left panel) and while on drug (oral plus suppository; right panel).

TABLE I

URINARY OUTPUT (μg/24 h)* OF 7α-HYDROXY-5,11-DIKETOTETRANORPROSTANE-1,16-DIOIC ACID IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED WITH INDOMETHACIN ORALLY AND ORALLY PLUS SUPPOSITORY

Patient	Sex	Before drug		Oral**		Oral** and suppository***	
		EI§	CI§§	EI	CI	EI	CI
1	M	5.34	5.60	2.76	2.66	2.34	2.41
2	F	2.40	2.68	0.95	0.77	0.81	0.89
3	F	3.73	4.64	1.50	1.59	0.96	1.18
4	M	15.3	15.5	6.20	7.03	4.56	6.42
5	M	24.5	28.0	6.53	9.50	5.00	6.98
6	F	7.92	9.35	3.79	4.35	3.54	4.66
7	F	7.18	7.46	1.08	1.07	3.36	3.26
8	M	16.9	20.1	3.02	4.32	3.73	5.90
9	F	6.63	6.56	1.53	2.09	2.13	3.38
10	F	12.17	13.53	8.68	8.96	2.33	2.83

*Averaged result from two injections.
**25 mg 3 times daily for 3 weeks.
***100 mg each night for 3 weeks.
§ Electron impact.
§§ Chemical ionization.

the urinary output was reduced following administration of indomethacin. These results are similar to the findings of Hamberg [1] and the Vanderbilt group [3, 10] on the effect of oral administration of anti-inflammatory drugs upon urinary levels of PGM.

ACKNOWLEDGEMENTS

The authors are grateful to Professor John Oates and Dr. H. Seyberth for their generous advice concerning the isolation procedure used in the PGM assay.

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Journal of Chromatography, 181 (1980) 90—94
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Note

Gas chromatographic retention indices of twenty metabolically important acylglycines as trimethylsilyl derivatives

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(First received March 20th, 1979; revised manuscript received September 13th, 1979)

Conjugation with glycine is an important mechanism for excretion of aromatic carboxylic acids in normal human metabolism. These aromatic acids are either produced in endogenous metabolism or derived from exogenous sources such as drugs. For instance, hippuric acid (benzoylglycine) is a major constituent of acid extracts of normal human urine. Salicylate is excreted in part as a glycine conjugate, *o*-hydroxyhippuric acid (*o*-hydroxybenzoylglycine) [1]. Glycine conjugates of other hydroxysubstituted aromatic acids have been found in human urine [2].

Recently glycine conjugates of carboxylic acids with short aliphatic chains have been found in the urine of patients with certain inborn errors of organic acid catabolism. These glycine conjugates include: isovalerylglycine in isovaleric acidemia [3], β-methylcrotonylglycine in β-methylcrotonyl CoA carboxylase deficiency [4], propionylglycine in propionic acidemia [5], and suberylglycine in dicarboxylic aciduria [6]. In addition, tiglylglycine has been detected in three inborn errors of isoleucine metabolism [7]. *n*-Hexanoylglycine has been identified in urine from patients with Jamaican vomiting sickness [8, 9] and urine from a patient with ethylmalonic aciduria [10]. *n*-Butyrylglycine has been identified in urine of hypoglycin-treated rats [11]. The detection of these unusual glycine conjugates is essential in the diagnosis of these organic acidurias. Therefore, it is of clinical importance to provide chromatographic and mass spectroscopic data on glycine conjugates of normal and pathological carboxylic acids for profiling urinary organic acids in normal subjects and in patients with metabolic diseases. The possibility exists that a new organic aciduria may be discovered in the future by identification of "new" acylglycines.

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In profiling urinary organic acids, two types of derivatization can be utilized. One is the methylation of carboxylic groups [9, 12] and the other is trimethylsilation [13, 14]. Both of them have been extensively used. We have previously published gas chromatographic (GC) retention indices [15] and mass spectral data [16] of the methyl esters of twenty metabolically important acylglycines. More recently, however, the trimethylsilylation procedure is being more extensively utilized than the methylation method because of the carcinogenic and explosive nature of reagents for the methylation method.

We report here methylene unit values on OV-1 and OV-17 columns of trimethylsilyl (TMS) derivatives of twenty acylglycines of known or potential biological significance. Seventeen of them were synthesized in our laboratory. Mass spectroscopic studies of TMS derivatives of these acylglycines will be published elsewhere.

MATERIALS

Acetylglycine and hippuric acid were obtained from Sigma (St. Louis, Mo., U.S.A.). *o*-Hydroxyhippuric acid was purchased from Aldrich (Milwaukee, Wisc., U.S.A.). The other acylglycines were synthesized in our laboratory as described previously [15]. Anakrom U, Anakrom ABS, OV-1 and OV-17 were purchased from Analabs (North Haven, Conn., U.S.A.). The *n*-hydrocarbon standards were obtained from Analabs and Eastman Organic Chemicals (Rochester, N.Y., U.S.A.). Tri-Sil BSA Formula P was from Pierce (Rockford, Ill., U.S.A.).

METHOD

Trimethylsilation

The method used is similar to that used for urinary organic acid analysis [13]. Under these conditions, two peaks are detected for each acylglycine due to mono- and di-TMS derivatives or di- and tri-TMS derivatives in the case of the hydroxylated aromatic glycine conjugates. Since we intended to report the methylene unit values for both potential TMS derivatives, we did not use conditions that gave a single derivative for each acylglycine [17].

Gas chromatography

Two GC columns, 5% OV-1 and 10% OV-17 columns were used in this study. The support used for these columns was Anakrom (80—90 mesh) that had been base washed, acid washed and silanized either by ourselves or by the vendor. OV-1 and OV-17 were used in 6 ft. × 2 mm silanized glass columns in a Varian 1800 gas chromatograph. Nitrogen was the carrier gas at a flow-rate of 10 ml/min. The column oven temperature was programmed at 4°/min from a starting temperature of 80°.

For the determination of methylene unit (MU) values, the TMS-acylglycines were mixed with hexane solutions of either even- or odd-chain *n*-hydrocarbons. MU values were calculated by linear interpolation from two adjacent hydrocarbon peaks. For the two columns used, when hydrocarbon retention times were plotted against carbon number, curves were obtained which deviat-

TABLE I

METHYLENE UNIT VALUES FOR TRIMETHYLSILYL DERIVATIVES OF ACYLGLYCINES

Compound	5% OV-1 column		10% OV-17 column	
	Mono-TMS derivative	Di-TMS derivative	Mono-TMS derivative	Di-TMS derivative
Acetylglycine	12.53	13.57	14.86	14.86
Propionylglycine	13.34	14.17	15.37	15.37
n-Butyrylglycine	14.16	14.79	16.24	15.88
n-Valerylglycine	15.14	15.59	17.23	16.67
n-Hexanoylglycine	16.11	16.48	18.17	17.48
Isobutyrylglycine	13.72	14.08	15.60	15.16
Isovalerylglycine	14.64	15.10	16.56	16.02
α-Methylbutyrylglycine	14.51	14.91	16.41	15.80
Acrylylglycine	13.33	14.04	15.55	15.35
Methacrylylglycine	13.88	14.42	15.92	15.52
Crotonylglycine	14.82	15.13	17.18	16.52
Vinylacetylglycine	14.02	14.71	16.23	15.96
Tiglylglycine	15.49	15.49	17.69	16.76
β-Methylcrotonylglycine	15.39	15.63	17.60	16.97
2-Furoylglycine	16.17	16.47	18.97	18.33
Hippuric acid	18.06	17.85	21.10	20.04
Phenylacetylglycine	18.66	18.42	20.67	21.74
	Di-TMS	Tri-TMS	Di-TMS	Tri-TMS
o-Hydroxyhippuric acid	20.47	19.54	23.03	21.01
p-Hydroxyhippuric acid	22.02	21.25	24.56	22.78
p-Hydroxyphenylacetylglycine	21.82	21.82	24.72	23.48

ed insignificantly from linearity for any 2-MU range. MU values shown in Table I are the average of triplicate runs. A typical chromatogram is shown in Fig. 1.

Gas chromatography—mass spectroscopy

Gas chromatography—mass spectroscopy (GC—MS) was done with a Varian MAT 111 mass spectrometer coupled to a Varian 1400 gas chromatograph. Silanized glass columns (6 ft. × 2 mm) filled with the same batches of packing as described above were used. Helium was the carrier gas at a flow-rate of 15 ml/min. The injector, separator and inlet line were kept at 250°.

RESULTS

MU values for TMS derivatives of the acylglycines are listed in Table I. All of the acylglycines gave two peaks on at least one of the columns used. The two peaks were found to be mono- and di-TMS (di- and tri-TMS for the hydroxylated aromatic acids) derivatives by GC—MS analysis. The carboxylic and phenolic groups apparently react rapidly during the derivatization reaction while the amide nitrogen is trimethylsilated more slowly.

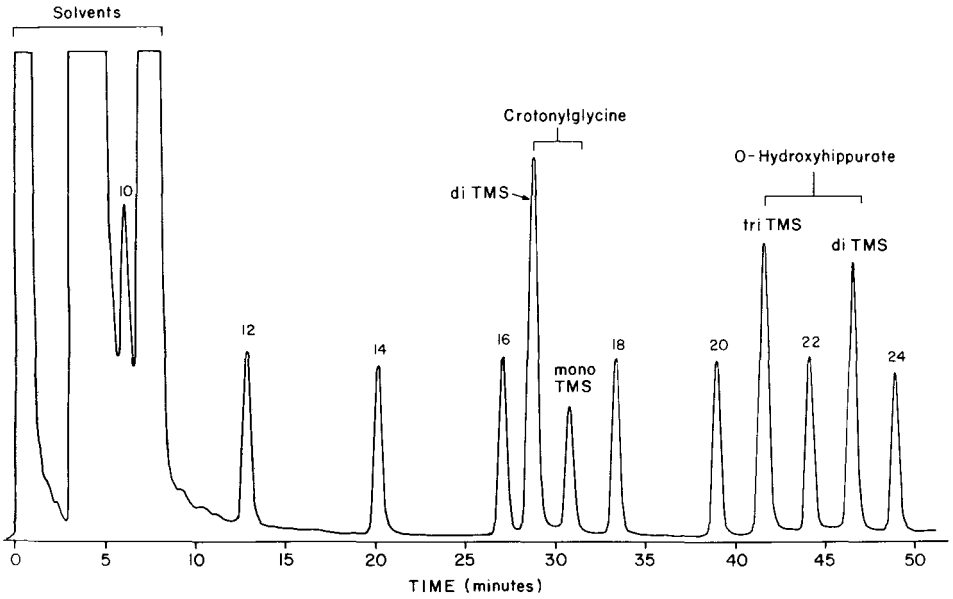

Fig. 1. Gas chromatogram of a mixture of crotonylglycine-TMS, *o*-hydroxyhippurate-TMS and even-number *n*-hydrocarbon standards. The column (2 mm × 180 cm) was packed with 10% OV-17. Temperature was programmed from 80° at a rate of 4°/min.

Di- (or tri-) TMS derivatives of some acylglycines are poorly resolved on either column. For instance, di-TMS butyrylglycine and di-TMS vinylacetylglycine elute close together on both columns and so do di-TMS valerylglycine and di-TMS tiglylglycine. The separations are better for these pairs as mono-TMS derivatives. In contrast, tiglylglycine and β-methylcrotonylglycine are better separated as di-TMS derivatives than as mono-TMS derivatives. *o*-Hydroxyhippuric and *p*-hydroxyhippuric acids are well resolved both as di- and tri-TMS derivatives on either column.

DISCUSSION

GC analysis of urinary acid extracts plays a key role in the detection of patients with organic acidurias. GC—MS analysis is usually used to identify unusual peaks. Occasionally, however, the mass spectral data are insufficient for identification due to incomplete separation of the components of an acid extract of urine. In such cases, precise GC characterization of the peak of interest can be of great value, if data for authentic standards are available as presented in this report. These GC retention indices are particularly useful for laboratories which are not equipped with GC—MS apparatus. Since the identification of the first organic aciduria, isovaleric acidemia, in 1966 [18], urinary organic acids in normal subjects [19] and in patients with various metabolic diseases [7] have been well characterized. It is our experience that with an extensive list of accurate GC retention indices of these normal and pathological metabolites on OV-1 and OV-17 columns, most of the known

diseases of organic acid metabolism could be readily identified without GC—MS. In these metabolic diseases, acylglycines are often the key metabolite for the diagnosis, but few of these acylglycines are commercially available. Previously, O'Neil-Rowley and Gerritsen [17] reported MU values of TMS derivatives of eight acylglycines. Mono- and di-TMS derivatives, however, were not identified in their study. As the first stage of a systematic study, we report here the MU values of TMS derivatives of twenty acylglycines. An extensive list of other organic acids is currently being compiled in our laboratory to be reported in the near future.

Trimethylsilylation is a commonly used derivatization method in urinary organic acid analysis. TMS derivatives are made by simple procedures using relatively less hazardous reagents. Especially when preceded by oxime formation, trimethylsilylation is applicable to acidic urinary metabolites with keto or aldehydic group [20]. The formation of two peaks with acylglycines is a problem, however. Formation of a single peak (di- or tri-TMS) is possible using a longer reaction time [17]. This reduces the complexity of chromatograms of samples containing acylglycines.

ACKNOWLEDGEMENTS

We wish to thank Mr. Bruce H. Baretz of our laboratory for the synthesis of 2-furoylglycine. This work was supported by a NIH grant (AM 17453) and a grant (1-378) from the National Foundation-March of Dimes.

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CHROMBIO. 427

Note

Determination of 6-diazo-5-oxo-L-norleucine in plasma and urine by reversed-phase high-performance liquid chromatography of the dansyl derivative

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(Received June 11th, 1979)

6-Diazo-5-oxo-L-norleucine (DON) is a glutamine antagonist and a potent inhibitor of L-asparagine synthetase [1, 2] and several L-glutamine amidotransferases concerned with purine biosynthesis [2, 3]. It has antitumor activity in a variety animal tumor system [4, 5]. Although DON has been used clinically [1, 4, 6], there has been no comprehensive study of the drug in man. Interest in DON has revived because of the effectiveness of the closely related azotomycin in certain types of cancer [7, 8] and because of the hope that inhibitors of L-asparagine synthetase might prevent the development of clinical resistance to therapy with L-asparaginase which has been linked to an increase in L-asparagine synthetase activity [9]. DON is currently being studied in Phase I clinical trials.

Microbiological procedures have been reported for the measurement of DON in plasma and urine [5, 6]. These microbiological assays are sensitive but lack specificity and several endogenous intermediary metabolites have been shown to affect the degree of inhibition of microbial growth produced by DON [5]. We report a specific and sensitive assay for DON based upon the separation of the dansyl derivative of DON from other dansyl derivatives in plasma and urine by reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

One ml of rabbit plasma containing DON was mixed with 2 ml of 5 m*M* dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride; Eastman Kodak, Rochester, N.Y., U.S.A.) in acetone. The mixture was incubated at 37° for 60 min in closed tubes and left at −20° for 60 min. Precipitated proteins were removed by centrifuging at 10,000 *g* for 10 min at −10°. Two ml of the

supernatant fluid were removed, frozen in a dry ice—acetone bath and lyophilized. The residue was dissolved in 0.2 ml of 50% acetone in water and 20 μl taken for chromatography. Urine was treated in a similar manner except that the pH was first adjusted to 9.0. It was not necessary to adjust the pH of plasma. Reversed-phase chromatography was carried out on a C_8-bonded Zorbax RP-8 column (4.5 × 250 mm) (DuPont, Wilmington, Del., U.S.A.) with 10% acetonitrile (Burdick & Jackson Labs., Muskegon Mich., U.S.A.) in 0.01 *N* sodium acetate buffer, pH 7.2 with a flow-rate of 1.7 ml/min and a column temperature of 55°. A Hewlett-Packard 1084B microprocessor-controlled high-performance liquid chromatograph and variable wavelength UV detector were employed. Eluting peaks were detected by their absorbance at 254 nm and peak areas integrated on a Hewlett-Packard 79850B liquid chromatograph terminal.

A logarithmic-ratio microbiological assay for DON using *Escherichia coli*/ ACB-C (supplied by Dr. W.J. Suling, Southern Research Institute, Birmingham, Ala., U.S.A.) as described by Cooney et al. [5] was employed for purposes of comparison.

DON was administered to male New Zealand white rabbits, weighting 2.5 to 3 kg, into a marginal ear vein over 30 sec. Blood was collected into heparinized tubes at various intervals from the other ear vein. Urine was collected from a catheter inserted into the bladder under light ketamine (Ketaject, Bristol Laboratories, Syracuse, N.Y., U.S.A.) anesthesia one hour prior to the study. The bladder was flushed at each collection with 5 ml sterile saline. Rabbits were placed in a metabolism cage for the collection of 24-h urine. Pharmacokinetic analysis of plasma drug levels was conducted using the SAS NLIN non-linear least squares regression analysis program [10]. DON (NSC-7365) was supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A., and stored desiccated at −20°.

