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CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biomedical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

(Continued overleaf)

Contents (continued)

Notes

ELECTROPHORESIS

A Survey of Techniques and Applications

edited by Z. DEYL, *Czechoslovak Academy of Sciences, Prague*

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PART A: TECHNIQUES

Z. DEYL *(editor)*
F.M. EVERAERTS, Z. PRUSÍK *and*
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CONTENTS:
Introduction. Chapters: 1. Theory of electromigration processes *(J. Vacík)*. 2. Classification of electromigration methods *(J. Vacík)*. 3. Evaluation of the results of electrophoretic separations *(J. Vacík)*. 4. Molecular size and shape in electrophoresis *(Z. Deyl)*. 5. Zone electrophoresis (except gel-type techniques and immunoelectrophoresis) *(W. Ostrowski)*. 6. Gel-type techniques *(Z. Hrkal)*. 7. Quantitative immunoelectrophoresis *(P.J. Svendsen)*. 8. Moving boundary electrophoresis in narrow-bore tubes *(F.M. Everaerts and J.L. Beckers)*. 9. Isoelectric focusing *(N. Catsimpoolas)*. 10. Analytical isotachophoresis *(J. Vacík and F.M. Everaerts)*. 11. Continuous flow-through electrophoresis *(Z. Prusík)*. 12. Continuous flow deviation electrophoresis *(A. Kolin)*. 13. Preparative electrophoresis in gel media *(Z. Hrkal)*. 14. Preparative electrophoresis in columns *(P.J. Svendsen)*. 15. Preparative isoelectric focusing *(P. Blanický)*. 16. Preparative isotachophoresis *(P.J. Svendsen)*. 17. Preparative isotachophoresis on the micro scale *(L. Arlinger)*. List of frequently occurring symbols. Subject Index.

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PART B: APPLICATIONS

Z. DEYL *(editor)*
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CHROMBIO. 1575

GAS—LIQUID CHROMATOGRAPHY REFERENCE METHOD FOR THE ASSAY OF URINARY CREATININE

L. HARTMANN*, G. DOREE and J.-P. AMBERT

Laboratoire de Chimie Clinique et de Biologie Moléculaire, Institut Biomédical des Cordeliers, 15 rue de l'École de Médecine, 75270 Paris Cédex 06 (France)

(First received July 19th, 1982; revised manuscript received November 11th, 1982)

SUMMARY

The gas—liquid chromatographic measurement of urinary creatinine described in this paper employs methylation, the use of a diethylene glycol succinate stationary phase, an internal L-hydroxyproline standard and a temperature of 180°C. The technique, which is specific and reproducible, is shown to be a reference method providing more precise and reliable results than a conventional colorimetric method. In addition, it can be used as a routine method because of its simplicity.

INTRODUCTION

Most techniques for the analysis of blood or urinary creatinine utilize the Jaffe reaction, whose principle is the development of an orange color in the presence of alkaline picrate [1]. The relatively non-specific nature of this reaction can be partially compensated by absorption on Fuller's earth [2] or determination of the kinetics of color development [3]. It thus appeared valuable to develop a specific and precise assay for urinary creatinine using gas—liquid chromatography (GLC).

MATERIALS AND METHODS

Material

Forty-seven urine samples were obtained from healthy subjects without antiseptic in the samples. Immediately after collection, each sample was acidified to pH 2 with 1 *M* HCl and maintained at 4°C overnight. Eventual urate precipitates were eliminated by centrifugation at 1000 *g* for 20 min and the supernatant was removed for assay.

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Instrumentation

A Carlo Erba Fractovap 2300 chromatograph equipped with a flame ionization detector was connected to a recorder. The column was a glass Pyrex tube, 2 m × 4 mm I.D. Hamilton microsyringes of 10 μl (No. 702 N) and 50 μl (No. 1705 N) capacity with crimped needles were used for injection.

Reagents

Standard solutions were freshly prepared: 0.0442 *M* creatinine in 0.1 *M* HCl; 0.076 *M* L-hydroxyproline in distilled water. Also, 1.73 *M* hydroxylamine hydrochloride in 0.1 *M* HCl was freshly prepared. Trimethylaniline hydroxide (MethElute, obtained from Pierce, Rockford, IL, U.S.A.) was 2 *M* in methanol. The stationary phase was Gas-Chrom Q (80—100 mesh) loaded with 15% (w/w) diethylene glycol succinate (DEGS).

OPERATING PROCEDURE

Gas—liquid chromatography

(1) Sample preparation (see Table I).

(2) Methylation: 20 μl of sample or standard were mixed with 40 μl of MethElute.

(3) Chromatography conditions: column temperature, 180°C; injector and detector temperature, 225°C; carrier gas (nitrogen), 50 ml/min at 120 kPa pressure; hydrogen, 45 ml/min at 90 kPa; air, 400 ml/min at 100 kPa; attenuation, 100 × 8; chart speed, 4.16 mm/min; volume injected, 5 μl.

TABLE I

SAMPLE PREPARATION

	Calibration			Test
	11 m*M*	22 m*M*	33 m*M*	
0.0442 *M* creatinine (ml)	0.2	0.4	0.6	—
Urine (ml)	—	—	—	0.8
0.076 *M* L-hydroxyproline (ml)	0.4	0.4	0.4	0.4
1.73 *M* hydroxylamine hydrochloride (ml)	0.2	0.2	0.2	0.2
0.1 *M* HCl (ml)	0.6	0.4	0.2	—

(4) After detector response to the solvent, the L-hydroxyproline peak and the creatinine peak successively appear on the chromatogram 8—10 min after injection (Fig. 1). After measuring peak heights, the creatinine/standard ratio was calculated. After plotting a standard curve, the creatinine concentration in a urine sample can be read directly off the abscissa. Within a 33 m*M* creatinine concentration, diluting the urine sample is not necessary.

(5) Repeatability was determined by successively injecting the same urine sample nineteen times.

(6) Reproducibility was determined by injecting ten successive preparations of the same urine.

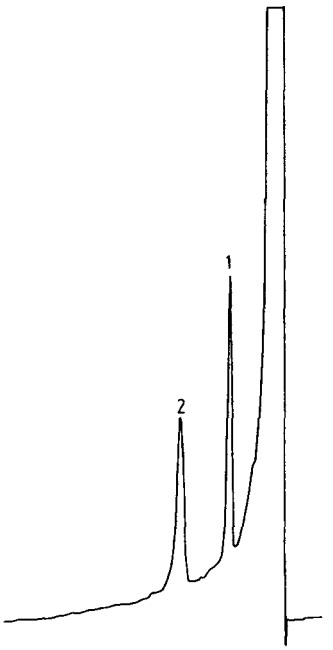

Fig. 1. Typical chromatogram obtained for the assay of urinary creatinine. Peaks: 1 = L-hydroxyproline standard (110 nmol); 2 = creatinine (38 nmol).

(7) Statistics were applied to the results obtained with both GLC and colorimetry. The means were compared with standard tests for paired data and correlations were calculated. The recovery rate mean is given with its confidence interval whereas that of repeatability and reproducibility trials are presented with the standard deviation.

Colorimetry

The method of Hare [2, 4], involving the Jaffe assay after absorption on Fuller's earth, was adopted.

APPLICATION

Both techniques were used to assay creatinine in 47 urine samples. In addition, extreme values were determined by diluting twenty samples with distilled water and by overloading fifteen samples with 0.0442 *M* creatinine.

The mean values obtained and their confidence intervals for each technique and each urine class are presented in Table II. The results obtained with GLC are slightly higher than those provided by colorimetry, but the confidence interval and standard deviation are of the same order. A comparison of the two assay methods with the paired data method is shown in Table III. The mean of the differences, which is significant for each batch of urine samples, increases with increasing creatinine concentration. The correlation coefficients and slopes of the regression lines, however, are of the same order (Figs. 2—4).

TABLE II

ASSAY RESULTS WITH GLC AND COLORIMETRY FOR NORMAL, DILUTED AND OVERLOADED URINE SAMPLES

Values are given in m*M*.

	Native urines (n = 47)		Diluted urines (n = 20)		Overloaded urines (n = 15)	
	GLC	Col.*	GLC	Col.	GLC	Col.
	19.82	17.60	17.17	16.01	29.20	27.61
	26.37	25.48	19.47	15.93	36.80	34.69
	5.75	5.66	4.86	5.22	34.33	33.27
	27.43	24.24	13.89	14.51	55.30	54.87
	7.70	9.73	13.80	13.09	16.28	17.70
	24.51	20.79	5.97	5.13	28.76	25.66
	7.08	6.63	1.32	0.97	28.93	20.35
	7.52	6.63	3.31	4.07	14.16	13.27
	18.76	15.57	5.08	5.48	19.64	19.64
	13.89	11.68	5.08	4.07	12.83	13.80
	12.92	12.65	14.07	13.62	18.05	13.80
	5.57	5.31	11.94	11.50	19.02	16.10
	6.01	5.48	6.41	6.19	19.64	19.64
	13.50	11.59	4.20	4.16	28.76	30.97
	17.87	15.93	5.53	5.39	10.84	9.91
	3.10	3.36	6.41	6.19		
	9.55	7.69	4.20	4.15		
	2.65	2.56	5.53	5.39		
	6.19	6.10	14.60	13.80		
	8.32	6.90	13.89	13.80		
	8.40	10.00				
	21.24	18.85				
	12.12	10.79				
	2.21	1.77				
	10.97	9.73				
	5.48	4.71				
	11.94	10.79				
	8.14	6.63				
	15.04	12.83				
	6.63	5.75				
	4.16	3.54				
	8.40	8.54				
	13.89	11.68				
	18.32	15.66				
	10.79	11.15				
	20.26	20.53				
	10.79	9.91				
	13.45	12.21				
	11.24	9.46				
	15.66	13.80				
	15.66	15.48				
	7.08	7.69				
	25.39	19.47				
	6.37	6.19				
	4.16	4.15				
	5.48	5.39				
	14.60	13.80				
Mean (m*M*)	11.75	10.68	8.84	8.43	24.84	23.41
Confidence interval (5%)	±0.22	±0.20	±0.29	±0.26	±0.75	±0.75
Standard deviation (± 2σ)	±13.10	±11.74	±10.70	±9.80	±23.20	±23.20

*Col. = colorimetry.