RESULTS AND DISCUSSION

The conditions of dansylation described in Experimental were determined to be optimum for the formation of the dansyl derivative of DON in plasma and urine. The dansyl derivative of DON was completely resolved from other dansyl derivatives in plasma as a peak eluting at approximately 30 min (Fig. 1). Dansyl chloride and less polar dansyl derivatives were removed from the column by gradually increasing the acetonitrile in the solvent mixture to 100% over 12 min. This procedure was necessary for consistent column performance. The assay could detect 1.0 μg DON per ml. A representative standard curve for DON added to plasma is shown in Fig. 2. Dansyl DON can also be detected fluorometrically with an excitation wavelength of 340 nm. DON was found to be relatively unstable in solution and all biological samples were frozen after collection and assayed within 24 h.

Plasma decay curves for DON following administration to rabbits at doses of 400 and 600 mg/m^2 (23 and 34 mg/kg) are shown in Fig. 3 (the anticipated starting dose for the clinical study is 300 mg/m^2). The HPLC assay gave slightly lower values for plasma DON at later time points but otherwise the two assays gave similar plasma decay curves. The lower limit of detection of DON in rabbit

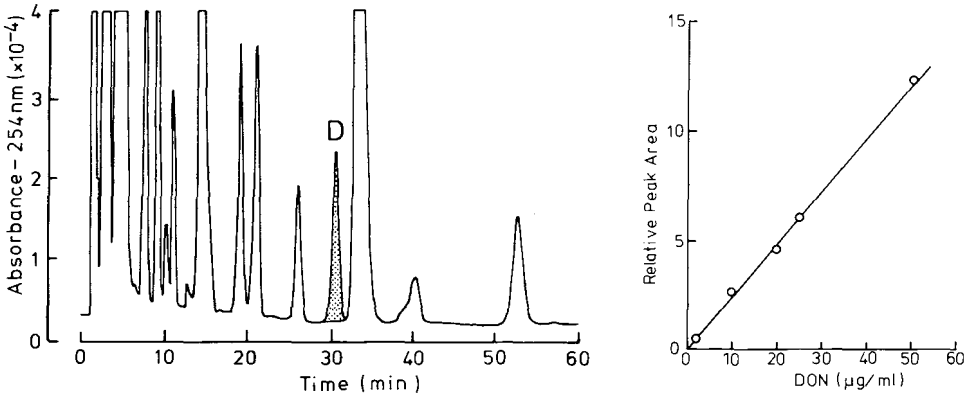

Fig. 1. Chromatogram of rabbit plasma to which DON had been added to give a concentration of 25 μg/ml. The plasma was derivatized and chromatographed by reversed-phase HPLC as described in the text. The peak formed by DON is shaded and identified by D. The baseline under the peak is the chromatographic pattern of the same plasma not containing DON.

Fig. 2. Standard curve for DON in plasma determined by reversed-phase HPLC.

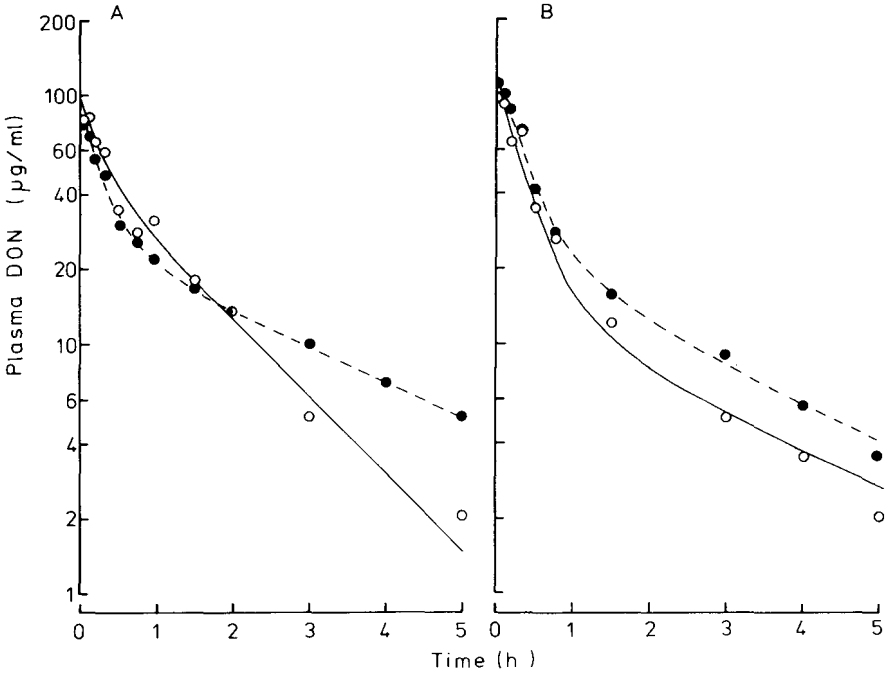

Fig. 3. Plasma levels of DON following the intravenous administration to rabbits of DON at doses of (A) 400 mg/m² (23 mg/kg) and (B) 600 mg/m² (34 mg/kg). ○, Determined by reversed-phase HPLC; ●, determined by the microbiological procedure. The lines are the computer plots of the data: —, for the HPLC data; — — —, for the microbiological data.

and human plasma by the microbiological procedure was 1.0 μg/ml, a sensitivity comparable to the HPLC assay. A ring of dense bacterial overgrowth immediately adjacent to the disk to which the plasma was applied limited the sensitivity of the assay. This overgrowth did not occur with mouse plasma which was used for the development of the original assay [5]. The removal of DON from the plasma measured by the HPLC assay was biphasic with mean pharmacokinetic parameters of $t_{1/2}^{\alpha}$, 11.1 min; $t_{1/2}^{\beta}$ 85.3 min; V_D^{α}, 284 ml/kg; V_D^{β}, 880 ml/kg and a clearance of 5.3 ml/min/kg body weight. No DON could be detected in the urine with either the chemical or microbiological assays.

Cooney et al. [5] using a microbiological assay, reported a monoexponential decline in plasma DON with an apparent half-life of ca. 30 min, following intraperitoneal administration of DON to mice. Studies by Magill et al [6], using a microbiological assay, showed biexponential plasma decay with a rapid distributive phase and a slower post-distributive phase with a half-life of 1—2 h in patients given DON intravenously. In the present study the agreement between the plasma concentration of DON as determined by the HPLC assay specific for DON and the microbiological assay suggest that in the rabbit, metabolites with growth inhibitory properties are not present in large amounts in plasma or urine. A slowly eluting peak (elution time ca. 52 min) was detected by the HPLC assay. The area of this peak, which has yet to be identified, increased with time.

Separation of dansyl amino acids by HPLC has been reported on ion-exchange resin [11], polyamide [12], silica [13], and more recently reversed-phase media [14]. None of these methods has been applied to amino acids in biological fluids. With the method we have developed, dansyl DON can be separated under isocratic conditons from other dansyl derivatives including amino acids within relatively short periods of time.

ACKNOWLEDGEMENTS

The excellent technical assistance of Ms. Margo Sternad is gratefully acknowledged. The assay method is based upon a procedure for the reversed-phase separation of dansyl amino acids developed by Jacque Elion and Kenneth Mann (unpublished work). The study was supported in part by Grant CM 97273A and Grant 15083 F.F., N.C.I., N.I.H., and a grant from the National Eagles Cancer Fund.

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Note

Method for the determination of tryptophan in serum and cerebrospinal fluid

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(Received June 25th, 1979)

Recently, high-performance liquid chromatography (HPLC), coupled with fluorimetric detection, has been shown to be useful for the analysis of indoles in physiological samples [1]. In this paper, a simple and rapid method is described based on the HPLC—fluorimetric system for the determination of tryptophan (Trp) in serum and cerebrospinal fluid (CSF).

EXPERIMENTAL

Equipment

The system consisted of a Spectra Physics 740B high-pressure pump, a Valco 7000 p.s.i. injection valve, a 100 mm × 4.6 mm I.D. column for the serum samples and a 150 mm × 4.6 mm I.D. column for the CSF samples, both packed with Nucleosil C_{18} (5 μm particle size), and a Schoeffel FS 970 fluorescence detector. The excitation wavelength was 282 nm; a 370-nm cut-off filter was used on the emission side. The mobile phase was a 10 mmol/l acetate solution (pH 4.0) containing 14% (v/v) methanol.

An Amicon Model 12 ultrafiltration cell and Diaflo XM50 filters were used for the ultrafiltration of serum.

Chemicals

Trp and α-methyltryptophan (αMTrp) were purchased from Sigma (St. Louis, Mo., U.S.A.). Standard solutions of Trp and αMTrp were prepared in water and stored at +4°. All other chemicals used were of an analytical grade.

Procedure

Human serum was obtained from healthy volunteers; human lumbar CSF was obtained from subjects under various neurological investigations.

To 1.2 ml of serum were added 45.1 nmol αMTrp and 0.1 ml of a 1.0%

sodium dodecylsulfate solution. The mixture was shaken and allowed to stand for 10 min. Thereafter, 1.2 ml of a 20% trichloroacetic acid solution was added. The mixture was shaken and centrifuged at 2000 *g* for 5 min. The supernatant was transferred to a clean tube and 10 μl were injected into the chromatographic system.

For the analysis of free Trp the albumin-bound fraction was separated by ultrafiltration. Serum (3.0 ml) was pipetted into the ultrafiltration cell. The system was rinsed with 5% carbon dioxide in nitrogen and a pressure of 75 p.s.i. was applied. When the first drop had passed, 50 μl of the filtrate were collected. A 10-μl aliquot was injected into the chromatographic system.

The CSF samples were shaken and 60 μl injected into the chromatographic system.

Quantitation was achieved by preparing standard samples in water. The standard samples were analysed as described above for CSF and serum.

RESULTS AND DISCUSSION

The chromatograms (Fig. 1) from the analysis of Trp (free and total) in human serum and CSF showed that no interfering peaks are present. The determination of total serum Trp involved precipitation of serum proteins. To correct for losses, αMTrp was used as an internal standard. For the analysis of the free Trp portion in serum, the ultrafiltrate was injected into the system without further treatment. Only a small volume (<5%) was allowed to pass the filter in order not to affect the equilibrium of free and bound Trp. The CSF required no treatment prior to injection.

The data from the determination of the reproducibility of individual analyses (Table I) showed that the total serum Trp and CSF Trp could be determined with experimental errors of less than 4%. The determination of the free Trp portion in serum showed a greater variability (11%), induced by the ultra-

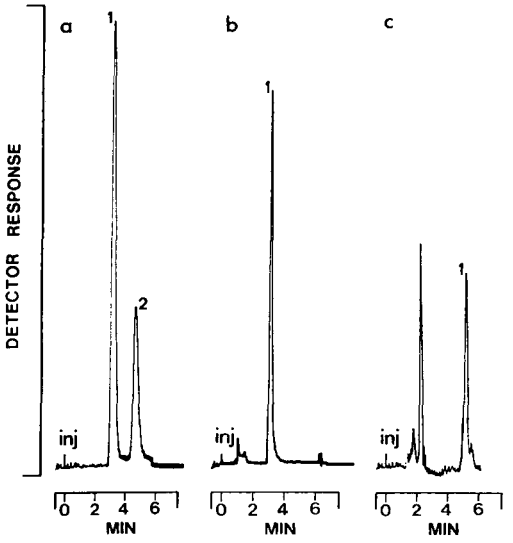

Fig. 1. Chromatograms from the analysis of (a) total serum Trp, (b) free serum Trp, and (c) CSF. Conditions were as given in the Experimental section. 1 = Trp; 2 = αMTrp.

TABLE I

REPRODUCIBILITY OF INDIVIDUAL ANALYSES

	Amount (nmol/ml ± S.D.)	%	*n*
CSF	2.04 ± 0.6	3.0	10
Serum (free)	17.9 ± 2.0	11	8
Serum (total)	68.3 ± 2.2	3.3	10

filtration. The levels reported here are in agreement with those in earlier publications [2].

In conclusion, the sensitivity and specificity of the HPLC–fluorimetric system allowed a simple and rapid method to be developed for the determination of Trp in serum and CSF.

ACKNOWLEDGEMENT

This work was supported by grants from the Swedish Medical Research Council (04041) and the National Institute of Health (MH 12007) to Bo Holmstedt.

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Journal of Chromatography, 181 (1980) 103—107
Biomedical Applications

CHROMBIO. 430

Note

Simultaneous analysis of ATP, ADP, AMP, and other purines in human erythrocytes by high-performance liquid chromatography*

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(Received June 25th, 1979)

The nucleotide profile of human erythrocytes is very simple when compared with nucleated mammalian cells. Having little or no pyrimidines, the human erythrocyte nucleotide profile consists exclusively of purines. Since DNA is not synthesized, only ribonucleotides are detected. The adenine ribonucleotide pool, consisting of ATP, ADP, and AMP, is both abundant and important for erythrocyte function and survival. The concentration of ATP is normally 1200—1700 μmoles/ml packed erythrocytes [1—3]. ADP and AMP concentrations are generally 5—8 and 50—100 times less than ATP concentrations, respectively. The guanine ribonucleotide pool (GTP, GDP, and GMP) as well as other purine pools, are much smaller than the adenine ribonucleotide pool in metabolically normal erythrocytes.

In our studies of metabolism and mechanism of action of antitumor agents, we found it desirable to monitor erythrocyte pools of purine nucleotides, nucleosides, and bases simultaneously. Purine nucleotides, but not nucleosides or bases, are resolved well on strong anion-exchange columns [4—6], and therefore a reversed-phase technique was developed which gives good resolution of ATP, ADP, and AMP, as well as partial resolution of other purine constituents. The ionic strength, pH, and gradient program of the mobile phase buffers were optimized for maximum resolution and precision, and minimum analysis time. Samples can be analyzed every 30 min, with relatively little solvent used.

* Taken from a thesis submitted by P.D.S. to The University of Texas System Health Science Center at Houston Graduate School of Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

** To whom correspondence should be addressed.

EXPERIMENTAL

Instrumentation

A Waters Model ALC-204 liquid chromatograph equipped with two Model 6000A pumps, a U6K injector, and a Model 660 solvent programmer, all of Waters Assoc. (Milford, Mass., U.S.A.) was used for all analyses.

Separations were achieved on a reversed-phase μBondapak C_{18} column (4 mm × 30 cm) also from Waters Assoc.

The detector was a Varian Vari-Chrom (Varian Assoc., Palo Alto, Calif., U.S.A.). Peaks were electronically integrated with a Varian CDS-111 integrator and printed out on a Varian Model 9176 recorder.

Materials

All purine standards and crystalline potassium dihydrogen phosphate were purchased from Sigma (St. Louis, Mo., U.S.A.). Potassium monohydrogen phosphate was purchased from J.T. Baker (Phillipsburg, N.J., U.S.A.). Glass distilled methanol was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Blood was obtained by venipuncture of healthy volunteers.

Buffer preparation

Buffers are diluted from a 1.0 *M* stock solution of 0.6 *M* K_2HPO_4 and 0.4 *M* KH_2PO_4.

Buffer A. Buffer A is prepared by diluting 100 ml of the stock to 1 l with distilled water. The pH is adjusted to 6.0 with concentrated phosphoric acid, and the solution is filtered through a Millipore 0.4-μm filter (Millipore, Bedford, Mass., U.S.A.).

Buffer B. Buffer B is prepared by diluting 100 ml of the stock solution to 750 ml with distilled water. The pH is adjusted to 6.0 and filtered as described above. To the filtrate is added 250 ml glass distilled methanol with constant stirring. The buffer is stirred for several hours at room temperature before use.

Standard solutions

All 17 purine standards are individually dissolved in appropriate solvents to a concentration of 3 m*M*. A mixture is obtained by combining an equal volume of each.

Sample preparation

Aliquots of thoroughly washed erythrocytes are packed by centrifugation at 12,000 *g* for 10 min at 4° on a Sorvall RC2-B refrigerated centrifuge. The packed cells are extracted for 30 min on ice with 2.5 volumes of 0.56 *N* perchloric acid (PCA). The insoluble material is removed by centrifugation, and the supernate is collected and neutralized with 10 *N* KOH. The $KClO_4$ is discarded after centrifugation and the resulting supernate is analyzed directly. Concentrations of purine constituents are determined by the peak area per nmole relationship of known concentrations of purine standards.

Chromatographic conditions

System I. For complete analysis of purine pools, a 30-min concave gradient

(curve 10 on the solvent programmer) from 0% Buffer B to 100% Buffer B is run at 1 ml/min. The eluent is monitored at 254 nm, with full scale deflection set at 0.2 absorbance units when 50-μl aliquots of the erythrocyte extracts are applied. After 40 min the column is re-equilibrated in 10 min by immediate reversal to 100% Buffer A.

System II. For a more rapid analysis of adenine ribonucleotide pools, Buffer A is pumped isocratically for 10 min at 1 ml/min followed by isocratic application of Buffer B for 10 min at 1 ml/min. The column is then re-equilibrated as above in 10 min.

RESULTS

Fig. 1 shows the separation of 17 purine standards using System I. Fig. 2 shows the separation of the purine standards using System II. With either system, the retention times of all compounds that are eluted in the first 15 min

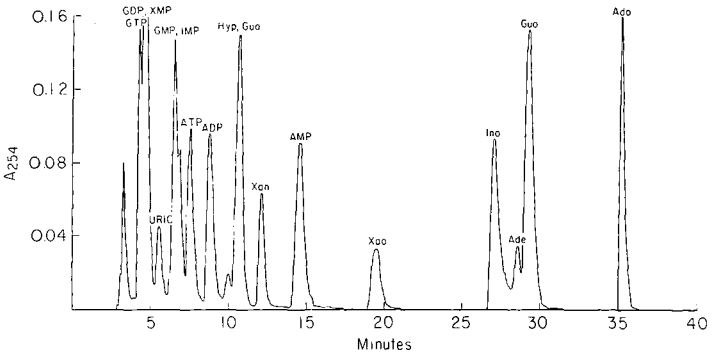

Fig. 1. Separation of approximately 3.5 nmole each of 17 purine standards using System I (20 μl injected).

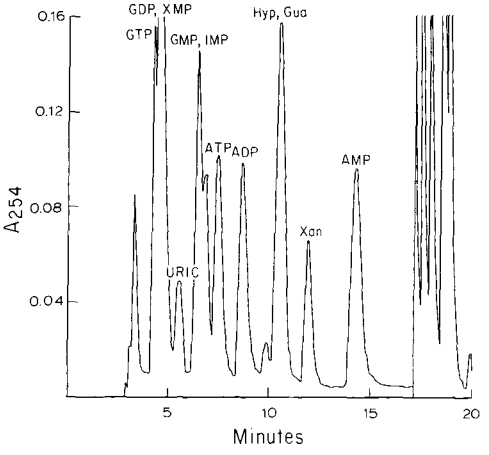

Fig. 2. Separation of approximately 3.5 nmole each of 17 purine standards using System II (20 μl injected).

are identical. Poor resolution of the later eluting compounds occurs with System II. Several compounds, including the guanine ribonucleotides, co-elute with other purine standards as experimental conditions are optimized for analysis of adenine ribonucleotides and common nucleosides such as inosine and adenosine.

A typical nucleotide profile of erythrocytes from a fresh specimen of venous blood drawn from a healthy subject is seen in Fig. 3. System II is used for this chromatogram, since little if any of the later eluting compounds are found in metabolically normal erythrocytes. ATP, ADP, and AMP levels are approximately 1440, 225, and 35 μmoles/ml of packed cells, respectively.

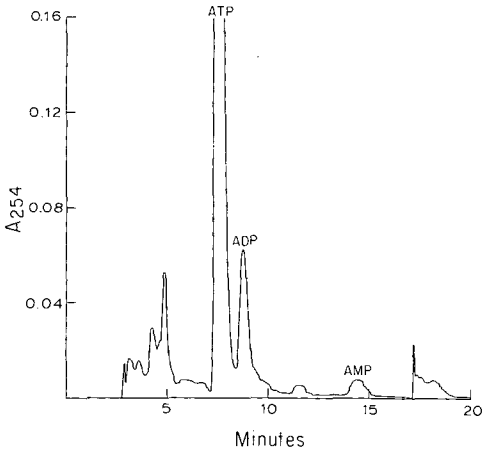

Fig. 3. Fractionation of 50 μl of PCA-soluble material obtained from human erythrocytes using System II.

DISCUSSION

The high-performance liquid chromatography technique described in this paper has several advantages. The relatively slow flow-rate enables multiple analysis without solvent change. Although two buffers are required, virtually no baseline drift occurs and almost all UV-absorbing compounds are removed from the column following each run. PCA extracts of erythrocytes can be routinely monitored at 0.2 a.u.f.s., which appreciably reduces electronic noise. Although several of the purine standards co-elute, none co-elute with ATP, ADP, or AMP, the nucleotides of interest. The method is fast and reliable for estimating adenine ribonucleotide concentrations in erythrocytes, and could be applied to many enzyme assays involving purine constituents.

ACKNOWLEDGEMENT

This work is supported by NCI Contract CM-87185.

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Biomedical Applications

CHROMBIO. 425

Note

Differentiation of the polysaccharide side-chains of glycoproteins by a fingerprinting technique: heterogeneity of human gastric mucin

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(Received June 12th, 1979)

Mucin glycoproteins, secreted into the gastrointestinal tract, differ in their cellular source, and are known to differ in their structure and composition. As biosynthesis is also controlled by genetic factors, differences are also found (from individual to individual) in glycoproteins secreted by the same cell type; the blood group inhibitory activity of the glycoproteins secreted by the gastric mucosa offers a well-known illustration of these differences. In order to study their physiological significance, a rapid method to scan the structural differences has been developed, based on the fact that the mucins are characterized by an O-glycoside linkage of the polysaccharide side-chain to the protein backbone. In alkali, the sugar chains are released by β-elimination, but are then subject to further uncontrolled degradation by base-catalysed "peeling" reactions [1—3]. Under suitably controlled conditions, however, a series of reduced oligosaccharides, as well as hexitols [3] and unsaturated alditols can be identified.

Recently, a two-dimensional chromatographic method was developed for the separation of oligosaccharides bearing acidic groups [4, 5]. The method has now been extended to permit the radioactive labelling of the neutral non-reducing oligosaccharides as well, in the products of the alkaline hydrolysis. These fingerprint techniques have been applied to the study of the polysaccha-

ride side-chains of human glycoproteins, obtained from gastric aspirates of A, B and O blood group donors. The results indicate that there are several differences, both in structure and in chain length, not only in terminal prosthetic groups but also in the inner core region of the carbohydrate moiety of these glycoproteins.

MATERIALS AND METHODS

The gastric juice was neutralised in vivo with phosphate buffer. Human mucins were separated by filtration and extensive washing from other soluble glycoproteins, as described elsewhere [6], from gastric juice aspirated from normal fasted subjects. Blood group and secretor status were determined in each case. Samples of lyophilized mucins from four or five donors of each blood group were pooled for analysis.

Partial alkaline degradation of the mucins was carried out as described previously [4]. Ten milligrams of mucin were stirred for six days at room temperature in 1 ml of 0.33 *M* KBH_4 in 1% sodium hydroxide. The reaction mixture was neutralized with Amberlite CG-120 (H^+). Fingerprinting of the alkaline hydrolysis products was carried out on 20 cm × 20 cm silica-coated thin-layer sheets (Schleicher & Schüll, Dassel, G.F.R., Selecta 1500). Oligosaccharide mixtures containing 0.2—1 μg of carbohydrate constituents were applied. The electrophoretic step was carried out in acetic acid—pyridine—water (10:1:89, v/v; pH 3.8) buffer at 10—20 V/cm for 2—3 h. The dried plates were chromatographed perpendicular to the electrophoretic migration with either solvent A, *n*-propanol—nitromethane—acetic acid—water (7:2:2:2, v/v), or solvent B, ethanol—nitromethane—acetic acid—water (5:3:3:3, v/v), by the ascending method until the solvent reached the upper edge of the sheet. The carbohydrate constituents were detected by sulphuric acid char or by the orcinol—sulphuric acid reagent [5, 7].

[^{14}C] Cyanohydrin formation

The reduced and neutralized alkaline degradation products of the mucins containing 0.1—0.2 μg of hexoses, as determined by the orcinol reaction, were dissolved in 50 μl of water, and 50 μg of galactose oxidase (Sigma, St Louis, Mo., U.S.A.) in 125 μl of phosphate-buffered saline (PBS) pH 7.0 were added. The mixture was incubated for 3 h at 37°. Forty microcuries of $K^{14}CN$ (specific activity 57 mCi/m*M*) in 25 μl of water were then added. The mixture was allowed to stand overnight at room temperature, then evaporated to dryness in a vacuum desiccator over KOH after the addition of one drop of 10% aqueous acetic acid. The residue was dissolved in 0.1 *M* acetic acid and evaporated again to dryness in vacuo. This operation was repeated three times to remove traces of radioactive HCN. The final residue was dissolved in 50 μl of water; 1—0.5 μl were deposited for fingerprinting and 1 μl was counted in a scintillation counter. Control experiments in which (a) the alkaline degradation product, or (b) the galactose oxidase was omitted, were performed with every series to detect radioactive artefacts. No artefacts, however, were detected between the starting point and the migration level of the disaccharides in the chromatographic step, although several non-specific radioactive spots were found in the

monosaccharide region of the control experiments. The fingerprints of the labelled oligosaccharides were exposed for 2—3 days to Kodak Kodirex films and developed with Kodak LX 14 developer. Samples of known oligosaccharides were applied to the chromatograms as markers.

Exclusion chromatography of the alkaline degradation products of the mucins was carried out on Sephadex G-25F columns (1 cm × 90 cm) eluted with 0.1 *M* acetic acid. The hexose level of the effluent was continuously monitored by the orcinol—sulphuric acid reagent in a Technicon device. The experiments were carried out in triplicate and the means of the quantities in each of the areas were compared (Fig. 1).

RESULTS

The fractionation of the alkaline degradation products on Sephadex G-25 columns indicate that the distribution of molecular weights of these products from donors of each group is similar, but differs in relative proportions (Fig. 1, Table I). Thus the ratio of the low-molecular-weight oligosaccharides to the higher molecular products, (peaks 3 + 4)/(peaks 1 + 2), is 0.55, 0.6 and 0.72 in the mucins of A, O, and B secretors, respectively. This finding, in itself inconclusive, suggests, however, that considerable differences must exist between the sugar chains of these glycoproteins.

Fingerprinting of the alkaline degradation products also shows marked differences between the glycoproteins of the A, B and O secretor groups (Fig. 2a and b). A satisfactory two-dimensional separation of the neutral and acidic reduced oligosaccharides liberated during the peeling reaction was obtained by this technique. The non-degraded macromolecular carbohydrate components exhibit electrophoretic mobility but are not displaced by the solvents used in the chromatography. The acidic oligosaccharides obtained from B and O secretor groups seem to be similar (Fig. 2). The separation of the neutral oligosac-

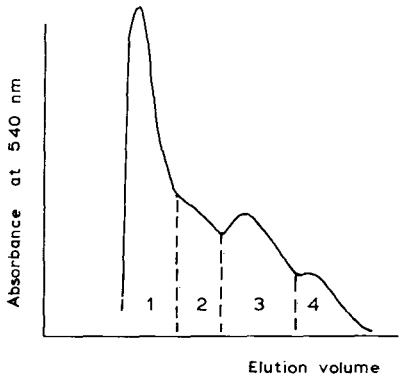

Fig. 1. Elution profile of the reduced alkaline degradation product (2 mg) on a Sephadex G-25F (1 cm × 90 cm) column. Eluent: 0.1 *N* acetic acid. The sugar content of the eluate was continuously monitored by the orcinol—sulphuric acid reagent using a Technicon device. The peak areas were compared by cutting out and weighing the region corresponding to the eluted fractions 1, 2, 3, and 4 (results, see Table I).

TABLE I

PERCENTAGE OF TOTAL HEXOSE FOUND IN EACH FRACTION ELUTED BY EXCLUSION CHROMATOGRAPHY OF THE ALKALINE DEGRADATION PRODUCTS OF GASTRIC MUCINS OBTAINED FROM A, B, AND O BLOOD GROUP DONORS *

Results are given as mean ± S.E.M. (n = 4).

Fraction	Percentage total carbohydrate (hexose)					
eluted**	Group O		Group A		Group B	
1	41.3 ± 1.8	1***	46.1 ± 2.5	1***	40.5 ± 1.2	1***
2	21.7 ± 1.0	0.51	18.6 ± 0.9	0.40	17.6 ± 1.0	0.43
3	26.7 ± 1.0	0.64	25.0 ± 1.1	0.56	31.0 ± 1.0	0.76
4	10.8 ± 0.9	0.26	9.4 ± 1.0	0.20	10.9 ± 1.1	0.27

* See Fig. 1.
** See Fig. 2.
*** Means values related to fraction 1.

charides is better when using solvent A for the chromatography step than solvent B.

The radioactive labelling reaction is based on cyanohydrin formation of the KCN with the aldehyde groups obtained by the action of the galactose or galactosamine of the sugar chain [8]. The cyanohydrin formation of the aldehydes proceeds quantitatively over several hours [10]. The chromatographic separation of the radioactive cyanohydrin derivatives of the oligosaccharides (Fig. 3) is superior to the separation of the parent oligosaccharides (Fig. 2a and b). This increased sensitivity can be used to differentiate products at both high and low concentration by varying the amounts applied. Thus, in Fig. 3, the neutral components are overloaded, whereas there are sufficient quantities of the radiolabelled minor components of acidic oligosaccharides to enable detection and confirmation of the results obtained in the fingerprinting of the parent oligosaccharides (Fig. 2). The similarity of the acidic oligosaccharides from B and O secretors is also confirmed by the autoradiograms (Fig. 3).

DISCUSSION

Gastric gel mucin comprises two-thirds by weight of the total non-dialysable solids in gastric juice, derived for the most part from epithelial and mucous neck cells of the gastric mucosa. Analysis has revealed fucose, galactose, N-acetylglucosamine and N-acetylgalactosamine in both fractions while the acidic fraction also contains sialic acid and sulphate. The polysaccharides are attached to a polypeptide core by O-glycoside linkages [9]. Thus, the alkaline degradation products of these mucins should be comparable to those obtained from the blood group substances [2, 3].

The fingerprints of the oligosaccharide from the alkaline borohydride degradation products and from their cyanohydrin derivative indicate that the reaction mixtures obtained from the A, B, and O secretor groups contain different

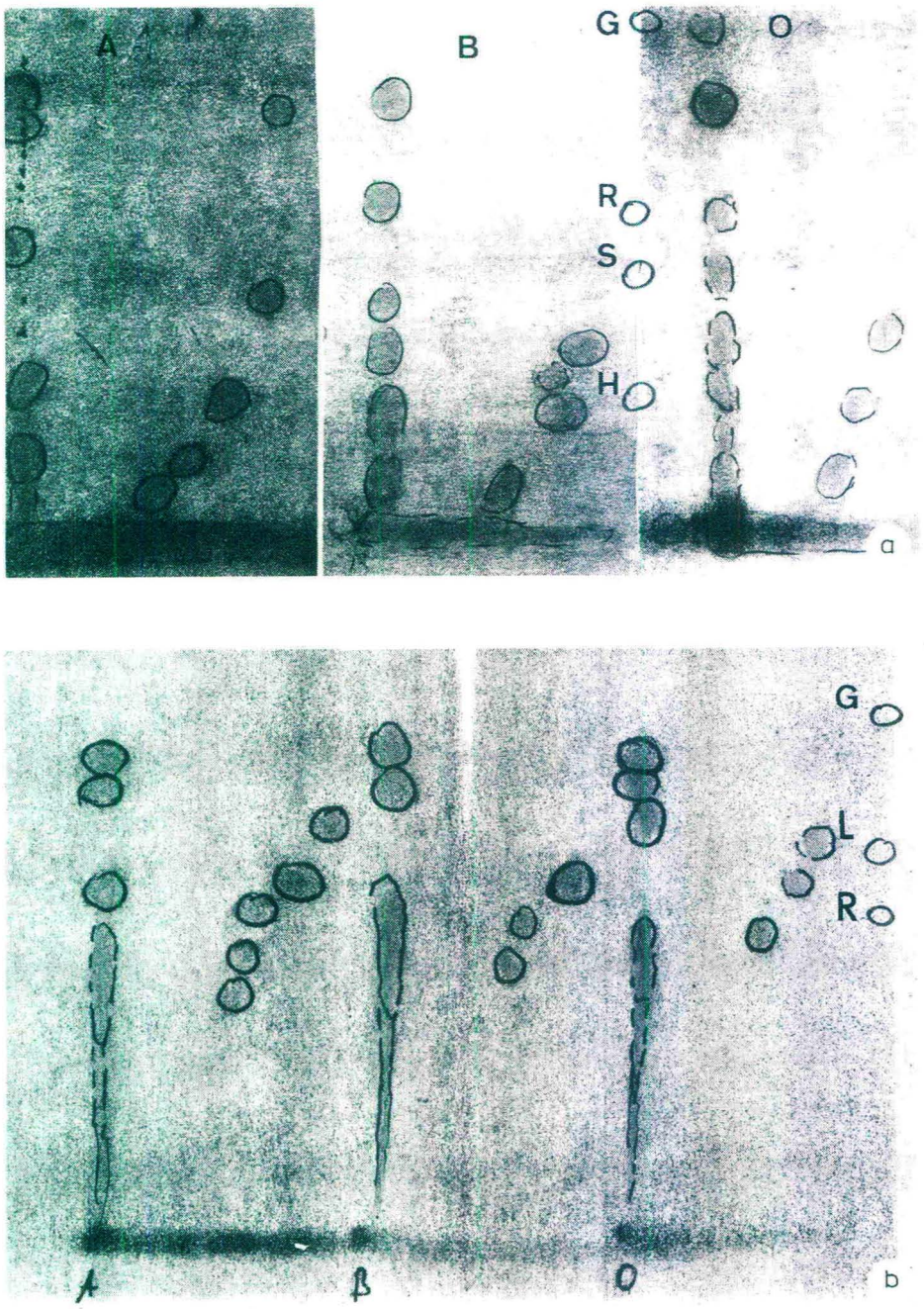

Fig. 2. Fingerprints of the alkaline degradation products on silica thin layers (Schleicher & Schüll, Selecta 1500, 20 cm × 20 cm) of the insoluble gastric mucins obtained from A, B, and O secretor groups. Ordinates: (a) chromatography in solvent A, and (b) in solvent B. Abcissae: electrophoresis in pyridine—acetate (pH 3.5). Detection: orcinol—sulphuric acid. S = Stachyrose, R = raffinose, L = lactose, G = galactose, H = glucosyl-galactosyl-hydroxylysine.