TABLE III

STATISTICAL COMPARISON FOR PAIRED DATA BETWEEN THE GLC AND COLORIMETRIC ASSAY METHODS FOR URINARY CREATININE AMONG THE THREE CLASSES OF SAMPLES ASSAYED

	Native urines (n = 47)	Diluted urines (n = 20)	Overloaded urines (n = 15)
Mean of differences (mM)	1.13	0.52	1.99
Reduced deviation	5.08	2.96	2.69
Difference (S = significant at 5% level)	S	S	S
Correlation coefficient	0.96	0.99	0.98
Slope of regression line	1.07	1.08	0.98

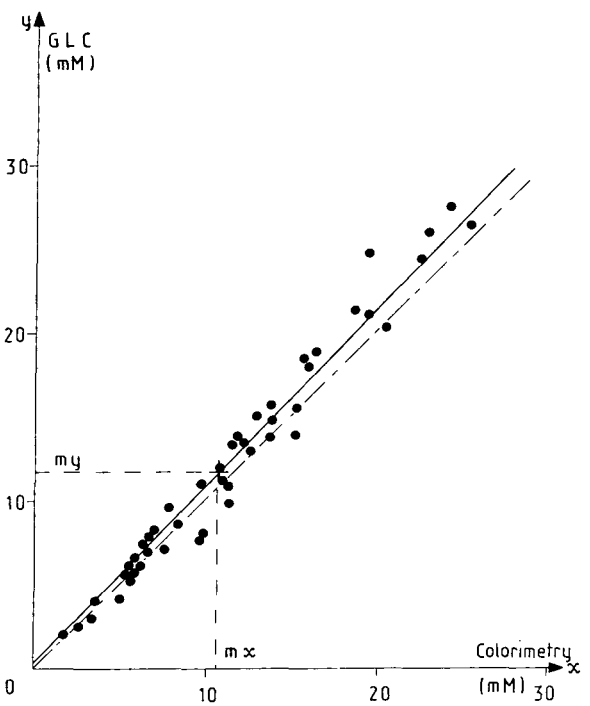

Fig. 2. Correlation between urinary creatinine results obtained with GLC (y-axis) and colorimetry (x-axis). Regression line (——), $Y = 1.07\ X + 0.03$, $r = 0.96$, $\alpha < 1\%$. Bisector (- — - — -).

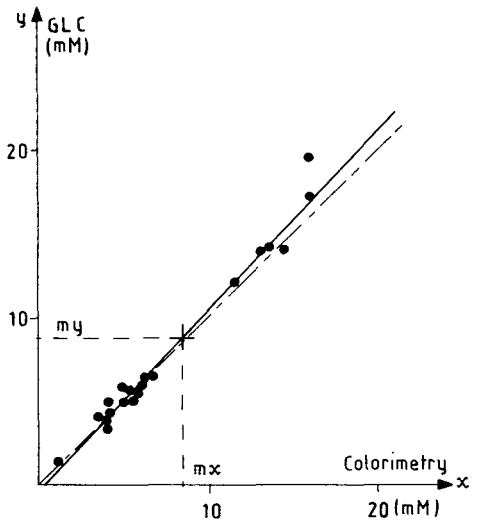

Fig. 3. Correlation between creatinine results for diluted urines measured with GLC (y) and with colorimetry (x). Regression line (——), $Y = 1.08\ X - 0.03$, $r = 0.99$, $\alpha < 1\%$. Bisector (- — - — -).

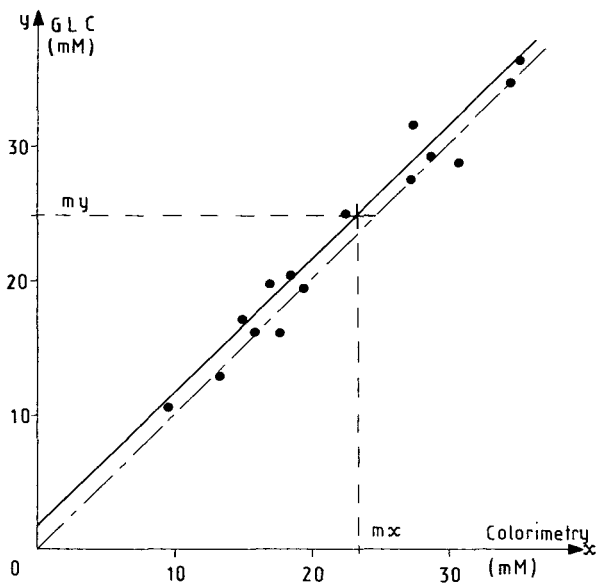

Fig. 4. Correlation between creatinine results in overloaded urines measured with GLC (y) and with colorimetry (x). Regression line (——), $Y = 0.98\ X + 0.214$, $r = 0.98$, $\alpha < 2\%$. Bisector (- — - — -).

The results from the repeatability and reproducibility trials are presented in Table IV.

Finally, 31 urine samples were subjected to GLC before and after a carefully weighed creatinine overload was achieved. Relevant results are given in Table V.

They correspond to a mean recovery rate of 98.09 ± 0.96%, for a 5% confidence interval.

TABLE IV

REPEATABILITY AND REPRODUCIBILITY TRIALS

	n	Mean (m*M*)	S.D.	C.V. (%)
Repeatability	19	11.2	0.4	3.6
Reproducibility	10	13.7	0.2	1.5

TABLE V

RECOVERY DATA

Trials	Native urines (m*M*)	Overload (m*M*)	Overloaded urines (m*M*)	Recovery (%)
1	7.96	4.42	12.17	95.2
2	11.50	3.45	14.71	93.0
3	5.97	5.40	11.50	102.4
4	8.41	5.58	13.72	95.2
5	9.73	4.37	13.94	96.3
6	5.09	3.36	8.30	95.5
7	5.09	4.57	9.62	99.1
8	14.82	4.11	18.80	96.8
9	11.72	1.88	13.50	94.7
10	11.50	4.46	16.03	101.6
11	12.61	5.13	17.70	99.2
12	3.32	3.89	7.08	96.7
13	8.63	4.19	12.61	94.9
14	12.16	5.00	17.03	97.4
15	20.53	9.38	29.82	99.0
16	7.30	4.44	11.72	99.5
17	2.65	3.25	5.97	102.1
18	4.42	4.23	8.63	99.5
19	4.86	5.22	9.95	97.5
20	4.42	4.32	8.85	102.4
21	18.36	3.36	21.68	98.8
22	9.90	2.67	12.60	101.1
23	12.16	9.73	21.68	97.8
24	5.30	2.65	7.75	92.5
25	4.86	2.76	7.52	96.4
26	3.76	3.55	7.30	99.7
27	4.87	3.40	8.18	97.3
28	10.62	3.58	14.16	98.9
29	13.70	13.26	27.17	101.6
30	14.60	2.32	16.81	95.5
31	6.86	6.44	13.49	103.0

METHODOLOGICAL CONSIDERATIONS

Chemical transformation of creatinine

Before GLC can be performed, creatinine must be converted into a stable and volatile derivative. Initially, silylation with N,O-bis(trimethylsilyl)acetamide (BSA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was attempted, either alone or with small quantities of trimethylchlorosilane (TMCS), used as an acid catalyst. Under these conditions, four to six peaks were obtained, regardless of the stationary phase, temperature and incubation time used.

As recommended by Poole et al. [5], hydroxyl functions were first blocked with hydroxylamine hydrochloride, but improvement was not sufficient.

As previously reported for GLC assays of urea [6], hot nitric acid in ethanol was unsuccessfully attempted, except for very high creatinine concentrations, which gave good results.

Acylation with acetic anhydride was attempted without success. Methylation with 0.2 *M* trimethylaniline hydroxide was chosen. By first reacting samples with a well-defined quantity of hydroxylamine hydrochloride, this reagent generated a single GLC derivative of creatinine.

Choice of internal standard

The criteria for this standard were a cyclic compound with both hydroxyl and amine functions, providing a single peak well separated from the injection and creatinine derivative peaks under the present operating conditions. L-Hydroxyproline was fully in agreement with these requirements. In the normal state, the concentration of this compound in urine is too low to be detected by this technique. In bone diseases giving rise to a significant elevation in urinary hydroxyproline excretion, creatinine assay might be erroneous. In this case, a control injection should be performed without adding hydroxyproline.

Choice of stationary phase and temperature

The stationary phase was chosen by injecting the methylated creatinine derivative with temperature programming from 50°C up to the critical phase temperature. Trials were performed with 3% OV-17 silicone on Gas-Chrom Q, 8% ethylene glycol succinate (EGS) on Gas-Chrom Q, 5% OV-225 silicone on Gas-Chrom P, 5% Apiezon L grease on Chromosorb G, 5% and 15% DEGS on Gas-Chrom Q. Using the latter phase, methylated creatinine could be detected at around 170°C.

At 180°C, 5% DEGS enabled two well-separated peaks of L-hydroxyproline and methylated creatinine to be obtained. When the stationary phase was increased to 15%, the peaks were delayed in the column and so precise quantitative determinations of low creatinine concentrations could be reached.

Interference

Our study only concerned compounds likely to change the Jaffe reaction. Glucose added at a rate of 55 m*M* gives two peaks well separated from that of

creatinine and hydroxyproline. Acetylacetone and phenylpyruvate peaks are hardly different from the solvent one.

Our working conditions did not enable us to study drug interference.

CONCLUSIONS

Urinary creatinine assay with GLC constitutes a reference method on account of its specificity and reproducibility. It is also usable in routine applications as a result of its simplicity. The results obtained are slightly higher than those provided by the colorimetric method. This technique is thus recommended to be used for 24-h diuresis and glomerular filtration rates.

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CHROMBIO. 1560

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF α-KETO ACIDS IN PLASMA WITH FLUOROMETRIC DETECTION

TOKISHI HAYASHI*, HIRONORI TSUCHIYA and HIROSHI NARUSE

National Center for Nervous, Mental and Muscular Disorders, 2620, Ogawa-Higashi-Machi, Kodaira, Tokyo (Japan)

(First received May 28th, 1982; revised manuscript received November 2nd, 1982)

SUMMARY

This paper describes a sensitive high-performance liquid chromatographic method for the quantitative determination of α-keto acids in plasma using a fluorescence detector. This method is about ten times more sensitive than that reported in a previous paper. Only 50 μl of plasma are needed for the determination of α-keto acids. However, *p*-hydroxyphenylpyruvic acid could not be analysed because the quinoxalinol derived from it does not exhibit fluorescence.

INTRODUCTION

Sensitive and reliable methods are essential to biochemical and clinical investigations. Many methods [1—6] have been reported for the determination of α-keto acids using hydrazone formation. 2,4-Dinitrophenylhydrazine has been most widely used. Recently, Ohmori et al. [7] reported a sensitive gas chromatographic method to determine these substances using pentafluorophenylhydrazine. However, the derivatization reactions employed in these methods produced *syn—anti* isomers and then complicated the chromatographic separation.