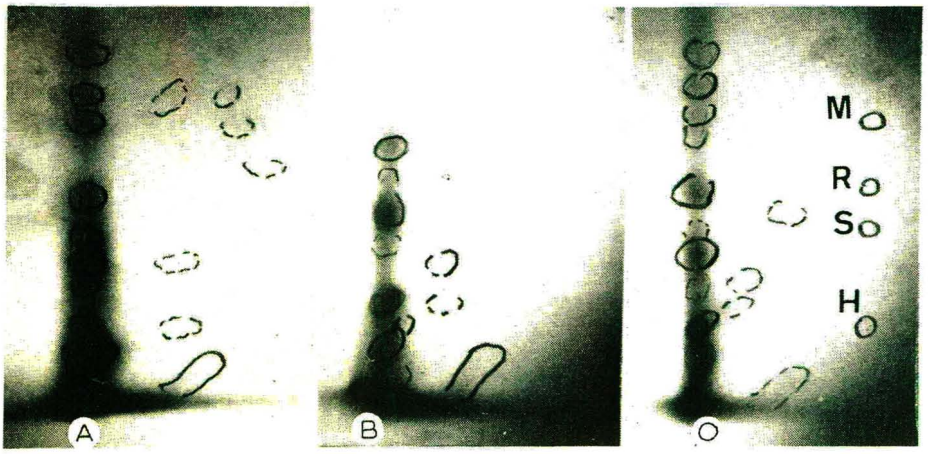

Fig. 3. Autoradiograms of the fingerprints of the cyanohydrin derivatives of the alkaline degradation products of the insoluble gastric mucins, obtained from A, B, and O secretor groups. Abcissae: electrophoresis (see Fig. 2). Ordinate: chromatography in solvent A. Abbreviations as in Fig. 2; M = maltose.

oligosaccharides. This finding implies that the major chain of these three types of mucin has to be different.

The glycoproteins used in these experiments have intense blood group activity, inhibiting the agglutination of erythrocytes by the appropriate antisera at dilutions as low as 0.1 μg/ml. A major terminal group of the polysaccharide side-chains must correspond therefore with the known structure of the blood group substances of the individual prosthetic groups. As the difference between the fingerprints involves several oligosaccharides it seems that not only the end-groups of the polysaccharides chains but also some interior sugar sequences have to be different. The results of the gel filtration experiments indicate also a different degree of fragmentation of the carbohydrate chains, implying structural differences in the interior of the carbohydrate fraction.

The fact that only slight differences were found in the overall sugar composition of the three glycoprotein complexes [6] studied, indicates that the fingerprinting of the oligosaccharides obtained by alkaline degradation of the mucins is required as a much more sensitive method for the detection of structural differences between the carbohydrate chains of these substances. However, more work is needed using oligosaccharides of precisely known structure before more exact conclusions can be drawn from the chromatographic data on the variations of the inner core region of the polysaccharide side-chains of these mucins.

ACKNOWLEDGEMENT

This work was supported in part by Grant MA 4058 from the Medical Research Council of Canada to D.W.-E., by the CNRS, GR No. 40, and by the University of Paris-Val de Marne, Créteil, France, to E.M.

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Journal of Chromatography, 181 (1980) 115—119
Biomedical Applications

CHROMBIO. 444

Note

Comparative statistical study of assay methods using mass fragmentography and gas chromatography with nitrogen detection for determination of the tetracyclic antidepressant mianserin in human plasma

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(Received July 2nd, 1979)

To determine drugs or their metabolites at low levels in biological fluids, sensitive and selective assay methods are required. In general, it is assumed that mass spectrometric methods offer a high degree of specificity and sensitivity. Application of assay methods using mass spectrometric detection can, however, be hampered because not everyone has access to mass spectrometry, the equipment is costly, is susceptible to occasional break-downs, and requires in general more attention from the average laboratory technician for the method to be employed on a routine basis. For the tetracyclic antidepressant drug mianserin (Org GB 94, the lab. code, is used to denote the salt mianserin hydrochloride), a mass fragmentographic method has been described [1,2]. The assay method includes extraction, plasma extract clean-up by liquid chromatography and quantitation by gas chromatography—mass spectrometry (GC—MS). A novel assay method for mianserin [3] using gas chromatography and a nitrogen-sensitive detector (GC—NPD) applies only extraction followed by injection of the raw plasma extract into the GC—NPD system for quantitation. The merits of the simplified GC—NPD assay method with respect to the GC—MS method have been discussed [3].

The present paper deals in detail with the statistical evaluation of data obtained after application of both assay methods to plasma samples from patients receiving mianserin treatment, and to blank plasma samples spiked with mianserin.

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MATERIALS AND METHODS

Assay procedures

Mass fragmentography (GC—MS). The assay method used is in essence the same as described earlier [1,2]. The method has been slightly modified according to the assay procedure for the determination of mianserin in dog plasma [4].

Gas chromatography with nitrogen sensitive detection (GC—NPD). The assay method as described recently [3] has been used.

Samples analyzed for comparison

Spiked samples. Over a period of six months, three artificial preparations A, B and C were made by spiking human plasma free of drug (blank) with approximately 20 ng of mianserin per ml plasma.

Clinical plasma samples. Patients' plasma samples from a clinical mianserin study with 20 mg three times daily or 60 mg nightly [5] have been used.

During mianserin analysis using GC—MS or GC—NPD, each series of measurements consisted of analysis of blank plasma samples, calibration samples, spiked samples, and clinical samples in a random order.

RESULTS AND DISCUSSION

Intra-laboratory variation in the GC—NPD and GC—MS methods

The intra-laboratory variation in both assay methods can be judged from the repeated determinations of the spiked samples of which a number ranging from 1 to 10 was included in each daily series of plasma samples to be analyzed.

In discussing the precision of a method it is important to distinguish between within- and between-day variation. If the same spiked sample is measured repeatedly on each different day using the same method, the logarithm y of the individual observation is assumed to be built up as follows:

$$y = \mu + \alpha_{\text{between days}} + \epsilon_{\text{within day}} \qquad (1)$$

where

$\alpha_{\text{between days}}$ = random error, inherent to all y values that might be observed on the day considered. It is assumed to be randomly drawn from a population of such errors with zero mean and variance σ^2 ($\alpha_{\text{between days}}$). This is the variance responsible for the day-to-day variation.

$\epsilon_{\text{within day}}$ = individual random error, independent from observation to observation and assumed to be randomly drawn from a normally distributed population of individual random errors with zero mean and variance σ^2 ($\epsilon_{\text{within day}}$).

A useful measure for the combined variation of a method is the total variance:

$$\sigma^2_{\text{total}} = \sigma^2(\alpha_{\text{between days}}) + \sigma^2(\epsilon_{\text{within day}})$$

This is the variance in a population of y values, each obtained on a different day. In this population the mean (or expected value) $E(y)$ will be:

$E(y) = E(\mu + \alpha_{\text{between days}} + \epsilon_{\text{within day}}) = \mu$

which shows the meaning of μ in the model eqn. 1.

From each set of observations obtained from one spiked sample on successive days using one method, estimates s^2 ($\alpha_{\text{between days}}$), s^2 ($\epsilon_{\text{within day}}$) and s^2_{total} were calculated from the analysis of variance.

For each method the estimates $s^2(\alpha_{\text{between days}})$ as well as the estimates $s^2(\epsilon_{\text{within day}})$ were combined over the three preparations, the latter combination following the standard method for pooling mean square estimates of the same variance [6]. For the combination of the three estimates $s^2(\alpha_{\text{between days}})$ the reciprocal of the estimated variance of $s^2(\alpha_{\text{between days}})$ was used as weighting factor. The combined value for s^2_{total} was obtained by addition. Finally, all estimated variances of y (= log observed plasma level) were converted to relative standard deviations (or coefficients of variation) of the observed plasma level itself, denoted as $s_{\text{rel. between days}}$, $s_{\text{rel. within day}}$ and $s_{\text{rel. total}}$ (see Table I).

TABLE I

RESULTS CALCULATED FROM DIFFERENT ARTIFICIAL PREPARATIONS A, B AND C, WHICH WERE INCLUDED IN EACH DAILY SERIES OF PLASMA SAMPLES TO BE DETERMINED

Means are geometric means. d.f. = degrees of freedom.

	A	B	C	Combined
GC—NPD method				
Total No. of observations	37	14	41	
No. of measuring days	8	4	11	
$s_{\text{rel. within day}}$	5.4 %	2.4 %	4.2 %	4.5 % (69 d.f.)
$s_{\text{rel. between days}}$	4.2 %**	6.2 %***	5.6 %***	5.2 %
$s_{\text{rel. total}}$	6.8 %	6.6 %	7.0 %	6.9 %
Weighted mean observed (ng/ml)	18.11	20.80	19.76	
S.D. of this mean (ng/ml)	0.37	0.60	0.34	
GC—MS method				
Total No. of observations	20	10	22	
No. of measuring days	6	3	3	
$s_{\text{rel. within day}}$	4.8 %	3.0 %	7.4 %	6.0 % (40 d.f.)
$s_{\text{rel. between days}}$	4.5 %*	1.8 %//	√—5.4 %//	2.6 %
$s_{\text{rel. total}}$	6.6 %	3.5 %	7.0 %	6.5 %
Weighted mean observed (ng/ml)	18.61	21.15	20.54	
S.D. of this mean (ng/ml)	0.33	0.52	0.42	

//Level of significance $P > 0.05$.
*Level of significance $0.05 > P > 0.01$.
**Level of significance $0.01 > P > 0.001$.
***Level of significance $0.001 > P$.

Clearly the total scatter of the two methods, as applied in our laboratory, is virtually the same, but there is a rather marked difference in the scatter components: the GC—NPD method shows the smallest within-day fluctuations but, in contrast to the GC—MS method, very pronounced day-to-day errors.

Systematic deviation between the results obtained by GC—MS and GC—NPD

Table I also shows the mean and its standard error of each spiked sample per method. Each mean was calculated as a weighted mean of the day means, using the reciprocal of the estimated variance of the day mean as a weighting factor. As can be seen, the difference of any two weighted means pertaining to the same preparation is small in comparison to the standard error of these means. The weighted mean of the three differences is calculated to be 0.50 with a standard error of 0.33, again suggesting no significant difference.

For a better analysis of a possible existing systematic difference between the GC—MS and GC—NPD assay method, 74 human plasma samples from a clinical mianserin study [5] were analyzed by GC—MS (including clean-up by liquid chromatography) as well as by GC—NPD. The mianserin steady state levels encountered during this study were within the range of 6 to 120 ng of mianserin per ml plasma. In Fig. 1, the plasma levels of each sample determined by the two different assay methods are represented by a dot, using logarithmic scales in order to equalize the scatter over the whole range. The straight line minimizes the sum of squares of the perpendicular distances from the points

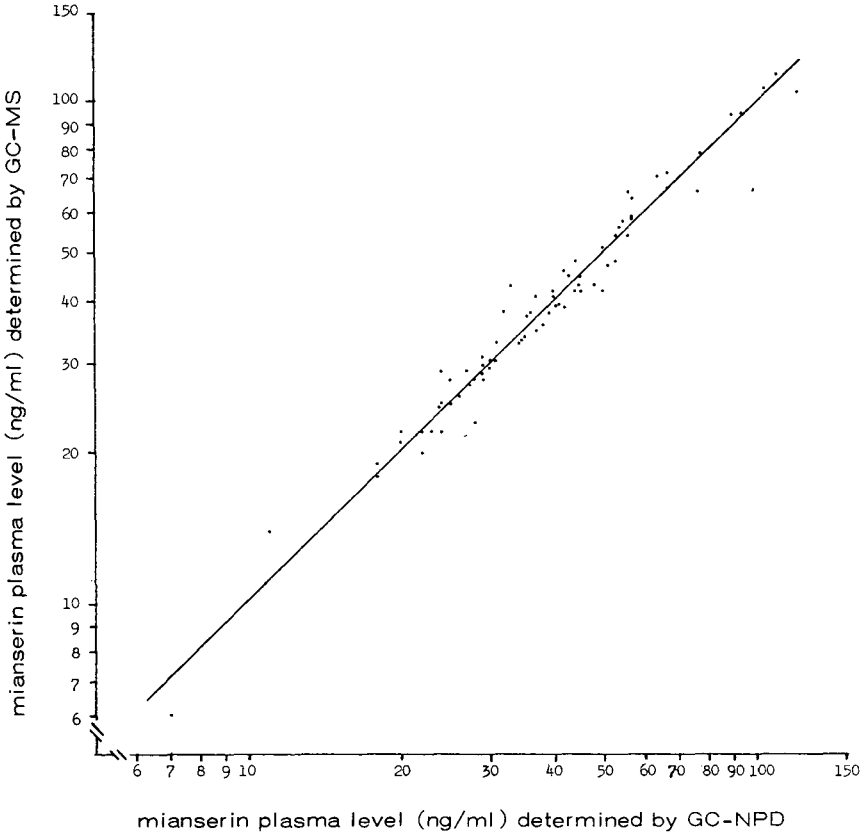

Fig. 1. A plot of the mianserin levels of 74 plasma samples from a clinical study determined by GC—NPD and GC—MS. The straight line minimizes the sum of squared perpendicular distances from the points to the line.

to the line. Its slope (in log units/log unit) is 0.992, very close to 1 and therefore compatible with the assumption that a systematic deviation, if any, from unity in the ratio between the results of analysis applying both assay methods, does not change with the plasma level in the range considered.

Analysis of variance of the observed plasma levels (in log units) reveals that the geometric mean ratio GC—NPD/GC—MS = 0.995 with 95% confidence limits of 0.977—1.014. Therefore, systematic deviation between the two assay methods, if existing at all, can only be very small.

CONCLUSIONS

For the determination of mianserin in human plasma, it is demonstrated that the GC—MS and GC—NPD assay methods are virtually the same with respect to the overall precision. A statistically significant day-to-day variation of the GC—NPD assay method is found. Because at present no explanation for this phenomenon can be given, it is recommended to analyze a particular plasma sample with the GC—NPD assay method on different days whenever possible. Averaging the results will lead to a still better estimate of the actual mianserin plasma level.

By application of both assay methods to plasma samples from depressed patients, no systematic difference between the results in mianserin plasma levels is observed.

Owing to the simplicity of the assay procedure, the GC—NPD method has advantages over the GC—MS assay method for routine mianserin plasma level determinations.

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Biomedical Applications

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Note

Reversed-phase high-performance liquid chromatographic assay for cefoxitin in proteinaceous biological samples

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(Received June 19th, 1979)

Cefoxitin is a semi-synthetic derivative of cephamycin C (q.v.) under government control (Canada) as an experimental antibiotic agent. It is a 7-α-methoxyl, 7-thienyl-acetamido cephalosporanic acid. The methoxy group at the 7-α position of the 3-cephem nucleus gives cefoxitin an increased resistance to β-lactamase produced by some bacteria. Organisms that are sensitive to cefoxitin but resistant to most other cephalosporins (at concentrations of less than 50 mg/ml), include indole-positive *Proteus morganii* and *Serratia marcescens* [1].

This method was developed to accurately quantitate low concentrations of cefoxitin in canine serum and cerebrospinal fluid (CSF). The method described also works well for accurate determinations of cefoxitin in small samples of human serum (100 μl). Other methods have been published for cefoxitin [2, 3] but none have included measurements in proteinaceous fluids.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatographic (HPLC) system used was the Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A solvent delivery system, Model 450 variable wavelength detector, Model U6K injector, and a Hamilton 25-μl syringe (No. 805). A μBondapak C_{18} (10-μm particle size) reversed-phase column (30 cm × 3.9 mm I.D.) was used, with a Bondapak C_{18}/Corasil guard column in series (Waters Assoc.). The detector was connected to a Hewlett-Packard Model 3380A integrator.

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Materials

Methanol (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Cefoxitin standard was produced by Merck, Sharpe & Dohme Research Labs. (West Point, Pa., U.S.A.) as a sterile preparation of the sodium salt (mefoxin) and supplied by the Department of Pharmacy, St. Boniface General Hospital.

The purity of the cefoxitin was assayed by injecting 20 μl of the 100 μg/ml stock solution onto the column under assay conditions. After 45 min no other significant peaks other than that of the cefoxitin were found.

Samples of the canine sera and CSF were supplied by Dr. G. Stiver, Director of Infectious Disease Section, Dept. of Medicine, St. Boniface General Hospital (supported by grants from Merck Frosst Labs., Point Claire-Dorval, PQ. and Manitoba Medical Services Foundation Inc.).

Procedure

Methanol was added to an aqueous solution of 0.03% ammonium carbonate (15:85, v/v). This mobile phase was filtered through a membrane filter (0.2-μm pores) (GA-8 cellulose triacetate; Gelman, Ann Arbor, Mich., U.S.A.) and degassed by mixing with a magnetic stirrer while applying a vacuum. This mobile phase was stored at 4° and allowed to come to room temperature before use.

Stock solutions of cefoxitin were prepared in water to concentrations of 10 μg/ml and 100 μg/ml and stored frozen. Experimental standards were prepared by adding the appropriate amount of stock solution to blank samples to concentrations of 1, 10, 25, 50, and 100 μg/ml.

To one volume of the sample were added 3 volumes of anhydrous ethanol. The solution was mixed on a vortex mixer for 2 min and allowed to stand for 15 min at room temperature, during which time the mixture was again shaken twice. The sample was centrifuged at 3000 *g* for 15 min and the supernatant removed. The supernatant (20 μl) was injected onto the column. If the protein layer was disturbed during the removal of the supernatant, the entire mixture was re-centrifuged, as the protein particles quickly clog the microparticulate columns.