Quinoxalinol formation, on the other hand, which has been used in gas chromatography [8, 9] or liquid chromatography [10—12], provides a single main product for each α-keto acid. However, these methods were restricted in their application because there was no effective clean-up method for α-keto acids.

In a previous paper [13] we reported a high-performance liquid chromatographic (HPLC) method for the determination of α-keto acids in biological samples. This method includes an effective new clean-up and derivatization technique using a hydrazide gel column.

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This paper describes a sensitive HPLC method for the determination of α-keto acids in plasma using fluorometric detection.

MATERIALS AND METHODS

Apparatus

A Tri Rotar I high-performance liquid chromatograph equipped with a Model GP-A30 solvent programmer, a Uvidec-100 UV detector, an FP-110 fluorescence detector and an RC-225 strip-chart recorder (Japan Spectroscopic Co., Tokyo, Japan) was used in this work. The HPLC separation was carried out with a 250 mm × 4 mm I.D. stainless-steel column packed with LiChrosorb RP-8 (5 μm) using a balanced density slurry packing method. The column was covered with a column jacket. The HPLC operating conditions are given in Figs. 1 and 2.

Fluorescence spectra were measured with a Model RF-510 spectrofluorophotometer (Shimadzu Seisakusho, Kyoto, Japan).

Reagents

Sodium α-ketoglutarate (KGA), sodium α-ketoadipate (KAA), sodium pyruvate (PA), sodium α-ketobutyrate (KBA), *p*-hydroxyphenylpyruvic acid (PHPPA), sodium α-ketovalerate (KVA), sodium α-ketoisovalerate (KIVA), sodium α-ketoisocaproate (KICA), phenylpyruvic acid (PPA), sodium α-keto-β-methylvalerate (KMVA) and α-ketooctanoic acid (KOA) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). *o*-Phenylenediamine sulfate was purchased from Tokyo Chemical Industry Co., (Tokyo, Japan) and used after recrystallization from a mixture (1:1) of 1% aqueous sulfuric acid and ethanol. Hydrazide gel was prepared according to the method reported in the previous paper [13].

Reagent preparation

A stock standard solution of each α-keto acid was prepared separately at a concentration of 2 μmol/ml in water or 10% aqueous ethanol (PPA, PHPPA). A standard mixture of α-keto acids was prepared weekly by mixing the stock standard solutions and diluting with redistilled water so that it contained 80 nmol/ml of each α-keto acid. The internal standard solution was prepared in a similar manner (80 nmol/ml). *o*-Phenylenediamine solution was prepared daily by dissolving 40 mg of *o*-phenylenediamine sulfate and 100 μl of mercaptoethanol in 40 ml of 2 *N* hydrochloric acid.

Procedure for the determination of α-keto acids in human plasma

Fifty microliters of human plasma were vigorously mixed with 100 μl of the internal standard solution and 400 μl of methanol. The mixture was centrifuged at 10,000 *g* for 10 min. The supernatant was concentrated to about 100 μl on a rotary evaporator. Three drops of 0.1 *M* aqueous acetic acid and 1 ml of 0.1 *M* sodium chloride solution were added. The mixture was poured into a 150 mm × 5 mm I.D. glass column containing 0.3 ml of hydrazide gel. After the column was drained, the gel was washed with 5 ml of 0.1 *M* sodium chloride solution. The gel was then transferred to a test tube. Two milliliters of

o-phenylenediamine solution were added to it and the test tube was warmed in a water bath at about 80° C for 2 h. The mixture was then diluted with 8 ml of saturated sodium sulfate solution. The derivatives of the α-keto acids were extracted into 10 ml of ethyl acetate by shaking for 5 min. The organic layer was dried on anhydrous sodium sulfate and evaporated to dryness. The residue was redissolved with two drops of N,N-dimethylformamide and the solution was diluted with five drops of water. About 40 μl of the resulting solution were subjected to HPLC.

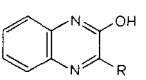
RESULTS AND DISCUSSION

In the previous paper [13] we reported on a HPLC determination of α-keto acids in human plasma using a new pretreatment method. This method was sensitive, selective and reproducible. Nevertheless, 500 μl of plasma were necessary for the exact determination. Therefore, it seemed worthwhile to develop a more sensitive method that could be applied to the analysis of α-keto acids in small plasma samples.

As fluorometry is known to increase the sensitivity of assay by several orders of magnitude, some investigators tried to develop a fluorometric method for the determination of α-keto acids. Spikner and Towne [14] developed a fluorometric method for the determination of α-keto acids based on the formation of quinoxalinol derivatives by a reaction between α-keto acid and *o*-phenylenediamine. Mizutani et al. [15] introduced 4′-hydrazino-2-stilbazole to the determination of α-keto acids. Takeda et al. [16] reported a fluorometric method based on the formation of fluorescent chelates with pyridoxamine and Zn(II) ion. However, HPLC methods using these reaction have not been reported as far as we know.

TABLE I

FLUORESCENCE SPECTRA OF QUINOXALINOL DERIVATIVES



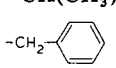
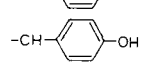
R	λ_{ex} (nm)	λ_{em} (nm)
$-CH_2CH_2COOH$	345	412
$-CH_2CH_2CH_2COOH$	355	413
$-CH_3$	350	412
$-CH_2CH_3$	352	412
$-CH_2CH_2CH_3$	356	412
$-CH(CH_3)_2$	354	410
$-CH_2CH(CH_3)_2$	359	411
$-CH(CH_3)CH_2CH_3$	353	410
	360	415
	—	—

Table I shows the fluorescence spectra of the quinoxalinol derivatives which were prepared according to the methods described in a previous paper [12]. These were measured after each quinoxalinol was dissolved in acetonitrile–0.1 *M* phosphate buffer (3:7). The quinoxalinol corresponding to PHPPA unfortunately did not show fluorescence under these conditions. For all the derivatives, the excitation (λ_{ex}) and emission maxima (λ_{em}) were observed at about 350 and 410 nm, respectively.

The upper chromatogram shown in Fig. 1 was obtained from a blank sample (redistilled water was used instead of human plasma and the internal standard solution) according to the procedure mentioned above using the fluorescence detector, and the lower one was obtained from a mixture of quinoxalinol derivatives. Chromatographic separation was carried out under the same conditions as those employed in the previous paper [13]. A strong peak was found on the chromatogram obtained from the blank sample. Its retention time was close to that of the derivative of KAA. The chromatographic separation of these compounds was further investigated. The results indicated a good separation, achieved merely by changing the concentration of tetrapropylammonium bromide in the first mobile phase down to 1/10.

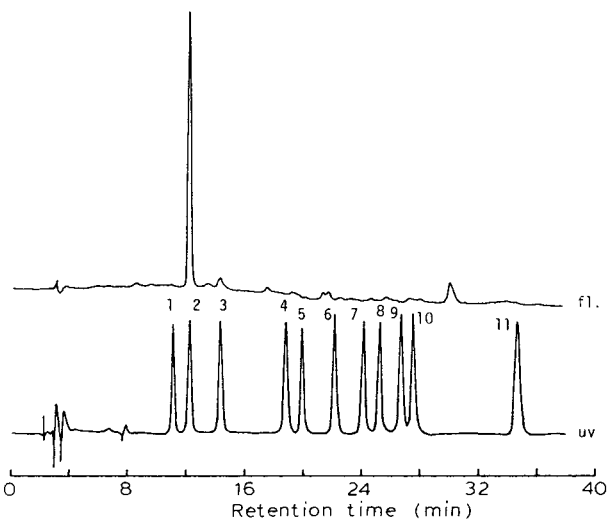


Fig. 1. High-performance liquid chromatograms obtained from blank sample (fluorescence detection) and standard mixture of quinoxalinol derivatives (UV detection). Operating conditions: column, 250 mm × 4 mm I.D., LiChrosorb RP-8 (5 μm); column temperature, 50°C. First solvent: acetonitrile—0.1 *M* aqueous tetrapropylammonium bromide solution—0.1 *M* sodium phosphate buffer (pH 7.0)—redistilled water (1:2:10:7). Second solvent: 80% aqueous acetonitrile. The gradient was prepared using a Model GP-A30 solvent programmer (convex 1, 64 min, 1 ml/min). Peaks: 1 = KGA, 2 = KAA, 3 = PA, 4 = KBA, 5 = PHPPA, 6 = KVA, 7 = KIVA, 8 = KICA, 9 = PPA, 10 = KMVA, 11 = KOA (internal standard).

Fig. 2 shows a typical chromatogram obtained from the standard mixture of α-keto acids. The chromatogram represented by a full line was monitored by the fluorescence detector and the other (broken) was obtained by the UV detector (340 nm). A good separation was obtained in 35 min. It was found

that peaks corresponding to KGA and KAA shift to shorter retention times, but that the other peaks are hardly affected by this change. These results suggest that the peak observed on the chromatogram of the blank sample might correspond to non-anionic compounds.

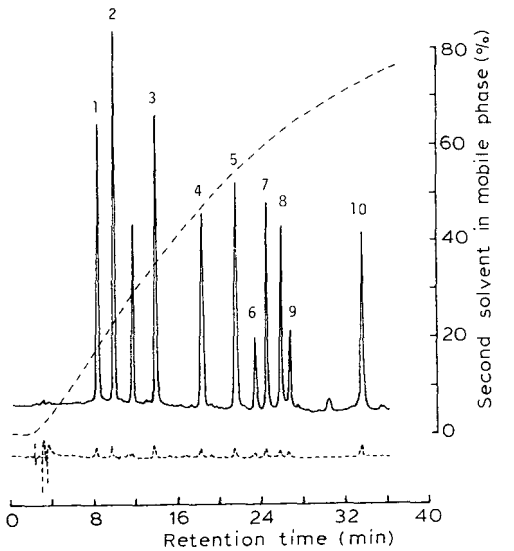


Fig. 2. High-performance liquid chromatograms obtained from a standard mixture of α-keto acids. Operating conditions: column, 250 mm × 4 mm I.D., LiChrosorb RP-8 (5 μm); column temperature, 50°C. First solvent: acetonitrile–0.01 *M* aqueous tetrapropylammonium bromide solution–(0.1 *M* sodium phosphate buffer–redistilled water (1:2:10:7). Second solvent: 80% aqueous acetonitrile. The gradient was prepared using a Model GP-A30 solvent programmer (convex 1, 64 min, 1 ml/min). (——), Fluorescence detection; (— — —), UV detection. Peaks: 1 = KGA, 2 = KAA, 3 = PA, 4 = KBA, 5 = KVA, 6 = KIVA, 7 = KICA, 8 = PPA, 9 = KMVA, 10 = KOA (internal standard).

The calibration graphs obtained using the recommended procedure are shown in Fig. 3. The ratios of each α-keto acid peak area to the internal standard peak area were plotted on the *y*-axis, and the amount of each α-keto acid on the *x*-axis. The curves were rectilinear for at least 0–4 nmol per sample.

Table II shows the percentage recoveries and the coefficients of variation obtained for eight replicate measurements according to the present method. These results are slightly inferior to those in the previous paper [13].

Fig. 4 shows the chromatograms obtained during the determination of α-keto acids in plasma from a classical maple syrup urine disease (MSUD) patient. Chromatograms A and B correspond to plasma samples before (at 14 days after birth) and during treatment, including an exchange transfusion and the use of low branched-chain amino acid milk (from 21 days after birth), respectively. The levels of α-keto acids in these plasma samples are shown with normal values in Table III.

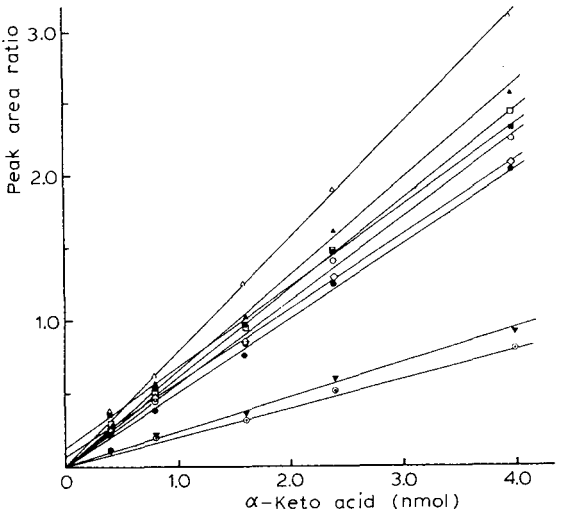


Fig. 3. Calibration graph for the determination of α-keto acids in human plasma. (△), KAA; (▲), KGA; (□), KVA; (■), PA; (○), KICA; (◇), PPA; (●), KBA; (▽), KMVA; (◉), KIVA.

TABLE II

RECOVERY OF α-KETO ACIDS* FROM HUMAN PLASMA

Concentrations are given in nmol per 50 μl.

Sample	KGA	KAA	PA	KBA	KVA	KIVA	KICA	PPA	KMVA
Spiked plasma									
1	4.00	3.75	7.28	3.69	3.41	4.20	5.12	3.46	4.83
2	3.92	3.68	7.06	3.37	3.28	4.45	4.60	3.60	5.43
3	3.80	3.42	7.18	3.61	3.52	4.35	5.01	3.75	5.09
4	4.07	3.89	8.26	4.00	3.74	4.25	5.44	3.90	4.87
5	4.51	3.88	7.47	3.86	3.52	5.35	5.76	3.79	5.30
6	4.33	3.88	6.76	3.71	3.52	4.60	5.23	3.73	4.96
7	4.31	3.81	7.20	3.84	3.54	5.05	5.65	4.00	5.78
8	4.42	3.76	7.23	3.55	3.36	4.70	5.10	3.81	5.04
Mean	4.17	3.76	7.31	3.70	3.49	4.62	5.24	3.76	5.16
C.V. (%)	6.2	4.1	6.0	5.4	4.0	8.7	7.1	4.5	6.3
Plasma blank	0.33	—	3.90	0.27	—	1.05	1.85	0.21	1.65
Amount added	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Recovery (%)	96	94	85	86	87	89	85	89	88

*For abbreviations see text.

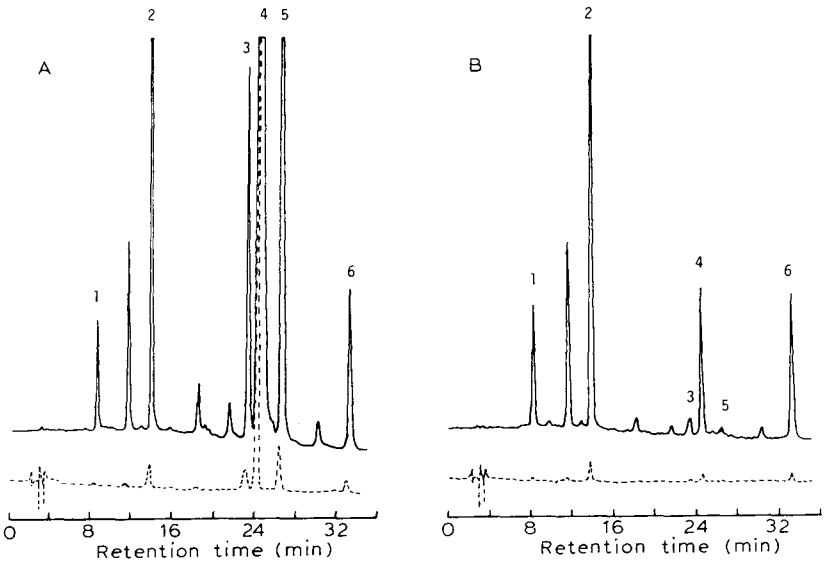


Fig. 4. High-performance liquid chromatograms obtained from plasma samples of an MSUD patient: (A) before treatment (14 days old); (B) during treatment (21 days old). (——), Fluorescence detection: (— — —), UV detection. Peaks: 1 = KGA, 2 = PA, 3 = KIVA, 4 = KICA, 5 = KMVA, 6 = KOA (internal standard).

TABLE III

α-KETO ACIDS* IN HUMAN PLASMA

Values are expressed in nmol/ml.

Sample (No. of samples)	KGA	PA	KIVA	KICA	KMVA
Normal (10)	6.0 ± 2.9**	58.8 ± 28.6	14.6 ± 5.6	38.8 ± 15.8	26.2 ± 8.5
Classical MSUD					
14 days	16.1	109	164	1230	327
21 days	18.2	96.2	10.6	31.2	3.6

*For abbreviations see text.
**± 2 S.D.

CONCLUSION

A sensitive method for the HPLC determination of α-keto acids in plasma has been developed. This method is about ten times more sensitive than that reported in the previous paper [13]. Only 50 μl of plasma are needed for the present method. However, PHPPA could not be analyzed using this method, and the coefficients of variation are slightly inferior to those of the previous method. Therefore, it is recommended that one of the two methods be selected depending upon the situation of the samples.

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CHROMBIO. 1555

DIRECT DETERMINATION OF 4-HYDROXY-3-METHOXYPHENYLACETIC (HOMOVANILLIC) ACID IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

RICHARD F. SEEGAL*, KARL O. BROSCH and BRIAN BUSH

Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201 (U.S.A.)

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SUMMARY

The improvement of high-performance liquid chromatographic analysis with electrochemical detection for urinary homovanillic acid is described. The method permits the chromatographic resolution of authentic homovanillic acid from coeluting interfering compounds in human and nonhuman primate, and rat urine. The electrochemically derived results are compared with post-column derivatized fluorescence results, and quality-control checks necessary to maintain assay precision in automated analysis are described.

INTRODUCTION

Subtle alterations in the concentrations of the major final metabolites of the catecholamine neurotransmitters dopamine and norepinephrine — 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) and dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylglycol (MHPG), respectively — reflect changes in neuronal activity [1—3] and are sensitive indicators of altered mood and emotional state [4—6]. Silbergeld and Chisolm [7, 8] have shown that alterations in urinary concentrations of HVA may indicate exposure to the known neurotoxin lead. If sufficient analytical precision can be achieved, it is possible that HVA measurement may be of general applicability to studies of the effects of environmental pollution on neurological systems. In such studies, a specimen which is simple to collect is almost mandatory; in this case urine is the specimen of choice.

The most promising technique for accurate and precise urine analysis is high-performance liquid chromatography (HPLC) [9]. However, published HPLC methods for urinary HVA level determination were initially developed to

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screen patients for neural crest tumors where differences in HVA concentrations between patients and controls are very large, i.e. 5—10 times [9, 10]. Imprecision appears to be attributable to the complexity of sample preparation particularly with gas chromatographic (GC) methods; inaccuracy is due to small chromatographic interferences which go unnoticed when high levels in pathological specimens are measured.

We report an HPLC assay for urinary HVA based on the direct injection of urine which avoids sample preparation errors and gives specificity for HVA in human, monkey and rat urine. It may be employed over the long term of a chronic toxicological exposure so that definitive neurochemical evidence of subtle neuronal changes can be observed. For simplicity and to facilitate automated injection, an isocratic mobile phase is used which had been shown to separate the majority of catecholamines and their metabolites [11].

Accuracy is demonstrated by comparison with data obtained by HPLC with fluorescent detection [12].

EXPERIMENTAL

Materials

4-Hydroxy-3-methoxyphenylacetic acid (HVA) was purchased as the free acid from Sigma (St. Louis, MO, U.S.A.), potassium dihydrogen phosphate (anhydrous) from J.T. Baker (Phillipsburgh, NJ, U.S.A.), and methanol (HPLC grade) from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Water was deionized, glass-distilled and stored in glass.

Mobile phase

The mobile phase was prepared by dissolving 13.61 g of potassium dihydrogen phosphate in 1 l of deionized, glass-distilled water to yield a 100 m*M* solution. The pH was adjusted accurately with 1 *M* hydrochloric acid before the addition of the organic modifier to avoid measurement in a non-aqueous solvent in which hydrogen ion activity is not precisely known. This solution (9 parts) was then mixed with methanol (1 part) and drawn under vacuum through a 0.45-μm Type HA filter (Millipore, Bedford, MA, U.S.A.) to filter and degas it.

Chromatography

Electrochemical assay. A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A chromatographic pump was used to deliver the mobile phase at a rate of 1.0 ml/min. Samples were injected with an automated sample injector (WISP Model 710A, Waters Assoc.) onto two serially connected Waters μBondapak C_{18} columns (30 cm × 3.9 mm, 10 μm non-spherical particle size) heated to 40° C. No pre-column was used. An LC-5 glassy carbon electrode (BioAnalytical Systems, West Lafayette, IN, U.S.A.) with a silver/silver chloride reference electrode was used to oxidize the compounds of interest at 0.80 V potential versus the reference electrode. The resulting signal was amplified with a polarographic analyzer (PAR 174, EG&G; Princeton Applied Research, Princeton, NJ, U.S.A.) and recorded on a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3385A automation system.