The flow-rate was 2.0 ml/min, detector wavelength 238 nm, with a sensitivity of 0.1 a.u.f.s. and a back pressure of 900 p.s.i. The integrator settings were: attenuation, 1; chart speed, 0.5 cm/min; area reject, 10^3 μV sec and slope sensitivity, 0.1 mV/min.

The peak areas were integrated in μV sec units and were plotted against the concentrations of the standards to give the calibration curve.

RESULTS

Chromatograms obtained from serum and CSF are shown in Fig. 1. Retention time for the cefoxitin was 4.8 min. No interfering peaks occurred near the cefoxitin peak in normal blank serum. In all cases the peaks were symmetrical, sharp, and well augmented from the baseline.

The concentration range of 1—100 μg/ml was linear with respect to the area (absorbance). The minimum detectable amount of cefoxitin was 2 ng in 20 μl, at this detector range.

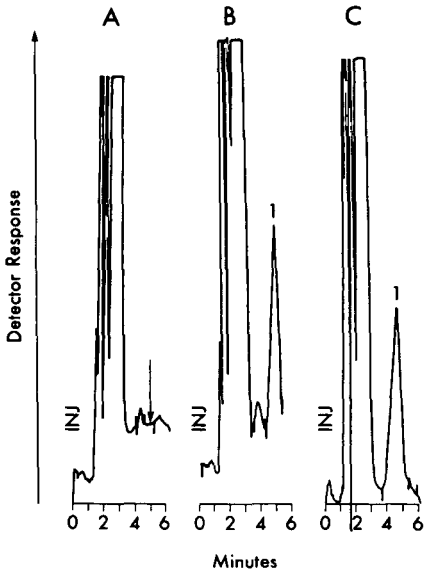

Fig. 1. Chromatograms of cefoxitin in canine serum and CSF. A, blank CSF sample (arrow indicates cefoxitin peak); B, serum sample and C, CSF sample. Cefoxitin concentration for B and C was 15 μg/ml.

Recovery from human serum was 92.5% ± 0.9% based on 10 samples for each standard concentration or 50 samples in total. Recovery studies on the canine samples could not be completed due to lack of samples but initial studies show a recovery of greater than 85%.

Ethanol was used to deproteinize the samples because it did not precipitate any of the protein-bound cefoxitin. Trichloroacetic acid is an excellent deproteinizing agent but because it lowers the pH the cefoxitin also precipitates out.

A reversed-phase HPLC method has been described for the assay of cefoxitin in human serum, canine serum, and canine CSF.

ACKNOWLEDGEMENTS

We are grateful to Mr. Dennis Cote, Dept. of Pharmacy, St. Boniface General Hospital for helpful discussions and Judi Doell for preparing the typewritten manuscript. The authors are supported by Medical Research Council of Canada Grant No. 321-3143-06 and funds from the Department of Surgery, University of Manitoba Faculty of Medicine.

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Biomedical Applications

CHROMBIO. 428

Note

High-performance liquid chromatographic micro-assay for chloramphenicol in human blood plasma and cerebrospinal fluid

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(Received June 14th, 1979)

Chloramphenicol (CAP) is an effective antibiotic whose use may be expected to increase [1]. The drug is associated with serious toxic side effects [2]. Some of these side effects and the therapeutic efficacy seem to be related to the circulating concentrations of the drug, and it is desirable, therefore, to monitor its concentration in biological fluids during therapy [3, 4].

Several different methods have been used in the analysis of chloramphenicol in biological fluids [5, 6], but high-performance liquid chromatography (HPLC) promises to be the method of choice [1]. However, the published HPLC procedures for the determination of CAP [1, 5, 7, 8], with one exception [7], do not use an internal standard, and rely instead on accurate volume transfers and on comparing the peak height of CAP to those of authentic standards for quantification. This is clearly a severe disadvantage in a high-volume routine clinical laboratory. A relatively large sample [0.5 ml] of plasma or serum is required in two of the published methods [1, 5], a serious limitation in pediatric work. The published method [7] which does use an internal standard also suffers from some disadvantages; the procedure involves extraction of the drug with an organic solvent followed by evaporation of the latter. The authors caution against heating during the evaporation, although no reason is given for this limitation. A variable-wavelength detector at 278 nm is used in the assay, and the procedure is lengthy.

The purpose of this communication is to describe a simple and rapid HPLC method for the determination of chloramphenicol in small-volume samples.

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EXPERIMENTAL

Chemicals

CAP was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). *threo*-2-Amino-1-(*p*-nitrophenyl)-1,3-propanediol and N-(β-hydroxy-*p*-nitrophenethyl)-acetamide were purchased from the ABC Library of Rare Chemicals, Aldrich (Milwaukee, Wisc., U.S.A.). Gentamicin sulfate and ampicillin were obtained from Sigma (St. Louis, Mo., U.S.A.). Chloramphenicol sodium succinate (Parke, Davis & Co., Ann Arbor, Mich., U.S.A.) and penicillin G (Squibb, Princeton, N.J., U.S.A.) were provided by the pharmacy of the University of Colorado Medical Center.

An aqueous standard solution of CAP was prepared containing 30 μg/ml CAP. Methanol and acetonitrile, glass-distilled grade, were obtained from Burdick & Jackson Labs., Muskegon, Mich., U.S.A. Ammonium phosphate, monobasic, was Baker-Analyzed reagent grade.

HPLC conditions

A Waters Assoc. HPLC system consisting of a Model U6K injector, a Model 6000A pump, and a Model 440 detector (254 nm) was used. The mobile phase was 20% methanol in water, 0.01 *M* in monobasic ammonium phosphate adjusted to pH 3 with concentrated hydrochloric acid. The mobile phase was pumped at 2.5 ml/min. The column was a Waters Assoc. μBondapak C_{18}, particle size 10 μm, 25 cm × 4 mm I.D. The retention times were: CAP, 8.8 min; internal standard, 5.2 min.

Assay procedure

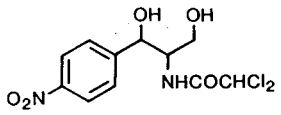
Aliquots of the standard, plasma, serum or cerebrospinal fluid, 50 μl, were placed in polypropylene 400-μl microcentrifuge tubes. Acetonitrile, 50 μl, containing 5 μg of the internal standard was added, the mixture was vortex mixed, and then centrifuged at 1500 *g* for 1 min. The supernatant (20—30 μl) was injected into the HPLC instrument with the detector set at 0.01 a.u.f.s.

Calculations

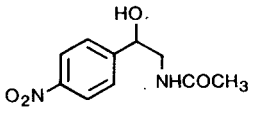
The concentration of CAP in the sample was obtained by comparing the CAP:internal standard peak height ratio from the sample to that of the 30 μg/ml aqueous CAP standard carried through the procedure.

RESULTS AND DISCUSSION

After evaluating several candidates, N-(β-hydroxy-*p*-nitrophenethyl)acetamide (I) was selected to serve as internal standard in the assay. Compound I is closely related in chemical structure to CAP, has a suitable retention time under the chromatographic conditions used, is readily available, and is inexpensive.



CAP



I

While CAP has an absorption maximum at 278 nm [9], the 254-nm fixed-wavelength detector in use in our laboratory was found suitable for the assay. It is also advantageous when using the same HPLC instrument for the analysis of several different drugs to use the same mobile phase if possible. This is because re-equilibration may take a relatively long time when mobile phases are changed. Therefore, the mobile phase selected for the determination of CAP is the same used in the analysis of other drugs (e.g., theophylline and acetaminophen) by HPLC in our laboratory.

In the procedure 50 μl of sample is treated with 50 μl of acetonitrile containing the internal standard. After brief centrifugation to separate the precipitated proteins, an aliquot of the supernatant is injected into the HPLC system.

Fig. 1a shows the chromatogram of the internal standard and authentic chloramphenicol. Plasma samples from individuals not exposed to the drug were carried tnrough the procedure with the slight modification that the acetonitrile added to the sample did not contain the internal standard. Chromatograms obtained in this fashion (Fig. 1b) showed no interference from endogenous compounds. Samples from patients on chloramphenicol therapy were also examined in the above manner, i.e., without internal standard, in order to ascertain that no metabolite of chloramphenicol interfered with the chromatography of the internal standard. No such interference was found. Fig. 1c shows a typical chromatogram obtained upon analysis of the plasma of a patient on chloramphenicol therapy. The procedure is suitable for the analysis of the drug in blood plasma, serum, and cerebrospinal fluid.

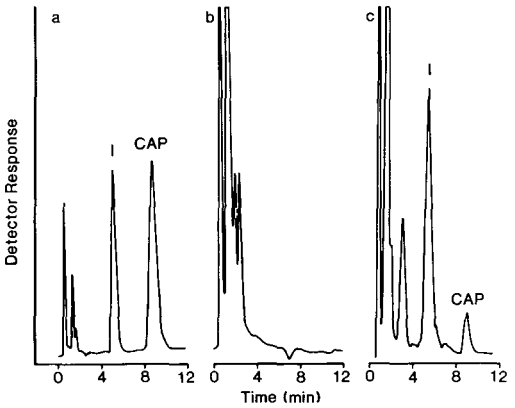


Fig. 1. (a) Chromatogram of authentic CAP and compound I. (b) Typical chromatogram obtained upon analysis of human plasma sample from an individual not exposed to CAP. Acetonitrile not containing internal standard was used to precipitate proteins. (c) Chromatogram obtained upon analysis of plasma from patient on CAP therapy. Concentration of CAP, 4.0 μg/ml. See Experimental for assay and chromatographic conditions.

Potential interference from several substances was examined. Chloramphenicol succinate, a frequently used dosage form of CAP, had a retention ca. 10 min longer than that of CAP and thus does not interfere. The N-deacylated derivative of CAP, *threo*-2-amino-1-(*p*-nitrophenyl)-1,3-propanediol, a major metabolite of CAP in man [9], had a retention time of 2 min, and therefore

does not interfere. The other major metabolite of CAP, a glucuronide conjugate [9], was not investigated, but in view of its polar nature, this conjugate would be expected to have a very short retention time under the reversed-phase conditions used. In addition, the plasma concentrations of such a metabolite may be very low. At any rate, no interference from metabolites was observed, as noted above.

Potential interference from other drugs was also investigated. Gentamicin, ampicillin, penicillin, theophylline, acetaminophen and salicylate were studied and were found not to interfere. Since some patients on CAP are sometimes administered anticonvulsant drugs for seizure disorders [10, 11] we also examined this class of drugs. Phenobarbital, phenytoin, primidone and cabamazepine were studied and were found not to interfere.

The assay procedure was developed for, and is linear in, the 1—60 μg/ml CAP concentration range. For routine use of the assay a single-point aqueous standard was found suitable.

The small sample size makes this procedure highly suitable for pediatric samples. The assay is rapid, and a sample can be carried through the procedure in 15 min.

Reproducibility was examined using nine replicate samples of pooled human plasma containing 10 μg/ml CAP. The mean value found was 9.0 μg/ml, with a coefficient of variation of 6.8%.

In conclusion, a rapid and simple microassay for CAP has been presented. The procedure uses the common 254-nm detector in an HPLC system under conditions also suitable for the determination of theophylline and acetaminophen.

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Biomedical Applications

CHROMBIO. 440

Note

High-performance paired-ion liquid chromatographic determination of bleomycin A_2 in urine

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(Received June 19th, 1979)

The antineoplastic antibiotic agent bleomycin was isolated from fermentation products of *Streptomyces verticillus* [1]. It has been shown to be effective against a variety of human neoplasms, particularly squamous cell carcinoma, lymphoma and testicular carcinoma [2—5]. The most significant value for this agent over other antineoplastic agents is a lack of bone marrow toxicity [6—8].

Various analytical methods have been used to assay a bleomycin mixture in biological fluids [2, 9—14]. However, all of these methods do not distinguish between the various components of the bleomycin mixture or their metabolites.

We recently reported a rapid method for the specific determination of bleomycin A_2 in plasma, using high-performance liquid chromatography (HPLC) with the paired-ion chromatographic technique [15]. In that method, trichloroacetic acid (TCA) solution was added to precipitate the plasma proteins prior to employing the chromatographic system. Since the strong interfering constituents present in urine are not protein in nature, the TCA precipitation is neither needed nor effective. A different pre-assay purification procedure was necessary and was developed. The method utilized a cartridge with reversed-phase packing as used in the analytical column to separate bleomycin from urine before chromatography.

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EXPERIMENTAL

Reagents and materials

Bleomycin A_2, bleomycin B_2-Cu (internal standard) and bleomycin mixture (Blenoxane®) were generously supplied by Bristol Laboratories (Syracuse, N.Y., U.S.A.). The sodium heptanesulfonate was obtained from Eastman Kodak Company (Rochester, N.Y., U.S.A.). Solvents were of HPLC grade. All other materials were reagent grade.

Stock solutions of bleomycin A_2 and bleomycin B_2-Cu were prepared by dissolving 10 mg of samples in 10 ml of deionized water. Urine standards were prepared by spiking control urine with appropriate small volumes of stock solutions.

Apparatus

A modular high-performance liquid chromatograph consisting of a constant-flow pump (Model M-6000A), a loop-type injector (Model U6K), a stainless-steel reversed-phase column (μBondapak C_{18}, 30 cm × 3.9 mm I.D., 10 μm particle size) and UV detector (Model 440) was purchased from Waters Assoc. (Milford, Mass., U.S.A.). The output from the detector was connected to a strip-chart recorder (Model 255, Linear Instruments, Irvine, Calif., U.S.A.). A guard column (3.9 mm × 6 cm) filled with pellicular reversed-phase packing (CO:Pell ODS) was obtained from Whatman (Clifton, N.J., U.S.A.). The cartridge (SEP-PAK C_{18} TM) which was used to separate bleomycin from urine was supplied by Waters Assoc.

Chromatographic conditions

The HPLC mobile phase was methanol—acetonitrile—0.0085 *M* sodium heptanesulfonate—acetic acid (30:10:59:1). A flow-rate of 2.0 ml/min was established. The resulting pressure was approximately 2500 p.s.i.

Assay procedure

To 1.0 ml of urine in a 12-ml conical centrifuge tube were added 40 μg of internal standard (40 μl of a 1.0 mg/ml aqueous bleomycin B_2-Cu solution). The samples were mixed and then 0.2 ml of the mixture was passed through a SEP-PAK C_{18} cartridge, which was prewashed with methanol and water. After washing with 2 ml of each of water, acetone, water and methanol, 2 ml of 0.02 *M* sodium heptanesulfonate in methanol solution were used to elute the cartridge. The effluent was collected and vortexed. Fifty microliters of the effluent were injected into the column.

The concentrations of bleomycin A_2 were determined from standard curves prepared by plotting the peak height ratios of bleomycin A_2 and internal standard against the spiked concentrations.

Recovery

The recovery of bleomycin from urine was determined by comparing the peak heights resulting from spiked urine samples and the peak heights obtained from direct injection of same amount of drug in aqueous solution.

Validation of the assay method

Aliquots of control urine were spiked with known amounts of bleomycin A_2 to give several concentrations. The samples were assayed in triplicate as previously described. The coefficient of variation (C.V.) and relative error for the assay were calculated.

Rabbit urine sample

Four female New Zealand White rabbits received 1—2 mg of bleomycin A_2 per kg in the marginal ear vein. After medication, the rabbits were placed in separate metabolic cages which were located in a quiet place. A regular diet and drinking water were supplied. The urine was collected through a funnel into a 250-ml Erlenmeyer flask which was surrounded with dry ice in an insulated foam box. After collecting the urine for 24 h, it was thawed at room temperature and then filtered. The filtrate volume was determined and mixed well. Aliquots of the 24-h urine were assayed as previously described. The percentage of bleomycin A_2 excreted unchanged in 24-h urine was calculated.

RESULTS AND DISCUSSION

In previous reported work on the HPLC determination of bleomycin A_2 in plasma, the interfering plasma proteins were removed by TCA precipitation prior to chromatography [15]. Since the strongly interfering constituents present in urine are not protein in nature, the TCA step was found not to be effective. In addition, because of its extreme hydrophilic property, bleomycin could rarely be extracted from urine. The use of the cartridge which was packed with similar packing materials to that present in the analytical column was found to be the most appropriate approach to separating bleomycin before assay.