Fluorescence assay. The fluorescence analysis was virtually identical to that of Rosano et al. [12]. The mobile phase consisted of 70 ml of acetonitrile and 2 ml of formic acid made up to 1 l in distilled water and delivered at a flow-rate of 1.0 ml/min. Urine was brought to pH 2.3 with 1 *M* hydrochloric acid, and injected on a single Waters μBondapak C_{18} column. The column effluent was reacted with an alkaline potassium ferricyanide reagent pumped into a 3-port T at 0.8 ml/min and reacted in a stainless-steel coil (2-ml injector loop from a Waters U6K injector) at room temperature. The fluorescent product was excited at 320 nm and the emission read at 420 nm on a fluorescence detector (Model 836, Dupont Instruments, Wilmington, DE, U.S.A.).

Standards and samples

Preparation of standard solutions. HVA was initially dissolved in glass-distilled, deionized water at a concentration of 100 μg/ml. Aliquots (1 ml) were then frozen at −80°C in polyethylene-capped microcentrifuge tubes (Markson Science, Del Mar, CA, U.S.A.). At the time of analysis a frozen aliquot was thawed and diluted to either 1 or 10 μg/ml with water.

Collection and preparation of urine samples. Urine was collected over a period of 24 h from humans into vessels containing 15 ml of 6 *M* hydrochloric acid and kept refrigerated; from monkeys (*Macaca nemestrina*) into icecooled containers; and from rats, into beakers under metabolism cages containing 1 ml of 3 *M* hydrochloric acid covered by 5 ml of paraffin oil (Fisher Scientific, Pittsburgh, PA, U.S.A.).

After collection, the pH of the urine was adjusted to < 2.5 with concentrated hydrochloric acid if necessary. It was then either filtered through Whatman No. 40 paper or centrifuged at 3200 *g* for 10 min. Aliquots (1 ml) were then frozen at −80°C until analysis. When thawed, each aliquot was diluted 10-fold with water and 20-μl injections were made with the WISP.

RESULTS

Successive rises in column temperature to 40°C showed an increase in theoretical plate efficiency for the mixture of standards and the apparent HVA peak in a normal human urine showed a shoulder, indicating the presence of an electroactive interferent (Fig. 1). Alterations in the pH of the mobile phase produced profound changes in the temporal relationship of HVA to potentially interfering compounds: the retention time of HVA was inversely related to the pH of the mobile phase within the ranges examined (Table I). Moreover, changes in the pH of the mobile phase caused the appearance and disappearance of interferents which were at times hidden under presumably pure HVA peaks. A pH of 4.00 and two columns in tandem gave a pure HVA peak (Fig. 2) on visual chromatographic evidence. The 6-ng peak shown in Fig. 2 yielded 22,000—25,000 integrator counts — clearly an HVA concentration one-tenth of that could be detected in a pure standard. However, in a complex chromatogram, sensitivity cannot be assessed so simply since occlusion of small peaks by large neighboring peaks must be considered. Because the concentration of endogenous HVA in urine of rats, human and non-human primates is in the range of 1—7 ng/μl, the detection limits in this assay pose no problem.

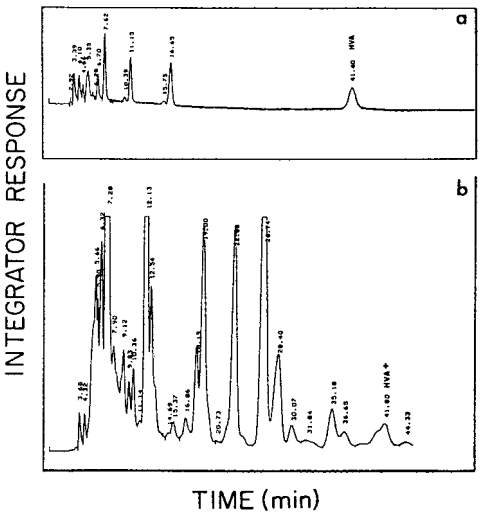

Fig. 1. (a) Chromatogram of a standard consisting of 6 ng of each of the following compounds: norepinephrine (5.35 min), dopamine (6.70 min), vanillylmandelic acid (7.62 min), MHPG (11.15 min), DOPAC (16.65 min), HVA (41.40 min). (b) Chromatogram of a 20-μl injection of filtered human urine diluted 1:10. Conditions: mobile phase, 100 m*M* KH_2PO_4 (pH 3.3) -10% methanol, at 40°C; electrolyzing voltage, +0.8 V vs. Ag/AgCl electrode. Note the appearance of a leading shoulder on the 41.80-min HVA peak, which indicates the presence of (an) interfering compound(s).

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS AND HVA CONCENTRATIONS OF TWO HUMAN URINES ASSAYED UNDER DIFFERENT MOBILE PHASE CONDITIONS

Mobile phase pH	Retention time (min)		Interfering peaks resolved	HVA (ng/μl)	
	HVA standard	HVA in urine		Urine with interfering peaks	Urine without interfering peaks
100 mM KH_2PO_4—10% methanol					
3.95	34.61	34.38	No	4.58	12.25
4.00	33.30	33.21	Yes	3.27	ND*
4.05	31.29	31.13	Yes	3.11	11.70
4.10	30.83	30.95	Yes	3.17	11.53
4.50	21.99	21.88	No	5.04	ND
60 mM KH_2PO_4—23% methanol					
3.00	10.10	9.96	No	4.74	12.43

*ND = Not determined.

Indeed, by selecting a higher gain on the amplifier the detection limits could be further lowered.

Numerical comparison of analyses of different urine samples supports the visual evidence that the HVA peak in Fig. 2 is pure. Table I shows the analysis

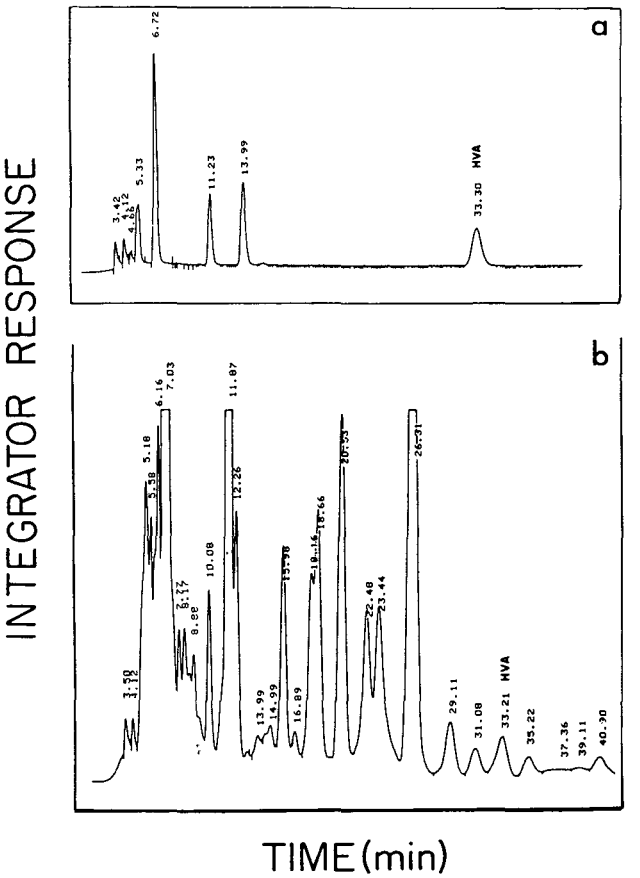

Fig. 2. (a) Chromatogram of the same standard as in Fig. 1a. Retention times are: norepinephrine (5.33 min), dopamine and vanillylmandelic acid (6.72 min), superimposed, MHPG (11.23 min), DOPAC (13.99 min), and HVA (33.30 min). (b) Chromatogram of a 20-μl injection of a human urine diluted 1:10. Conditions: mobile phase, 100 m*M* KH_2PO_4 (pH 4.0)—10% methanol, at 40°C; electrolyzing voltage, +0.8 V vs. Ag/AgCl electrode.

of two samples of urine, the first of a male laboratory scientist and the second of a patient who had a resected neuroblastoma. Notice the HVA analysis reaches a minimum at a pH near 4.05. Analysis of urine from five monkeys with a different mobile phase [13] yielded apparently higher HVA concentrations than those obtained under optimized conditions (Table II). Finally, chromatograms produced by the post-column derivatization method of Rosano et al. [12] determined the HVA level of the human urine used throughout this work to be 2.9 ng/ml and the neuroblastoma patient's urine to be 11.1 ng/ml, in good agreement with the result obtained at optimized pH (Table I).

Automated injection yielded the results shown in Fig. 3A, taken from a long series of 20 μl filtered, 1:10 diluted urine injections. A standard HVA solution was run after every fourth urine, and a control urine was run every eighth sample.

To avoid interference from non-polar electroactive compounds from preceding injections a hold period of 20 min is allowed after the emergence of

TABLE II

HVA CONCENTRATIONS (ng/μl) IN 24-h URINE SAMPLES FROM *MACACA NEMESTRINA* MEASURED UNDER TWO MOBILE PHASE CONDITIONS

Monkey name	Mobile phase		Overestimation (%)
	60 m*M* KH_2PO_4—23% methanol (pH 3.0)	100 m*M* KH_2PO_4—10% methanol (pH 4.1)	
Madison	7.98	3.94	102
	9.70	5.14	89
Mars	2.96	2.50	18
Adams	7.79	7.01	11
Earth	4.77	3.86	24
Venus	6.90	4.50	53

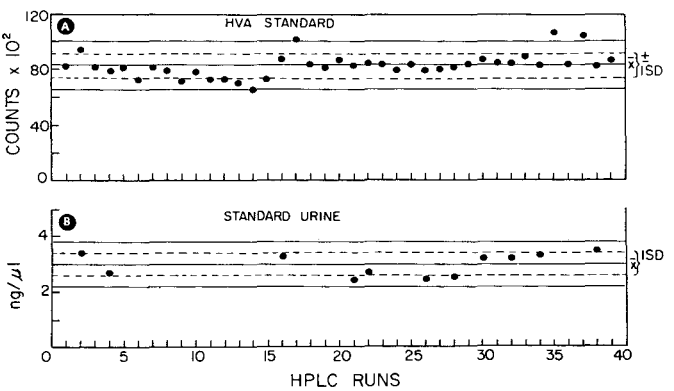

Fig. 3. (A) Integrator responses (counts × 10^3) for 36 μl of an HVA standard (1/6 ng/μl) injected after every fourth urine sample over a series of approximately 150 injections. The mean of all standard runs 1 × S.D. (— — —), and 2 × S.D. (——) are shown. (B) HVA concentration expressed as ng/μl for 20 μl of a control urine diluted 1:10 and injected every eighth sample.

HVA, before the next injection. Any irreversible build-up of non-polar material on the column slowly causes a reduction of retention time and a degradation in resolution. The column was flushed periodically with acetonitrile—water (3:2). This is illustrated in Fig. 4 after the automated runs of 3-9-82 and 3-19-82.

DISCUSSION

The mobile phase chosen for this work, 0.100 *M* aqueous potassium dihydrogen phosphate had been shown to separate a wide variety of catecholamine metabolites [11] and it would be expected to be nearly ideal as a support electrolyte for anodic polarography, being a small singly charged unoxidizable anion. Methanol was chosen as an organic modifier since we have found that it can be obtained electrochemically pure, and at concentrations

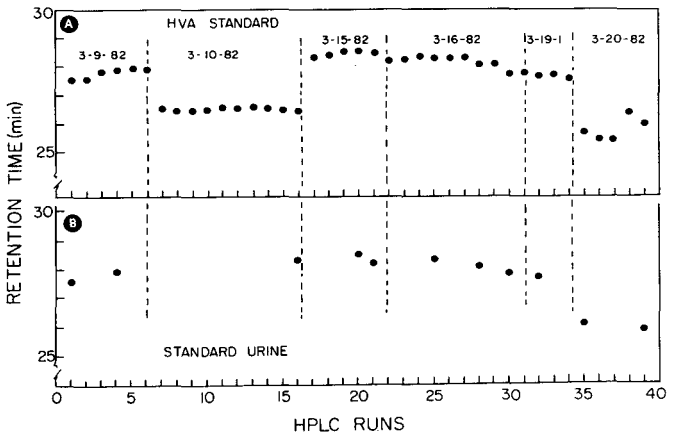

Fig. 4. (A) Retention time (min) for an HVA standard injected every fourth run over a series of approximately 150 runs. (B) Retention time (min) for the standard urine. All injection volumes were as described in Fig. 3.

less than 20% and voltages less than +1 V relative to the Ag/AgCl electrode, the standing current observed is tolerable in that it does not produce noticeable pump pulses at the amplification required for the analysis and can be offset with the standard polarograph we employ. A temperature greater than 40°C was not employed to avoid problems of compound stability in spite of the fact that chromatographic efficiency still continued to improve up to this temperature. Temperature has recently been shown to be an important separative factor [14, 15].

Slight drift in the retention time of HVA during long series of runs can be corrected by the automated recalibration system of the Hewlett-Packard reporting integrator. Larger changes in retention time between days can be corrected by slight adjustments of the pH of the mobile phase or slight changes in the buffer—methanol ratio, but large changes in the retention time of the HVA standard (as shown in runs 6—16 of Fig. 4A) are associated with poor resolution of HVA in the control urine and often indicate column deterioration.

The electrolyzing voltage was changed in 0.1-V steps from 0 to 1.2 V and approximate dynamic half-wave potentials for the catechol derivatives were found to be +0.35 V vs. the Ag/AgCl electrode, whereas the monomethoxy derivatives HVA, MHPG and vanillylmandelic acid and dynamic halfwave potentials of +0.6 V vs. the Ag/AgCl electrode in agreement with previous work in other support electrolytes [10]. Several of the waves showed polarographic maxima [16] unlike the voltammograms previously reported which were observed in the presence of an ion-pairing detergent which would have suppressed maxima. Hence, an oxidizing voltage of +0.8 V was chosen for analysis to ensure diffusion-controlled electrolysis. The electrolyzing potential is known to drift slowly if the salt bridge of the reference electrode is modified by the flowing mobile phase. However, our choice of a relatively high electrolyzing voltage has resulted in no noticeable drift in response as is illustrated by Fig. 3. Changes in the response of the oxidizing electrode caused by coating of the

surface with coeluting urine components are controlled by injecting an HVA standard at frequent intervals in an automated run (every fourth sample), and if the integrator responses for the HVA standard are diminished, the glassy carbon electrode is polished according to techniques described by Bio-Analytical Systems. Experience with these diluted urine samples has shown that repolishing is rarely required.

Care must be taken in the interpretation of results of HPLC electrochemical assays for urinary HVA. Symmetrical Gaussian-shaped peaks with retention times similar or identical to those of the standard are not sufficient proof that chromatographic separation has been achieved. Careful analytical development must include validation by additional techniques (e.g. HPLC with fluorescent detection or gas chromatography). Indeed, the particular post-column reaction and fluorescent detection of urinary HVA used here only responded to HVA and resulted in a selective chromatogram whose numerical values for HVA compared favorably with those obtained by electrochemical detection with a mobile phase at the optimum pH. Electrochemical detection was chosen for this work in spite of the selectivity of the fluorescence method because it is our intention to extend the method, with direct urine injections, to other dopamine metabolites in due course. A supply of control urine should be maintained frozen in convenient-sized aliquots, to provide a check on HVA retention time and chromatographic resolution from potential interferents.

Several organic solvent extraction techniques [9] were tried to improve chromatographic prints. Methods based on ethyl acetate, diethyl ether and toluene extractions all carried electroactive interferents to the HPLC analysis and greatly increased sample preparation time. The present method allows fifteen analyses to be carried out in a 24-h period; this includes all the quality control samples mentioned. One technician can prepare the samples in less than 1 h.

Accurate HVA measurements may be useful in monitoring nervous system function after human exposure to toxic agents. Changes in HVA concentrations, for example, provide an objective, clinically applicable means of assessing nervous system damage caused by chronic lead exposure. When this approach is used in conjunction with a peripheral monoamine oxidase inhibitor, such as debrisoquin sulfate [17, 18], an accurate estimate of central dopaminergic function can be obtained. This technique also provides an experimental means of obtaining multiple, nonstressful estimates of dopaminergic function.

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ANALYSIS OF POLYAMINES AND ACETYL DERIVATIVES BY A SINGLE AUTOMATED AMINO ACID ANALYZER TECHNIQUE

DIANE HADDOCK RUSSELL*, JEFFREY D. ELLINGSON and THOMAS P. DAVIS

Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ 85724 (U.S.A.)

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SUMMARY

In a single, rapid and precise analysis, monoacetylputrescine, N^8-acetylspermidine, N^1-acetylspermidine, putrescine, spermidine, and spermine can be separated using a five-buffer system on an automatic amino acid analyzer. This method allows, for the first time, the separation of all the known acetyl derivatives of putrescine and spermidine as well as the parent compounds in urine and tissues with a single automated procedure. The method has been applied to the analysis of biological samples from normal volunteers, cancer patients and a rat liver supernatant. Mass spectral confirmation was obtained for each compound.

INTRODUCTION

We have reported previously a single-step high-performance liquid chromatographic method for the analysis of acetylated polyamines [1]. Because of the possible clinical relevance of polyamines to evaluate the success or failure of cancer chemotherapy [2—6], we have continued to work on a method which would allow us to rapidly analyze multiple samples and which would separate both the acetyl derivatives and the parent compounds in a single, automated amino acid analyzer procedure. Not only are the two acetylspermidine derivatives made in cytoplasmic (N^1-derivative) and nuclear (N^8-derivative) compartments [7, 8], but the extent of conjugation for each polyamine as well as N^1/N^8 ratio may be of importance in characterizing tumor cell kinetics of common malignancies [9, 10]. A recent method reports separation of the urinary acetyl derivative of putrescine in a second procedure but does not separate N^1- and N^8-acetylspermidine derivatives [11].

Therefore, this method allows for the first time the separation of all the known acetyl derivatives of putrescine and spermidine in urine and tissues as

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well as the parent compounds in a single, automated chromatographic method requiring 83 min. Studies using this technique should be useful in evaluating alterations in acetyl derivatives in both urine and tissues.

MATERIALS AND METHODS

Apparatus

A model D-500 automatic amino acid analyzer (Dionex, Sunnyvale, CA, U.S.A.) equipped with a fluorescence detector (Aminco, Silver Spring, MD, U.S.A.) was used for analyses. The D-500 also included a strip chart recorder (Honeywell, Minneapolis, MN, U.S.A.) and a PDP/8M Digital computer (Digital Equipment Corp., Maynard, MA, U.S.A.) for peak retention time recording, integration, and instrument control. The analyzer was equipped with a column heating jacket designed to accommodate 3.2 mm O.D. columns where column temperature was determined by inserting a thermometer directly to the position of the column in the jacket. The D-500 has an automatic injection system capable of holding 80 samples.

The column used for the analyses was 11.5 cm × 1.7 mm I.D. packed with Aminex A-9 cation-exchange resin (> 8% crosslinked styrene—divinylbenzene copolymer, bead diameter 11.5 ± 0.5 μm) (BioRad Labs., Richmond, CA, U.S.A.). The column was packed from a slurry of resin in 0.2 *N* sodium citrate, 2.1 *N* sodium chloride, 2.5% ethanol, and 0.5% thiodiglycol, pH 5.6, at a pressure of 126 bars at ambient temperature.

Samples were prepared for analysis using 1.5-ml polypropylene microcentrifuge tubes (West Coast Scientific, Berkeley, CA, U.S.A.). A Beckman microfuge B (Beckman, Berkeley, CA, U.S.A.) was used for centrifugations.

A Radiometer Copenhagen PHM 83 pH meter (The London Company, Cleveland, OH, U.S.A.) was used in buffer preparation.

Chemicals

Only high purity chemicals were used in reagent preparation. Sodium citrate dihydrate (analytical reagent grade; Mallinckrodt, Paris, KY, U.S.A.), sodium hydroxide, boric acid, concentrated hydrochloric acid, sodium chloride (J.T. Baker, Phillipsburg, NJ, U.S.A.), ethanol (U.S.I., U.S.P., U.S. Industrial Chemicals, New York, NY, U.S.A.), thiodiglycol, and pentachlorophenol preservative (Pierce, Rockford, IL, U.S.A.) were used for the preparation of buffers.

Boric acid, potassium hydroxide, 2-mercaptoethanol, potassium thiocyanate (J.T. Baker), ethanol (U.S. Industrial Chemicals), and *o*-phthalic dicarboxaldehyde (OPA) (Aldrich, Milwaukee, WI, U.S.A.) were used in preparation of the *o*-phthalaldehyde reagent.