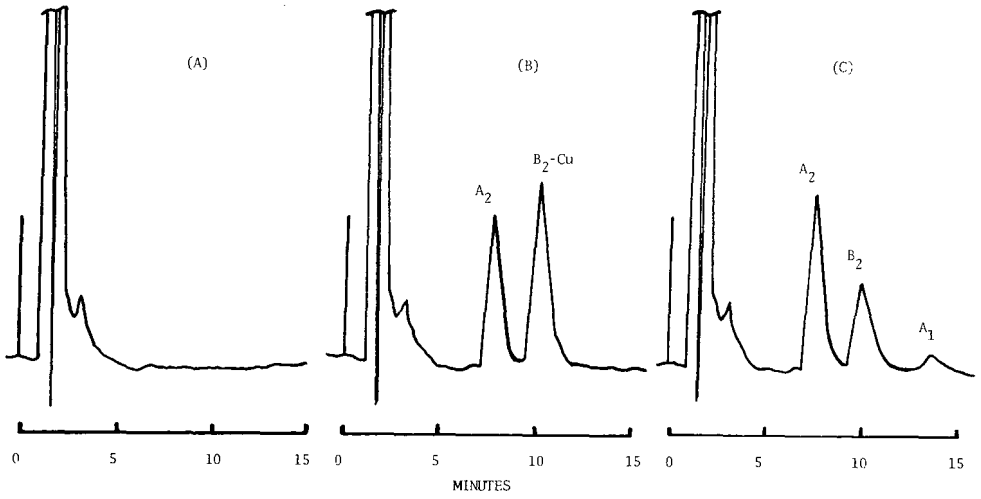

Fig. 1. Chromatograms obtained from HP-PIC assay of (A) control rabbit urine, (B) urine from a rabbit 24 h after receiving a single dose of bleomycin A_2. Peak for bleomycin A_2 was found to correspond to a concentration of 30 μg/ml. Peak for internal standard bleomycin B_2-Cu was 40 μg/ml. (C) Urine from a rabbit 24 h after receiving a single intravenous dose of bleomycin mixture 4 units/kg.

TABLE I

PRECISION AND ACCURACY FOR ASSAY OF BLEOMYCIN A_2 IN URINE

Actual concentration (μg/ml)	Experimental concentration [μg/ml; mean* (range)]	Coefficient of variation	Relative error (%)
Control	–	–	–
8.00	8.10 (7.19—9.54)	15.5	12.0
16.0	15.4 (14.1—17.1)	10.0	7.10
28.0	29.5 (28.3—31.0)	4.70	3.70
40.0	40.5 (40.0—41.0)	1.30	1.00
50.0	49.0 (46.7—52.0)	5.60	4.10
	Overall	(7.40)	(5.60)

* $n = 4$.

In a preliminary study, bleomycin was found to be adsorbed onto the cartridge from aqueous solution and could not be eluted from the cartridge by using either water or organic solvents such as methanol, acetonitrile, acetone or ethyl acetate. However, it could be easily eluted by small volumes of 0.02 *M* sodium heptanesulfonate in methanol solution. Though there is no definitive explanation to this adsorption phenomenon, most of the bleomycin was retained and all of the interfering substances were removed with the described procedures.

Chromatograms obtained from the analysis of control rabbit urine and urine containing bleomycin A_2 and bleomycin mixture are shown in Fig. 1. The chromatogram of control urine showed no interfering peaks. Under the assay conditions, bleomycin A_2 and bleomycin B_2-Cu had retention times of 7.8 and 10.2 min, respectively. The retention time of bleomycin B_2 is found to be identical to that of bleomycin B_2-Cu.

Excellent linearity (r>0.990) was observed for the standard curve over the 5—50 μg/ml range. The precision and accuracy for the assay of bleomycin A_2 are reported in Table I. The coefficient of variation (C.V.) ranged from 1.3 to 15.5%, with an average of 7.4%. The mean relative error was 5.6%.

The recovery of bleomycin A_2 from urine after a primary separation using new cartridges was found to be 60%. However, the recovery could be improved

TABLE II

PERCENTAGE OF BLEOMYCIN A_2 EXCRETED UNCHANGED IN RABBIT URINE 24 h AFTER SINGLE-DOSE INTRAVENOUS ADMINISTRATION OF BLEOMYCIN A_2

Rabbit No.	Dose (mg/kg)	Percentage of bleomycin A_2 excreted unchanged
1	1.0	50.5
2	1.0	54.8
3	2.0	56.6
4	2.0	67.0
	Mean ± S.D.	57.2 ± 7.0

by conditioning the cartridges with an ion-pairing reagent, 0.02 *M* sodium heptanesulfonate in methanol. In attempting to condition the cartridge, 2 ml of aliquots were passed through the cartridge followed by washing with 10 ml of methanol and 10 ml of water. This procedure was undertaken repeatedly. The recovery was found to be as high as 85% after the cartridge was conditioned with more than 60 ml of the methanolic ion-pairing reagent.

To demonstrate the utility of this method as an appropriate procedure for the determination of bleomycin A_2 in urine, urine obtained from rabbits that had received a single intravenous dose of the drug were assayed (Table II). The percentage of the bleomycin A_2 excreted unchanged in 24-h urine was calculated.

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Journal of Chromatography, 181 (1980) 132—134
Biomedical Applications

CHROMBIO. 438

Note

Reversed-phase high-performance liquid chromatography of phenylbutazone in body fluids

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(First received June 14th, 1979; revised manuscript received September 13th, 1979)

Phenylbutazone (PBZ) is an effective anti-inflammatory agent used widely in horses to improve their racing performance. PBZ has been detected in body fluids by spectrophotometry [1—4], high-performance liquid chromatography (HPLC) [5] and gas—liquid chromatography [6]. However, difficulty has been encountered in measuring minute amounts of PBZ such as those remaining 24—48 h after drug administration. An HPLC method using a reversed-phase column, which allows the detection of 50—100 ng of PBZ per ml in 0.5—1 ml of plasma, urine, saliva and sweat of horses is described.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a continuous-flow constant-volume delivery system (Model 6000A, Waters Assoc., Milford, Mass., U.S.A.), a U6K universal injector (Waters Assoc.) and a variable wavelength UV detector (GM 770, Schoeffel Instruments). A column (250 mm × 4.6 mm I.D.) packed with μBondapak C_{18} (Waters Assoc.) was included with the apparatus.

Reagents

The reagents were of analytical grade (Prolabo, Paris, France). Phenylbutazone was purchased from Vetoquinol (Lure, France).

Standards

A phenylbutazone standard stock solution was prepared containing 1 mg of PBZ per ml of methanol. The solution was diluted with mobile phase to obtain

1, 0.1, and 0.01 μg, respectively, in a constant injection volume of 10 μl. Standard solutions were stored at +4°.

Operating conditions

Analyses were performed with a mobile phase consisting of 2% glacial acetic acid in water—methanol (35:65, v/v). The flow-rate was set at 2 ml/min (inlet pressure of 2800 p.s.i.).

Before use the phase was degassed by applying a vacuum to the solvent reservoir for approximately 5 min. Detection of PBZ was achieved at 240 nm. The retention time of PBZ was 6 min and the system operated at an ambient temperature of 18—20°. A typical chromatogram for 10 ng of PBZ injected into the column with a detection sensitivity of 0.01 a.u.f.s. is shown in Fig. 1.

Extraction procedure

One millilitre of 1 *N* HCl and 10 ml of hexane were added to 0.5 ml of plasma, urine or sweat. The tubes were shaken for 30 sec and centrifuged for 10 min at 11,400 *g*. The organic phase was isolated by filtration on phase-separating paper (Whatman No. 1 Ps) and evaporated at 60° under a nitrogen gas stream to prevent oxidation. The residue was dissolved in 100 μl of elution solvent. Ten or 20 μl of this solution were injected into the column.

The recovery of the extraction procedure for both plasma and sweat was found to be 65 ± 2% with a concentration range of 100 ng to 10 μg.

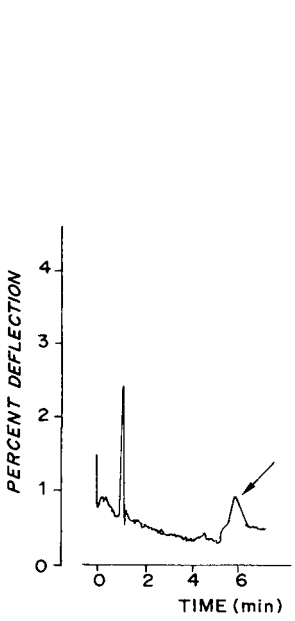

Fig. 1. Chromatogram corresponding to an injection of 10 ng of phenylbutazone.

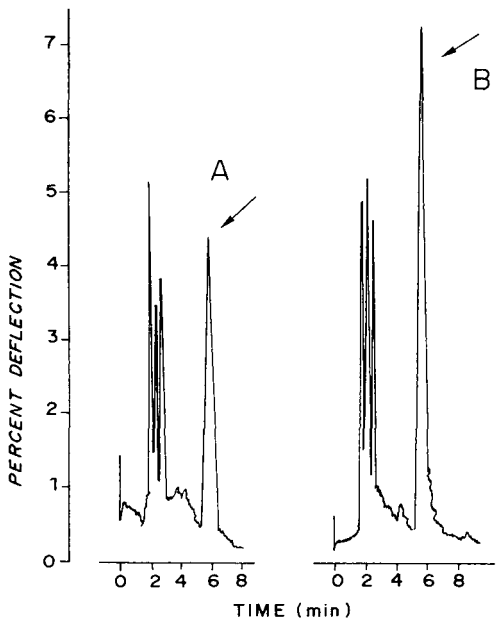

Fig. 2. Chromatogram of horse sweat containing phenylbutazone before (A) and after (B) addition of 50 ng of phenylbutazone.

RESULTS AND DISCUSSION

The method allows the determination of PBZ concentrations as low as 50 ng/ml in a variety of body fluids, especially sweat in racing horses. A typical chromatogram obtained by this method is shown in Fig. 2A from an extract of sweat collected immediately after a race from a horse of 500 kg body weight which, 18 h before, had received a tablet containing 2 g of PBZ.

Oxyphenylbutazone, the major metabolite of phenylbutazone, was also separated with a retention time of 3.2 min. However, the measured concentration in the sample remained low (probably due to its low solubility in hexane); polar organic solvents such as dichloromethane or diethyl ether could be used for a better extraction. Nevertheless, such a procedure would not be selective enough to detect low concentrations of PBZ without interference by other substances present in body fluids.

In summary, a specific and precise HPLC assay has been developed to permit estimation of minute quantities of PBZ in horses.

ACKNOWLEDGEMENT

This work was supported by a grant from the Haras.

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Journal of Chromatography, 181 (1980) 135—140
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Note

Quantification of oxprenolol in biological fluids using high-performance liquid chromatography

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(Received June 18th, 1979)

Oxprenolol, a β-adrenergic blocking drug, has recently been reported to be effective in the control of hypertension in human pregnancy [1]. It was shown to be equivalent to α-methyldopa in the control of maternal hypertension with improved placental and foetal growth. The disposition of oxprenolol in the maternal-placental—foetal system is under investigation in this laboratory. Unlike propranolol whose metabolite, 4-hydroxypropranolol, has been reported to be pharmacologically active [2], oxprenolol has no known active metabolite and so a method for the quantification of oxprenolol only was developed.

Several gas chromatographic (GC) methods have been reported for the quantification of oxprenolol in biological fluids [3—5]. These methods involve lengthy and laborious extractions followed by tedious and expensive derivatization procedures. Trifluoroacetic anhydride was the derivatizing agent used in all the methods reported and the di-trifluoroacetyl derivative of oxprenolol was detected using electron-capture detection following GC separation. The assay time for the methods reported by Jack and Riess [3] and Degen and Riess [4] was over 2 hours per sample. When Walle's method [5] was used with an additional back extraction step, the total analysis time was more than an hour for each sample.

This paper describes a simple and rapid high-performance liquid chromato-

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graphic (HPLC) assay for the quantification of oxprenolol in blood, plasma, urine or breast milk. HPLC offers advantages that (a) no derivatization steps are required prior to quantification and (b) oxprenolol is detected directly.

EXPERIMENTAL

Reagents and materials

Oxprenolol hydrochloride and alprenolol hydrochloride were gifts from Ciba-Geigy (Sydney, Australia) and Astra Chemicals (Sydney, Australia), respectively. The methanol (Ajax, Sydney, Australia) used for the chromatography was analytical grade and the water was glass distilled. The 0.005 *M* solution of 1-octylsulphonic acid, adjusted to about pH 3.5 with glacial acetic acid was prepared from a commercial reagent (PIC-B8, Waters Assoc., Milford, Mass., U.S.A.). The dichloromethane used for extraction was analytical grade and the diethyl ether (anaesthetic grade) was freshly distilled in glass every morning. All other reagents were analytical grade and were used without further purification.

All the glassware was cleaned with a chromic acid mixture and washed with distilled water. The extraction and evaporation tubes were then silylated with Siliclad (Clay Adams, Parisppany, N.J., U.S.A.), washed with distilled water and dried.

Drug extraction from biological samples

Aliquots of biological samples (3 ml of plasma or urine, 1 ml of blood diluted with 2 ml of water or 2 ml of expressed breast milk diluted with 1 ml of water) were pipetted into a 15-ml glass centrifuge tube fitted with a PTFE-lined screw cap and containing an aqueous solution of the internal standard (10 μg of alprenolol hydrochloride per 100 μl). After the addition of 300 mg of sodium chloride and 200 μl of 5 *M* aqueous sodium hydroxide, the mixture

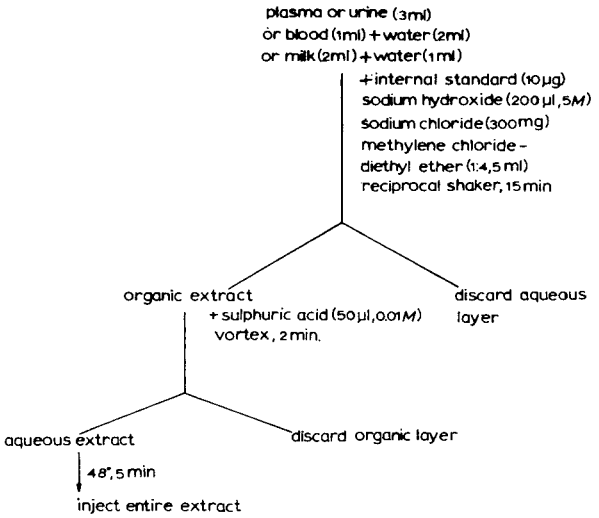

Fig. 1. Schematic diagram for the extraction of oxprenolol from biological fluids.

was extracted with 5 ml of methylene chloride—diethylether (1:4). To minimize emulsion formation, this extraction was done with gentle shaking on a reciprocal shaker for 15 min. After centrifugation at 1500 *g* for 5 min, the organic extract was transferred to an evaporation tube, a 7-ml glass tube with a 50-μl capillary at the base. After the addition of 50 μl of 0.01 *M* sulphuric acid, the evaporation tube was stoppered and shaken on a vortex mixer for 2 min. After centrifugation at 1500 *g* for 5 min the organic phase was aspirated and discarded. The aqueous phase left in the evaporation tube was then heated in a water bath at 48° for 5 min to evaporate off any residual organic solvent. The entire aqueous extract was then injected into the liquid chromatograph. Fig. 1 gives a schematic outline of the extraction procedure.

Chromatography

The HPLC system consisted of a Varian Aerograph Model 8500 high-performance liquid chromatograph, a Valco Valve injection port operated with a 50-μl loop, and a Spectra-Physics Model 770 variable-wavelength UV detector operated at 275 nm. The column (250 × 4.6 mm I.D.) was stainless-steel tubing packed with octadecylsilane-bonded silica gel having an average particle size of 10 μm (Whatman PXS10-25 ODS-2). The output from the detector was connected to a variable input potentiometric recorder (Linear Instruments, Model 355) operating at 5 mV full-scale deflection. Analyses were performed using a mobile phase of 0.005 *M* 1-octylsulphonic acid (1 vial of PIC-B8 per liter of solvent) in an aqueous solution of 67% (v/v) methanol. The flow-rate was 60 ml/h at an inlet pressure of 2000 p.s.i. The column was water-jacketed and the operating temperature was maintained at 30°.

Calibration and reproducibility

Known quantities of oxprenolol hydrochloride (equivalent to 30—630 ng of the oxprenolol base) were added to blank plasma samples. The samples were then assayed for oxprenolol. Calibration curves were constructed by plotting the peak height ratios between oxprenolol and the internal standard versus the amount of oxprenolol base added. To check the reproducibility of the analytical procedure, three different plasma calibration curves were constructed on three different days using human plasma samples from six different sources: a healthy male, a healthy female and four healthy pregnant women near term. Calibration curves using human urine, blood and expressed breast milk were similarly prepared using samples from at least two different sources.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, the retention times of oxprenolol and alprenolol were 6 and 8.1 min, respectively. Fig. 2 shows chromatograms of the extract of 3 ml of blank human plasma (Fig. 2a) and 3 ml of blank human plasma from another source to which were added oxprenolol hydrochloride equivalent to 569 ng of oxprenolol base (peak 1) and 10 μg of alprenolol hydrochloride (peak 2) (Fig. 2b). Similar chromatograms were obtained with extracts of urine, diluted blood and diluted human breast milk.

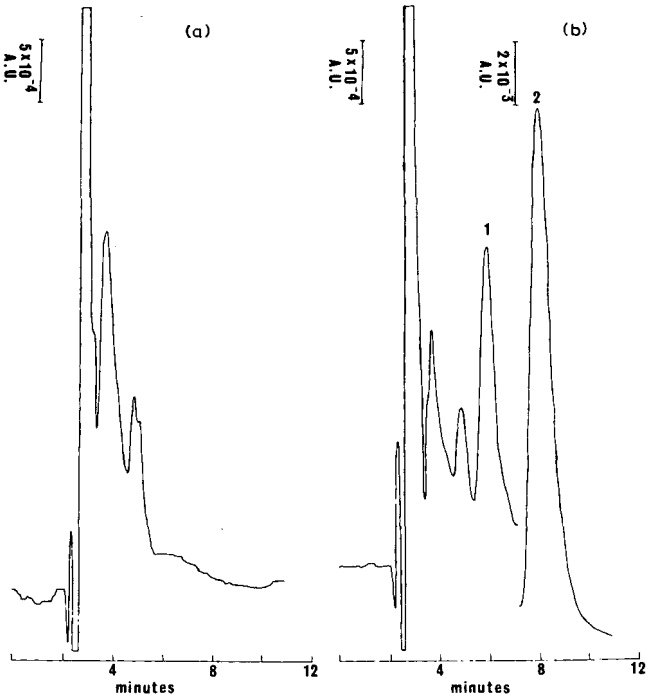

Fig. 2. Chromatograms of (a) blank human plasma and (b) blank human plasma from a different source to which were added oxprenolol hydrochloride equivalent to 569 ng of oxprenolol base (peak 1) and 10 μg of alprenolol hydrochloride (peak 2). Chromatographic conditions are given in text.