Sample preparation involved sulfosalicylic acid (Sigma, St. Louis, MO, U.S.A.) and sodium hydroxide (J.T. Baker).

Calibration standards

Putrescine, spermidine and spermine hydrochloride salts were purchased from Sigma. Acetylputrescine, N^1-acetylspermidine, and N^8-acetylspermidine were synthesized in our laboratory as previously described [1]. The concentration and purity of the acetyl-derivative stock solutions for use as analytical

standards were determined as follows: acetyl-derivative stocks were analyzed for the presence of non-derivatized polyamine. Aliquots of each stock were quantitatively hydrolyzed for 16 h in sealed tubes. An aliquot of each 6 *N* hydrolysate was diluted with distilled water to 0.6 *N* hydrochloric acid and analyzed for polyamine content against an analytical standard. These values were compared to the acetylpolyamine stock solutions and an analytical standard was prepared. The standard contained 200 pmole per 50 μl of acetylputrescine, N^1- and N^8-acetylspermidine, putrescine, cadaverine, spermidine, and spermine. The standard, prepared in 0.1 *N* hydrochloric acid, was found to be stable at the longest time assayed, 4 months, when stored at 4°C or below.

The relative fluorescence responses (RFR) were determined similar to relative weight responses (RWR) as described by Davis et al. [12]. The internal standard (IS) used was 3,3'-iminobispropylamine (IPA). The RFR of each polyamine (PA) and acetylpolyamine based on IPA was calculated as follows:

$$RFR_{PA/IS} = \frac{\text{area PA}}{\text{nmole/ml PA}} \times \frac{\text{nmole/ml IS}}{\text{area IS}}$$

The RFR values were determined by ten analyses of analytical standards of the polyamines and acetyl derivatives. The RFR can be used to control for assay variation.

Buffer preparation

All buffers were prepared and brought to volume prior to pH adjustment. Buffers 2—4 were adjusted to pH 5.6—5.7 using concentrated hydrochloric acid, while buffers 1 and 5 were adjusted using granular boric acid (Table I). Before use, all buffers were filtered and degassed through 0.45-μm filters (Millipore, San Francisco, CA, U.S.A.).

Reagent preparation

o-Phthalaldehyde reagent was prepared as follows: borate buffer was

TABLE I

BUFFER COMPOSITION AND PROCEDURE TIMING

All buffers contain 0.5% thiodiglycol with 4 drops pentachlorophenol per liter.

Buffer	Sodium citrate (*N*)	Sodium chloride (*N*)	Sodium hydroxide (*N*)	Ethanol (%)	pH	Time of procedure (min)
1*	0.125	0.0	0.05	0	10.2	0—38, injection at 25
2	0.2	0.4	0.0	2.5	5.7	38—43
3	0.2	1.2	0.0	2.5	5.65	43—59
4	0.2	2.1	0.0	2.5	5.6	59—73.5
5*	0.2	1.8	0.05	2.5	10.2	73.5—83

*All buffers adjusted to the proper pH with concentrated hydrochloric acid except those indicated with an asterisk were adjusted with boric acid.

prepared by initially adding 25 g potassium hydroxide to 800 ml deionized water; granular boric acid was then added to a final pH of 10.4 ± 0.02. The volume was brought to 1 liter with deionized water. Potassium thiocyanate (5.8 g), 4.5 ml 2-mercaptoethanol, 3 ml of 30% Brij solution, and 800 mg *o*-phthalic dicarboxaldehyde predissolved in 20 ml ethanol were added to the liter of buffer. The reagent was gently stirred and filtered through a 0.45-μm filter. After transfer to the reagent reservoir, the solution was purged with purified nitrogen to prevent oxidation and degradation. The reagent was stored at 4°C and was found to be stable for at least 7 days.

Sample preparation

Urine samples were collected in 15-ml screw-cap centrifuge tubes and stored at −20°C until preparation for analysis. Samples were then thawed at room temperature and 1 ml was transferred to a 1.5-ml microfuge tube where 100 μl 50% sulfosalicylic acid were added. After vortexing for 5 min, the sample was centrifuged at 8000 *g* for 10 min and 750 μl of the supernatant were transferred to another microfuge tube where 50 μl of a 4% sodium hydroxide solution were added. The sample was then placed on ice to complete precipitation prior to a 10-min centrifugation. After final centrifugation, the supernatant was decanted and stored at −20°C until analysis.

Ion-exchange chromatography

The deproteinized urine sample was thawed and vortexed prior to loading into a sample cartridge. The buffer program was designed as shown in Table I with buffer 1 running 25 min to equilibrate the column for injection. Acetylputrescine was eluted in buffer 1. Buffer 2 was used to lower column pH in preparation for the elution of polyamines in buffers 3 and 4. Both isomers of acetylspermidine and putrescine were eluted in buffer 3 whereas buffer 4 eluted cadaverine and spermidine. Spermine was eluted in buffer 5, which also served as an analytical column cleaning and regeneration buffer due to its high pH and high salt content.

Polyamines were quantitated by their relative fluorescence intensity at 455 nm (emission) with 340-nm excitation. Areas under the polyamine peaks were integrated and compared to areas of 200 pmole standard polyamine peaks. To assure quantitation accuracy, polyamine standards were chromatographed before and after each group of samples.

Peak identification

Polyamines and acetylpolyamines were identified based on retention times compared with analytical standards. Confirmation of identities was accomplished by mass spectrometry using a Finnigan Model 3300 mass spectrometer coupled to a Model 2300 INCOS data system (Finnigan, Sunnyvale, CA, U.S.A.).

RESULTS

Optimization of chromatography

In working with polyamines and ion-exchange chromatography, polyamines generally are separated from other physiological amines with a strategy exploiting their relatively strong cationic charge. The acetylpolyamines are somewhat less cationic than the parent compounds, which makes their chromatographic resolution from other physiological amines and basic amino acids more complicated. Acetylputrescine, in particular, posed this problem; when chromatographed at a moderate pH (5.6), acetylputrescine eluted earlier than arginine from the A-9 cation-exchange column. Another problem with using ionic buffers of low pH was that the two isomers of acetylspermidine, N^1 and N^8, co-eluted in one peak.

Elution with high pH (10.2) buffers yielded completely different results. Not only did arginine elute earlier than acetylputrescine, but N^8-acetylspermidine eluted earlier than N^1-acetylspermidine, as reported previously [1]. In order to resolve an unknown amine peak from N^8-acetylspermidine in urine, pH 5.7 and pH 5.65 buffers were employed. The separation of the acetylspermidine isomers was maintained.

Table I illustrates the 5-buffer separation of the polyamines and acetyl derivatives of putrescine and spermidine. The entire analysis, including equilibration, is completed in less than 85 min. The standard solution contained 200 pmole of each polyamine. A 11.5 cm × 1.7 mm I.D. column of A-9 cation-exchange resin (BioRad) was employed with a flow-rate of 19.2 ml/h. *o*-Phthalaldehyde reagent flow-rate was also maintained at 19.2 ml/h. Column temperature was maintained at 54 ± 1°C.

Minimum detection limit

The sensitivity of fluorescence detection allows a low detection limit of about 10—20 pmole for the polyamines and acetylputrescine with the limit for acetylspermidine (both isomers) being slightly higher, 20—30 pmole. The detection limit was calculated as the concentration of polyamine which yielded a fluorescence response equal to twice the noise level.

Retention times and RFR

The retention times and RFR, compared to the internal standard IPA, for the polyamines and the acetyl derivatives are shown in Table II. Retention times were determined for each fresh lot of buffers. Precision of retention times exhibited a 1% variability over a three-day period of analysis.

Precision of chromatographic analysis

Repeated injections of analytical standards gave an average relative standard deviation (R.S.D., %) for the acetyl derivatives of 3.2% or less. Table III shows the precision of analysis of polyamines and acetylpolyamine derivatives from eight independent analyses of a pooled urine sample. Reproducibility is good when the sample analyzed contains more than 20 pmole of the amine or its derivative. Precision of retention times in an analysis of ten different urine samples analyzed over a three-day period was less than 1% R.S.D. in all cases

TABLE II

RELATIVE FLUORESCENCE RESPONSE

Retention time (min)	Compound*	RFR	area/200 pmole peak**
44.43	IPA	1.0	1,309,000
9.48	AcPut	0.63	825,000
27.70	N^8AcSpd	0.74	968,000
30.77	N^1AcSpd	0.87	1,140,000
35.25	Put	1.13	1,475,000
41.08	Cad	1.48	1,940,000
47.75	Spd	1.53	2,000,000
53.90	Spm	0.79	1,030,000

*Abbreviations: IPA = 3,3'-iminobispropylamine; AcPut = acetylputrescine; N^8AcSpd = N^8-acetylspermidine; N^1AcSpd = N^1-acetylspermidine; Put = putrescine; Cad = cadaverine; Spd = spermidine; Spm = spermine.
**Arbitrary fluorescence units.

TABLE III

PRECISION OF ANALYSIS FOR POLYAMINES IN HUMAN URINE

Data are compiled from eight independent analyses on two different days of a pooled urine sample. Values in nmole/mg creatinine.

	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd
	15.5	2.3	5.6	0.4	<0.2
	15.2	2.3	5.6	0.4	<0.2
	15.0	2.2	5.5	0.4	<0.2
	15.0	2.3	5.6	0.4	<0.2
	14.1	2.3	5.7	0.4	<0.2
	15.4	2.2	5.6	0.3	<0.2
	15.4	2.3	5.8	0.4	<0.2
	15.7	2.4	5.9	0.3	<0.2
Mean	15.2	2.3	5.7	0.4	<0.2
S.D.	0.49	0.06	0.13	0.05	
R.S.D. (%)	3.2	2.8	2.3	12	

(Table IV). In other studies, variation in the composition of the sample did not alter the retention time. Regular comparison of the sample to the standard prevented any alteration from affecting accuracy.

Recovery of polyamines from pooled urine

Since no extraction is necessary for urine sample preparation, recovery is essentially 100%.

Linearity

All polyamines and acetyl derivatives of polyamines responded linearly at quantities ranging from 25 pmole to 1.2 nmole injected onto the column (Fig. 1). Polyamine concentrations were sufficient for detection without concen-

TABLE IV

PRECISION OF RETENTION TIMES OF POLYAMINE AND ACETYLPOLYAMINE DERIVATIVES IN HUMAN URINE

Data represent the mean of eight or more independent analyses that were carried out over a three-day period.