There was no endogenous peak interfering with the oxprenolol or alprenolol peaks in any case.

A summary of the calibration data is presented in Fig. 3. The calibration curves are linear and pass through the origin. The method allows reliable quantification of 30 ng of oxprenolol in plasma and thus provided a lower sensitivity limit of 10 ng/ml for a 3-ml sample.

Both benzene [4] and a mixture of dichloromethane—diethyl ether in the ratio of 1:4 [2, 3] have been used as the extraction solvent for quantification of oxprenolol. The extraction of oxprenolol from biological fluids using these two solvents and also chloroform, dichloromethane and diethyl ether have now been investigated. The dichloromethane—diethyl ether (1:4) mixture was found to be most suitable as it extracted the least number of endogenous compounds and it gave adequate recoveries for both oxprenolol and alprenolol from all biological fluids studied (Table I). The back extraction into a small volume of aqueous sulphuric acid removes any neutral endogenous compounds that may interfere with the analysis. Also, this step provides adequate concentration of the sample, making time-consuming evaporation steps unnecessary. However, the residual amount of diethyl ether present in the aqueous extract has to be removed prior to injection as it produces a negative signal which interferes with the oxprenolol peak.

TABLE I

RECOVERIES OF OXPRENOLOL AND ALPRENOLOL FROM VARIOUS BIOLOGICAL FLUIDS

Biological fluid	Oxprenolol (average percentage recovery)	Alprenolol (average percentage recovery)
Plasma	83	96
Urine	71	99
Blood	71	85
Milk	63	62

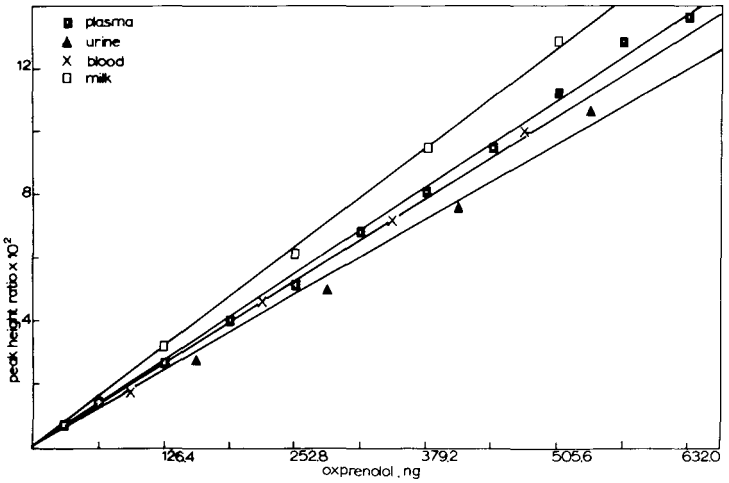

Fig. 3. Calibration curves for oxprenolol in various biological fluids.

Oxprenolol, being a strongly basic compound (pK_a = 9.2) is poorly retained on conventional reversed-phase HPLC systems. In our hands these systems resulted in skewed peaks and they also showed poor separation efficiency. The instability of silica gel at high pH (pH >8) does not allow ion-suppression by increasing pH.

An ion-pairing approach was tried when developing the chromatographic system. Alkylsulphonic acids were used as counter-ions to form neutral complexes with the protonated oxprenolol. The complex, being more lipophilic than oxprenolol, is better retained on a reversed-phase column and therefore gives better peak shape and improved separation. The lipophilicity and thus the retention characteristics of the ion-pair complex are determined by the length of the alkyl chain. Alkylsulphonic acids with pentyl, hexyl, heptyl and octyl side chains were investigated as counter-ions. Symmetrical peak shape as well as resolution of oxprenolol from alprenolol were achieved with all agents used. However, octylsulphonic acid gave the best resolution from

peaks related to endogenous substance. When pentylsulphonic acid was used, an endogenous compound with a retention time between oxprenolol and alprenolol appeared thus interfering with both peaks. On changing to alkylsulphonic acids with longer alkyl chains and increasing the methanol concentration to retain the retention of oxprenolol at approximately 6 min, the endogenous peak shifted towards the solvent front. With hexylsulphonic acid, it was completely resolved from the alprenolol peak but merged with the tail end of the oxprenolol peak. With heptylsulphonic acid, the order of elution of the endogenous peak and the oxprenolol peak reversed and the endogenous peak merged with the leading edge of the oxprenolol peak. With octylsulphonic acid, the endogenous compound was resolved from both oxprenolol and alprenolol. The methanol concentration was 67% (v/v) and the octylsulphonic acid was used at a concentration of 0.005 *M*. At the concentration of oxprenolol studied, there was no improvement of the chromatogram observed on doubling the octylsulphonic acid concentration.

Reproducible quantification of oxprenolol was achieved by using alprenolol as the internal standard. Alprenolol was chosen for its close similarity in chemical structure. Other β-blockers, metoprolol and propranolol, were also tried but alprenolol was found to be most satisfactory. The similar chemical and physical properties of alprenolol resulted in acceptable linearity of the calibration data in all biological fluids studied.

The HPLC method is rapid and accurate for the quantification of oxprenolol concentrations in plasma, blood, urine or breast milk. The sample preparation procedure is relatively simple and requires no evaporation or derivatization steps. The total analysis time for a single sample is less than 45 min. Other antihypertensive drugs, namely hydralazine, α-methyldopa and the thiazide diuretics, commonly used with oxprenolol, do not interfere with the analysis.

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Journal of Chromatography, 181 (1980) 141—146
Biomedical Applications

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Note

Determination of chlordiazepoxide and its metabolites in human plasma by reversed-phase high-performance liquid chromatography

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(Received July 2nd, 1979)

Many analytical methods of analysis have been developed in the past few years for determinating chlordiazepoxide and its metabolites in body fluids, using colorimetry [1], spectrophotofluorimetry [2,3], gas chromatography [4,5], differential pulse polarography [6], radioimmunoassay [7] and thin-layer chromatography with densitometry [8]. Each method has its limitations, permitting the determination either of chlordiazepoxide only and not of its metabolites or of only some of these.

With the advent of high-performance liquid chromatography (HPLC) and its rapid acceptance in the biomedical field, it is possible to solve the problem speedily, as has been predicted [5,9]. Recently two papers have appeared dealing with the determination of chlordiazepoxide and its metabolites in plasma or serum by means of reversed-phase HPLC [10,11]. The first [10] disregards two active metabolites of chlordiazepoxide, demoxepam and desmethyldiazepam, and uses the gradient technique, in our view unjustified. The second [11], the starting point for the method we have developed, uses a type of extraction that is unsuitable for demoxepam because of the low recovery (3.8%). Further this method of extraction does not allow for purification of the body fluids from their lipids, which damage the columns and reduce the reproducibility of the results. At least, the particle size of the chromatographic column support (5 μm) is somewhat critical and, in our view, not recommendable for routine work.

Recently, a basic paper [12] dealing with HPLC of Librium® and metabolites in human plasma, was published; this paper differs from ours, above all, in chromatographic conditions. In fact, both analytical methods rely on a double property common to many benzodiazepines to be chromatographed (unionized molecules) either in acid [12] or in alkaline medium (our paper).

We describe here a new method of determining chlordiazepoxide and its active metabolites in human plasma, that may be used for studying pharmacokinetics in man during long-term chlordiazepoxide therapy [13].

EXPERIMENTAL

Chromatographic system

The determinations were carried out on a chromatographic system made up as follows: Altex Model 110 A pump (Altex Scientific, Berkeley, Calif., U.S.A.), UV—visible (200—850 nm) Kontron-Uvicon 725 spectrophotometric detector (Kontron, Zurich, Switzerland) operating at a wavelength of 260 nm with 0.02 a.u.f.s., Rheodyne Model 712 injector with a 20-μl loop (Rheodyne, Berkeley, Calif., U.S.A.), an Hibar chromatographic column filled with LiChrosorb RP-18 (10 μm) (E. Merck, Darmstadt, G.F.R.) 250 mm × 4 mm I.D., and/or an Altex column filled with LiChrosorb RP-18 (10 μm), 250 mm × 3.2 mm I.D., and a precolumn filled with Corasil™ (37—50 μm) (Waters Assoc., Milford, Mass., U.S.A.). The detector was coupled through an interface to a Sigma 10 Data System chromatographic computer (Perkin Elmer, Norfolk, Conn., U.S.A.) and the calculations were made according to an internal standard method.

The eluent was a mixture of acetonitrile—0.1% ammonium carbonate (31:69), in isocratic conditions with a flow-rate of 2 ml/min (ca. 1300 p.s.i.).

In the above conditions the retention times of the substances of interest were: demoxepam ca. 4 min, desmethylchlordiazepoxide 5 min, nitrazepam (internal standard) 6 min 20 sec, chlordiazepoxide 7 min 45 sec, and desmethyldiazepam 11 min (Hibar column); demoxepam 2 min 20 sec, desmethylchlordiazepoxide 3 min, nitrazepam 3 min 40 sec, chlordiazepoxide 4 min 40 sec, desmethyldiazepam 6 min 30 sec (Altex column).

Reagents and drugs

Acetonitrile and *n*-hexane Lichrosolv were from E. Merck and ammonium carbonate, sodium hydroxide type RPE and diethyl ether for pesticides from C. Erba (Milan, Italy). The water was double-distilled and filtered through a 2-μm filter, Chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, N-desmethyldiazepam and nitrazepam, pharmaceutical grade, came from Hoffmann-La Roche (Basle, Switzerland).

Extraction of chlordiazepoxide and metabolites from human plasma

Into a screw-stoppered test tube (Sovirel 30, Paris, France) put 1 or 2 ml human plasma (from a pool of plasma from healthy subjects not treated with drugs for at least two weeks), adjust to pH 9 with 0.1 *N* sodium hydroxide (about 0.275 ml for 2 ml plasma), add 5 ml double-distilled water and homogenize by slow rotation. To the aqueous phase add 7 ml diethyl ether and extract mechanically for 10 min (Model K 30/300 three-dimensional agitator, Bicasa, Milan, Italy).

Centrifuge at 2500 *g* for ca. 3 min and carefully remove the ethereal phase. Re-extract the sample, proceeding as before. Combine the ethereal extracts and evaporate on a thermostatically controlled waterbath at 40 ± 1° in a light stream of pure nitrogen. Take up the residue with 100 μl of eluent mixture (for chromatography) containing 10 μg nitrazepam (internal standard), add 100 μl *n*-hexane, homogenize for 30 sec on a vortex mixer and then centrifuge at 1065 *g* for 2 min. Remove and discard the upper hexanic phase

(containing the lipids extracted from the plasma) with a 1-cm^3 syringe (special 2-in. 23 gauge needle, Becton-Dickinson, Toronto, Canada). Inject 20 μl into the chromatographic system, operating in the conditions described.

RESULTS AND DISCUSSION

We found a linear correlation between the concentration of the benzodiazepines and the ratio of the areas of the peaks, benzodiazepine/internal standard, in the range between 30 ng/ml and 3 μg/ml of the original plasma samples. For desmethyldiazepam the linearity range was between 50 ng/ml and 3 μg/ml. For lower concentrations it is advisable to start with 2 ml plasma and work at 0.01 a.u.f.s.; even at this sensitivity the instrumental noise is fairly low. The sensitivity limits for the drugs are the lower values of the linearity range (starting from 2 ml of human plasma samples).

This procedure is standardized for determinations on plasma samples from patients on long-term chlordiazepoxide therapy, but is also suitable for single 30-mg oral administration. Steady-state plasma values for individual benzodiazepines are published [6,14], obtained by other techniques, from patients undergoing chronic treatment (30 mg daily); we chose values similar to these

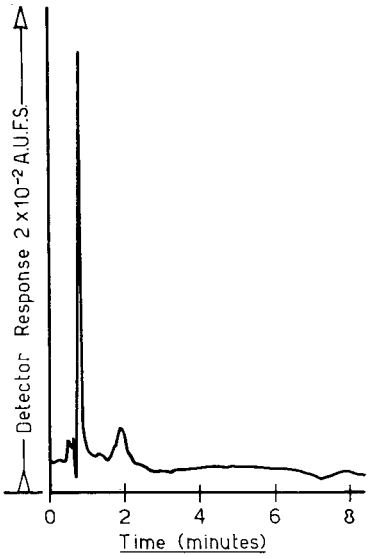
TABLE I

RECOVERY OF CHLORDIAZEPOXIDE AND ITS METABOLITES ADDED TO HUMAN PLASMA USING AN ALTEX COLUMN

Compound	Quantity added (μg/ml)	Recovery* (μg/ml)	Recovery** (%)
Chlordiazepoxide	1.20	1.190 ±0.028	99.0 ±2.3
	1.00	0.973 ±0.012	97.3 ±1.2
	0.60	0.599 ±0.007	99.9 ±1.19
	0.20	0.202 ±0.009	101 ±4.5
	0.10	0.103 ±0.0048	103 ±4.66
N-Desmethylchlordiazepoxide	0.40	0.3707±0.0068	92.7 ±1.84
	0.35	0.337 ±0.007	96.3 ±2.0
	0.30	0.271 ±0.005	90.3 ±1.85
	0.18	0.175 ±0.007	97.1 ±4.1
	0.12	0.106 ±0.0047	88.27±4.43
Demoxepam	0.45	0.341 ±0.0085	76 ±2.5
	0.30	0.220 ±0.0041	73 ±1.84
	0.20	0.153 ±0.004	76.4 ±2.6
	0.08	0.065 ±0.0018	81.6 ±2.83
	0.05	0.0422±0.002	84.5 ±4.72
N-Desmethyldiazepam	0.40	0.392 ±0.019	98 ±4.8
	0.30	0.2937±0.0093	98 ±3.2
	0.20	0.1935±0.0116	96.8 ±6

*Mean of 5 determinations, ± S.D. calculated on 5 plasma samples having the same nominal concentration.

**Mean recovery (%) ± S.D.



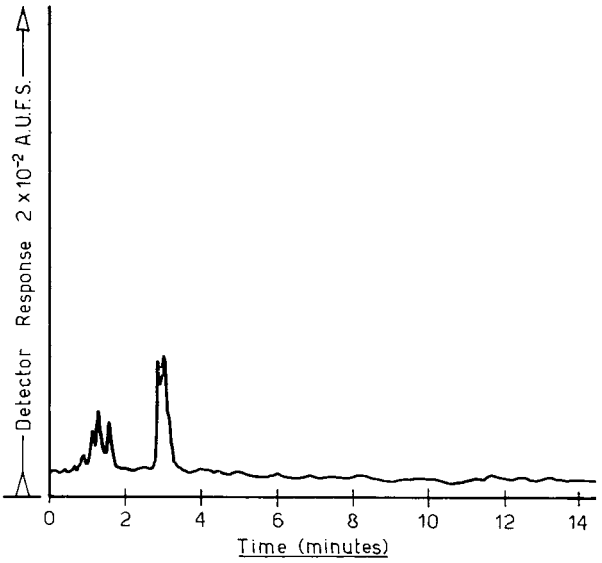
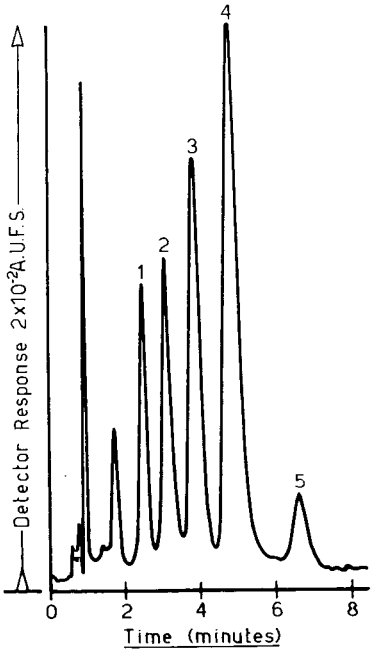


Fig. 1. Chromatogram of an extract of control human plasma using an Altex column.

Fig. 2. Chromatogram of an extract of control human plasma using an Hibar column.



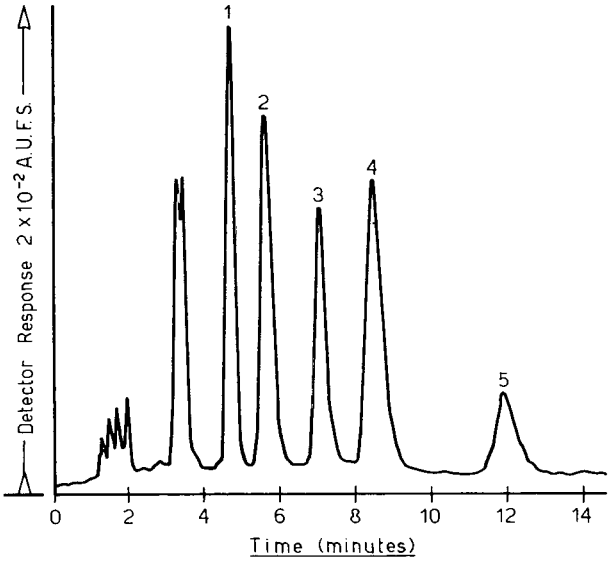


Fig. 3. Chromatogram of authentic standards recovered from control human plasma: 1, demoxepam (0.4 μg/ml); 2, desmethylchlordiazepoxide (0.4 μg/ml); 3, nitrazepam; 4, chlordiazepoxide (1 μg/ml) and 5, desmethyldiazepam (0.4 μg/ml); column, Altex.

Fig. 4. Chromatogram of authentic standards recovered from control human plasma: 1, demoxepam (0.45 μg/ml); 2, desmethylchlordiazepoxide (0.35 μg/ml); 3, nitrazepam; 4, chlordiazepoxide (0.48 μg/ml) and 5, desmethyldiazepam (0.4 μg/ml); column, Hibar.

for simulating plasma concentrations close to the real ones. When this method is employed to determine chlordiazepoxide and its metabolites after a single 30-mg dose of Librium it is necessary to bear in mind the fact that demoxepam and desmethyldiazepam are present at rather low concentrations about 30 h after administration [6], here the sensitivity can be increased by reducing the final volume of extract from 100 to 50 μl and/or using a larger loop. Table I gives the results. Also, some examples of chromatograms are given (Figs. 1—5).

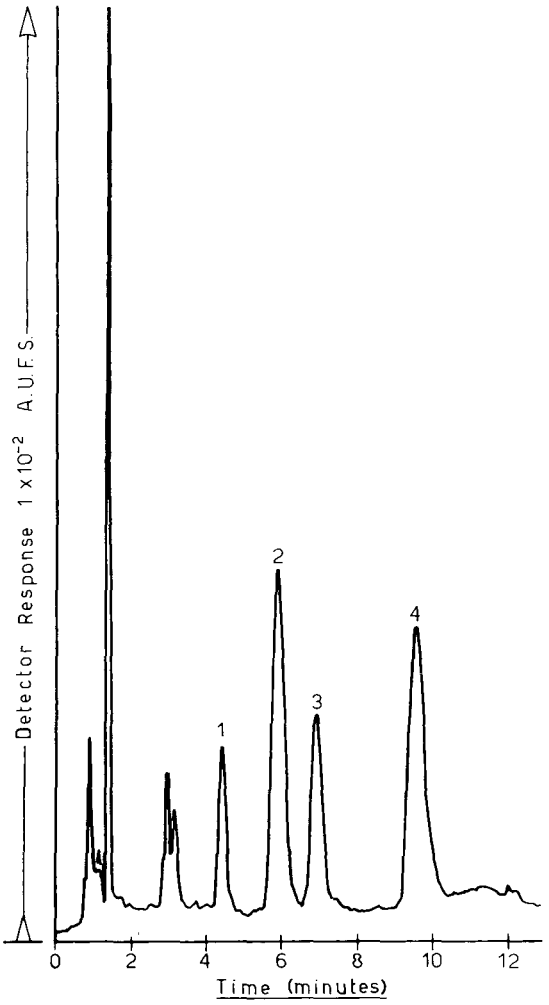


Fig. 5. Chromatogram of a human plasma extract from a patient having received a single oral administration, 30 mg chlordiazepoxide, taken at 36 h. Peaks: 1, demoxepam; 2, desmethylchlordiazepoxide; 3, nitrazepam; 4, chlordiazepoxide. Column, Hibar.

In conclusion, we consider that reversed-phase HPLC is at present the most suitable method of analysis for determining chlordiazepoxide and its metabolites in body fluids because of its specificity, sensitivity, simplicity and speed of execution.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. P. Heizmann (Hoffmann-La Roche) for criticism and suggestions to this paper, Mr. G. Cappelletti for technical collaboration, Mrs. C. Marchi for typing this manuscript and Mr. R. Quaggio for drawing the figures.

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Journal of Chromatography, 181 (1980) 147—152
Biomedical Applications

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Note

New rapid assay of theophylline in plasma by isotachophoresis

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(Received June 27th, 1979)

Theophylline (1,3-dimethylxanthine) is a key drug in the treatment of asthma, because of its effectiveness as a bronchodilator. The therapeutic value depends on the concentration in the blood. Levels of 10—20 μg/ml are considered to be therapeutic plasma concentrations. The rate of elimination from the circulation varies markedly among individual patients. Children and smokers eliminate theophylline fast. Levels higher than 20 μg/ml result in unacceptable side-effects. A "standard" dosage will, in many cases, result in overdosage or insufficient therapy, and consequently analysis of the theophylline concentration in plasma is necessary to ensure adequate therapy. For these analyses a rapid quantitative method is necessary which, since the patients are often children, should require only a small volume of plasma.

Existing methods commonly used for theophylline analyses are spectrophotometry, gas—liquid chromatography (GLC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and the enzyme multiplied immunoassay technique (EMIT). The spectrophotometric methods [1—3] require relatively large amounts of plasma (1—3 ml) and have low specificty due to interference by both endogenous and exogenous substances, such as caffeine in coffee and tea and theobromine in chocolate. Gas chromatographic methods [4, 5], which eliminate the interference by such substances and by many other drugs, have been developed. Plasma volumes of 100 μl are sufficient, but the extraction and derivatization steps give an over-

all analysis time of about 30—45 min even though the time for the GLC separation is only a few minutes. Quantitative TLC has been evaluated recently [6, 7], but it is time consuming if not used batch-wise. To obviate the need for derivatizing the theophylline, a number of HPLC procedures have been proposed during the last few years [8—16]. HPLC requires some type of sample preparation, such as extraction, and/or protein precipitation followed by centrifugation to remove solid material. The volumes of plasma needed are about 100 μl. The direct injection of serum onto a guard column has recently been described [17]; the volume of sample then required was only 10 μl, but the column had to be repacked frequently. EMIT requires only simple sample preparation [18]. The analysis time is short, but the reagents are expensive.

Since most of the above procedures are cumbersome and laborious, we investigated the possibility of using isotachophoresis for the analysis of theophylline. The isotachophoretic technique has been described by several authors — Haglund [19], Arlinger [20], and recently in an extensive review by Everaerts et al. [21]. It was shown that theophylline could be separated and readily quantified with this technique without laborious pretreatment of the sample.

EXPERIMENTAL

Isotachophoretic conditions

The isotachophoretic analyses were performed with an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a 23-cm capillary tube. Initially the leading electrolyte used was 5 m*M* HCl adjusted with Tris (Sigma, St. Louis, Mo., U.S.A.) to pH 8.4 to give a broad separation range. Later on, a narrow separation range was obtained with 5 m*M* glycyl-glycine (Fluka, Buchs, Switzerland), adjusted to pH 8.4 with Tris, as leading electrolyte. In both cases 0.2% (w/v) hydroxypropylmethylcellulose (HPMC, Methocel 90 HG, 15,000 cps; Dow Chem. Co., Midland, Mich., U.S.A.) was added to the leading electrolyte to minimize electroendosmosis. The terminating electrolyte was initially 10 m*M* glycine (Sigma) adjusted to pH 9.0 with $Ba(OH)_2$, but was later changed to 5 m*M* L-serine (Sigma) adjusted with $Ba(OH)_2$ to pH 9.5.

The analyses were run at a constant current of 100 μA, and the total analysis time was about 10 min. The transmission at 254 nm was recorded at a chart speed of 6 cm/min. The capillary was thermostatted to 10°.

Standard solutions

Theophylline, theobromine and caffeine were obtained from Sigma.

Standard solutions of theophylline in water were prepared at concentrations of 10, 20, 40, 60 and 80 mg/l. The stock solutions were used for making the calibration curves according to the procedure given below.

Calibration curve of theophylline in plasma

The plasma calibration curve was obtained by mixing 50 μl of plasma with 50 μl of theophylline from each of the five standard solutions and adding 100 μl of 38% (w/v) polyethylene glycol 6000 (PEG). The final plasma concentrations of theophylline were then 2.5, 5, 10, 15, and 20 mg/l. The solution was mixed thoroughly for half a minute. After centrifugation for about 5 min

at 1000 *g*, the clear supernatant was decanted and 2 μl were analysed. Care must be taken not to inject any precipitate, since this will affect the analysis result. The samples were injected by means of a microlitre syringe, equipped with a stop on the needle to ensure injection at a fixed position at the border between the leading and terminating electrolytes.

Plasma samples from patients

When assaying plasma samples from patients, 50 μl of plasma were mixed with 150 μl of PEG. A standardized volume of 4 μl was analysed. Samples containing less than 25 pmoles of theophylline were re-injected at a volume of 8 μl, and samples with more than 125 pmoles were re-run with a volume of 2 μl, to ensure more reliable values.

RESULTS AND DISCUSSION

Capillary isotachophoresis has been described as a useful technique even for quantitative determinations of picomole amounts. It was shown by Arlinger in 1974 [22] that the height of the UV signal of an ATP zone remains constant until the zone length falls below the aperture diameter of the UV cell. For narrower zones, there is a linear relationship between UV peak height and the amount of the compound injected. Svoboda and Vacík [23] and Wielders [24] have discussed the basic theory of the response of the UV detector when analysing very small amounts of sample which give zone lengths of less than the slit width of the detector. It was shown that a linear relationship exists between the height of the UV peak and the amount of sample within a certain interval. The possibilities of the "UV spike" method are demonstrated in Fig. 1, which is a plot of peak height versus amount and concentration of theophylline in plasma. It can be deduced from this standard curve that amounts of theophylline between 25 and 125 pmoles can be accurately determined. UV-absorbing compounds adjacent to the trace component zone of interst may

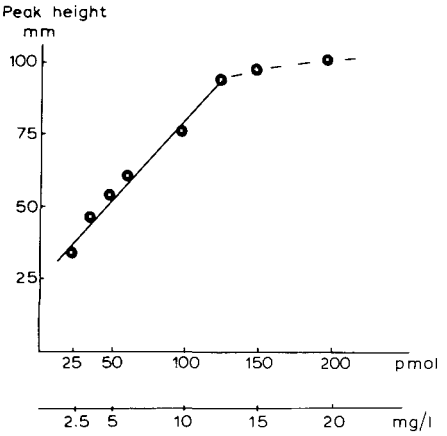

Fig. 1. Plot of peak height versus amount and concentration of theophylline in plasma; 2 μl of each theophylline concentration were injected. Glycyl-glycine was used as leading electrolyte and serine as terminating electrolyte. For further details, see Experimental.

influence the accuracy of measuring the peak height. Therefore, a low and constant level of impurities in the electrolytes and standard solutions is very important. To be able to detect trace amounts of theophylline in a complex mixture such as plasma or serum, it is necessary to remove interfering proteins by a precipitation step. Several precipitating agents were tried. PEG was found to be superior to the others. PEG is easy to handle, not too viscous at 38% w/v, does not increase the analysis time by adding extra ions and precipitates most of the proteins with a mobility similar to that of theophylline.

Fig. 2a illustrates the analysis of 250 pmoles of theophylline. A 50-μl volume of plasma was spiked with 50 μl of 0.2 m*M* theophylline, and 100 μl of PEG were added to precipitate the proteins; after centrifugation 5 μl of the supernatant were injected into the Tachophor. However, as can be seen from the UV record, some UV-absorbing compounds with a mobility similar to that of theophylline appear on either side of the theophylline zone. These UV absorbing compounds were found to influence the accuracy when quantifying the theophylline. Therefore, to minimize the influence of these UV-absorbing compounds, a number of non-UV-absorbing ions with mobilities very close to that of theophylline were tried as discrete spacers.

Fig. 2b illustrates the same experiment as in Fig. 2a, but with 10 nmol of glycyl-glycine added as spacer ion. The glycyl-glycine is shown to have a mobility close to the theophylline, and most of the UV-absorbing compounds are spaced apart from the theophylline zone by it.

In the experiment illustrated in Fig. 2c 10 nmol of serine has been added to

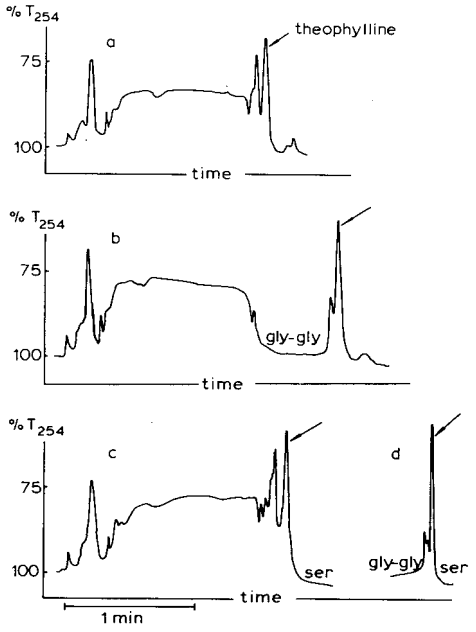

Fig. 2. Isotachophoretic analysis of a mixture of plasma, theophylline and PEG (used to precipitate most of the proteins). The arrow indicates the theophylline peak. In (a) HCl was used as the leading ion and glycine as the terminating ion; in (b) glycyl-glycine was added to the mixture; and in (c) serine was added to the mixture. In (d) glycyl-glycine was used as the leading ion and serine as the terminating ion; the sample mixture was the same as in (a).

the plasma sample. At the pH at which this separation is performed, the serine has a net mobility just below that of theophylline, and the small UV-absorbing peak appearing just after the theophylline peak (see Fig. 2a and b) has now been spaced apart by the serine.

The separation system can obviously be optimized by using glycyl-glycine as leading ion and serine as terminating ion. In this way a highly discriminating system has been developed, which is illustrated in Fig. 2d. In this electrolyte system neither theobromine nor caffeine will interfere, as they lie outside the narrow, selective mobility range. Standard calibration curves (Fig. 1) were made with plasma spiked with theophylline and PEG, using glycyl-glycine as the leading ion and serine as the terminating ion; some typical plasma analyses are shown in Fig. 3. The standard curve had to be checked each time new electrolytes were made up, because of differences in the amount of impurities in the electrolytes.

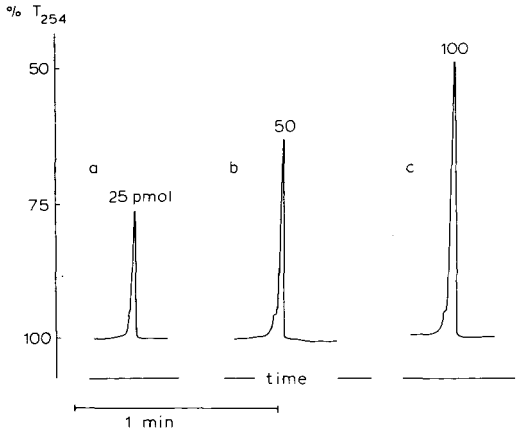

Fig. 3. Typical isotachopherogram used to prepare the calibration curves of a mixture of plasma, theophylline and PEG. Leading electrolyte was 5 m*M* glycyl-glycine and terminating electrolyte was 5 m*M* serine. Analysis time 10 min. For further details, see Experimental.

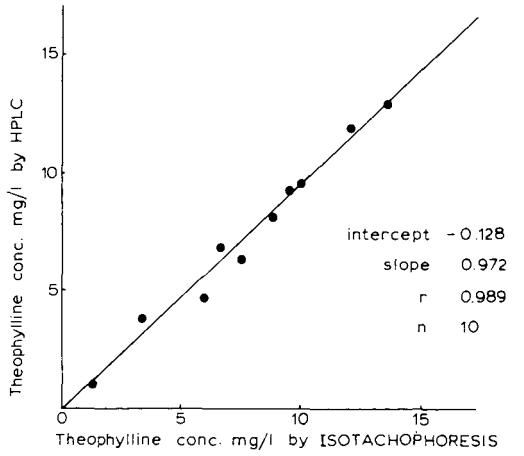

Fig. 4. Correlation of theophylline concentration in ten patient plasma specimens as measured by isotachophoresis and by HPLC.

The precision of the isotachophoresis method has been calculated from a standard calibration curve (see, for example, Fig. 1, where eight different concentrations of theophylline were measured four times each). The standard deviation was ± 2.7 mm. Samples giving peak heights below 30 mm (29% of the full UV-absorbance level of theophylline) ought to be re-run at the double volume to give a more reliable result.

Plasma samples from patients receiving theophylline therapy were analysed and quantitated using the standard curve. Ten plasma samples from patients were run twice and the mean value was compared with the mean value of double runs of the same sample analysed on HPLC according to the method of Jusko and Poliszczuk [13]. The isotachophoresis and HPLC techniques compare very well, as can be seen in Fig. 4.

CONCLUSIONS

Analytical isotachophoresis in capillaries has the unique advantage of combining accurate quantitative information with a short analysis time and high sensitivity. The only pretreatment of the plasma samples which is necessary is a simple precipitation and centrifugation. The versatility of the method makes it possible to select discriminating electrolytes, to prevent interference from related xanthines such as theobromine and caffeine.

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