	Retention time (min)					
	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd	Spm
$\bar{x}$	9.53	27.63	30.72	35.20	47.68	53.73
σ	0.04	0.06	0.03	0.03	0.03	0.02
R.S.D. (%)	0.41	0.21	0.10	0.08	0.06	0.03

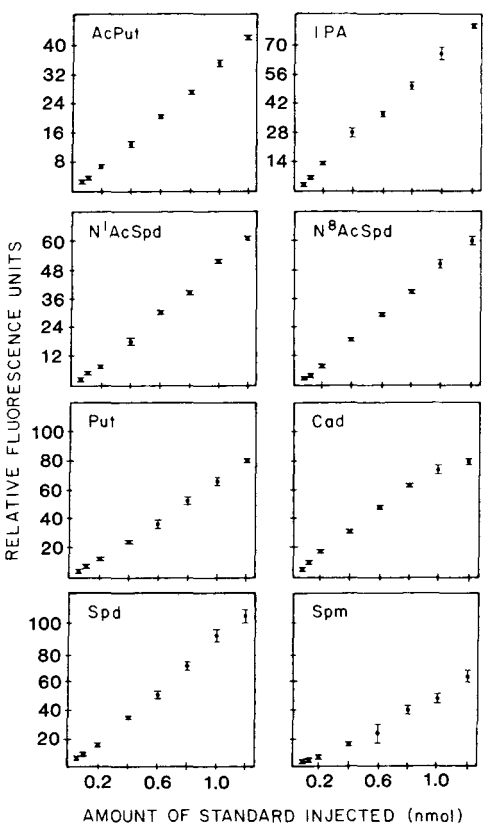

Fig. 1. Linearity of polyamines and acetylpolyamine derivatives over a range from 25 pmole to 1.2 nmole. Abbreviations: AcPut = acetylputrescine; IPA = 3,3'-iminobispropylamine; N^1AcSpd = N^1-acetylspermidine; N^8AcSpd = N^8-acetylspermidine; Put = putrescine; Cad = cadaverine; Spd = spermidine; and Spm = spermine.

tration of the urinary or tissue specimen, and in some cancer patients, urinary acetylputrescine was present in very high concentrations.

Analysis of polyamines and acetylpolyamines in human urine

The described ion-exchange chromatography method has been applied to the analysis of various human urine samples. A chromatogram illustrating standard solutions of polyamines and acetylpolyamines, a chromatogram of a normal human urine, and a rat liver analysis after carbon tetrachloride injection are shown in Fig. 2. Samples of eight different human urines were analyzed for polyamine and acetylpolyamine content and quantitated per mg creatinine. The values are shown in Table V. Table VI illustrates the urinary values of three patients with ovarian carcinoma before and after surgery.

TABLE V

POLYAMINE AND ACETYLPOLYAMINE CONTENT OF EIGHT NORMAL HUMAN URINE SPECIMENS

Data in nmole/mg creatinine.

Subject	Sex	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd	Spm
SH	F	20	2.0	3.0	2.8	N.D.*	<0.2
CI	F	19	1.6	4.7	0.8	N.D.	N.D.
GS	F	14	1.4	3.7	0.7	<0.2	<0.2
SC	M	15	1.5	3.0	0.4	N.D.	<0.2
RS	M	16	1.9	3.8	1.2	N.D.	N.D.
CP	M	19	1.1	2.7	0.8	N.D.	<0.2
JR	M	12	1.1	2.0	0.7	N.D.	<0.2
JE	M	10	1.6	2.6	0.5	<0.2	<0.2
Range		10—20	1.1—2.0	2.0—4.7	0.4—2.8		
$\bar{x}$		15.6	1.5	3.2	1.0		
S.E.M.		0.45	0.2	0.3	0.1		

*N.D. = nondetectable.

TABLE VI

ACETYLPOLYAMINE AND POLYAMINE CONTENT OF THREE PATIENTS WITH OVARIAN CARCINOMA BEFORE AND AFTER SURGERY

Data in nmole/mg creatinine.

Subject	AcPut	N^8AcSpd	N^1AcSpd	Put	Spd	Spm
ZH presurgery	26	1.0	9.8	1.4	N.D.*	N.D.
ZH postsurgery	40	3.2	43	5.2	N.D.	N.D.
ZH postsurgery	42	1.3	18	5.8	N.D.	N.D.
MA presurgery	16	0.7	9.8	0.4	N.D.	N.D.
MA postsurgery	16	2.3	21	1.7	N.D.	N.D.
EM presurgery	20	1.8	7.9	N.D.	N.D.	N.D.

*N.D. = nondetectable.

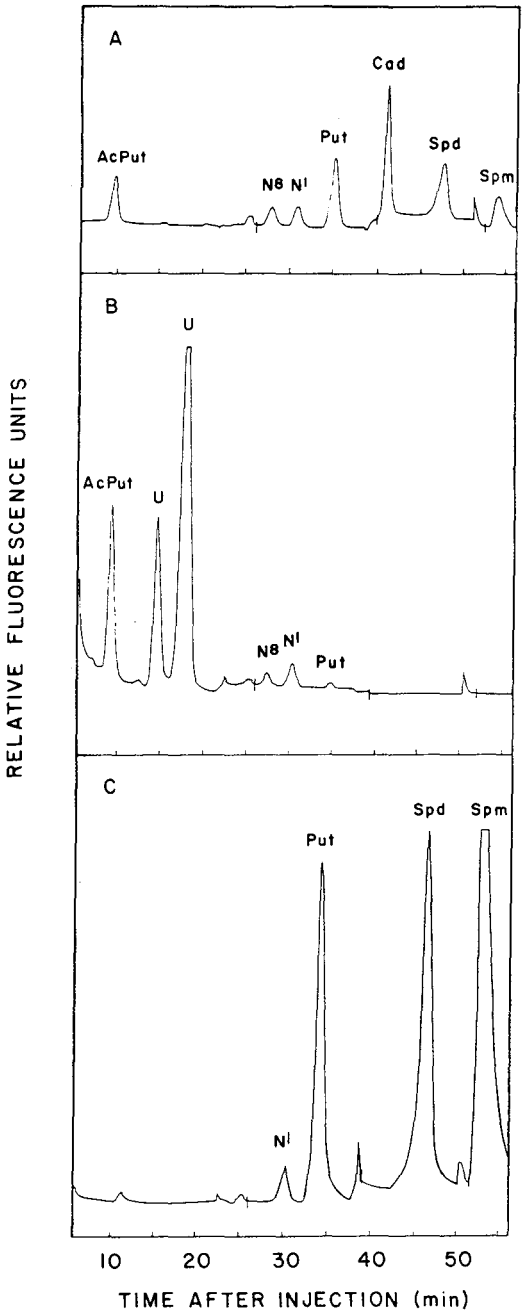

Fig. 2. (A) Chromatogram of calibration standards of acetyl derivatives and polyamines. A standard containing 200 pmole of each amine in 50 μl of 0.1 *N* hydrochloric acid was analyzed as described in Materials and Methods. (B) Chromatogram of a normal urine. Note the almost complete absence of nonconjugated polyamines. (C) Chromatogram of rat liver 6 h after carbon tetrachloride injection (2 mg/kg, intraperitoneally). This procedure results in N^1-acetylspermidine accumulation [13]. Abbreviations as in the legend to Fig. 1.

DISCUSSION

This is the first reported automated method that allows for the separation of both acetyl derivatives of polyamines found in urine and tissues and the free polyamines in a single chromatographic procedure. This is of importance because the urinary excretion of polyamines appears to reflect accurately disease activity in cancer patients as well as a partial or complete response to cancer therapy [3]. Further studies of possible specific patterns of N^1- and N^8-acetylspermidine excretion compared to total excretory polyamines now can be conducted with this methodology.

The major excretory polyamines have been identified as acetylpolyamine derivatives [9, 11, 14—32]. Spermine appears to be excreted mainly in a free, nonconjugated form [22], although a trace amount of N^1-acetylspermine has been detected in a mouse tissue [24]. Abdel-Monem and Ohno [9] were the first to suggest that the N^1- to N^8-acetylspermidine ratio might be altered in cancer patients. In a study of patients with leukemia, 13 of 15 patients had an elevated N^1- to N^8-acetylspermidine ratio when compared to normal volunteers. This suggested that quantitation of isomeric monoacetylspermidine derivatives might provide more information about disease activity and response to therapy. The two isomers were found in a nearly 1:1 ratio in the urine of healthy human volunteers [19, 20]. Seiler et al. [22] studied urinary acetylpolyamine excretion in two male melanoma patients and found that in one patient the acetylputrescine excretion was normal, N^8-acetylspermidine excretion was slightly elevated, and N^1-acetylspermidine excretion was several-fold higher than in healthy male controls. In the other patient, monoacetylputrescine, N^1- and N^8-acetylspermidine were excreted in elevated amounts as compared to controls. Prussak and Russell [1] found elevated monoacetylputrescine in the urine of three cancer patients and elevated N^8-acetylspermidine compared to N^1-acetylspermidine in two of the three patients. Therefore, it appears that extensive studies of intracellular and extracellular acetylpolyamine derivatives are required in order to establish whether specific functions exist for the two acetyl isomers of spermidine related to pathology. This should be possible with the instrumentation of the method reported in this communication.

Utilizing this method we found that acetylated derivatives of putrescine and spermidine comprised 90—95% of the total excreted polyamines in a series of urine specimens collected from normal controls (Table V). Of interest, the excretion of N^1-acetylspermidine in three ovarian carcinoma patients before and after surgery was higher in every instance than its excretion in normal controls (Table VI). After surgery there appeared to be an elevation also in unconjugated putrescine.

Follow-ups of studies of specific acetyl excretory patterns in pathology may be important since the N^1- and N^8-acetylspermidine derivatives are formed in different compartments of the cell [22]. Substrates for the nuclear N-acetyltransferase reaction are histones and polyamines including putrescine [7, 25, 26]. In the presence of spermidine, N^8-acetylspermidine is the only spermidine derivative formed by the nuclear enzyme [22]. The substrates of the cytosolic enzyme are spermine and spermidine, forming the N^1-monoacetyl

derivatives of these compounds [24, 27]. Putrescine and histone are not substrates for this enzyme. Pretreatment of a rat with carbon tetrachloride increases the cytosolic enzyme activity and the formation of N^1-acetylspermidine [13, 24, 27, 28]. The nuclear N-acetyltransferase activity, apparently not induced by carbon tetrachloride, can be stimulated in the kidneys of rats after administration of growth hormone and ACTH [29].

The importance of the formation of N^1-acetylspermidine in the cytoplasm may relate to its ability to be converted to putrescine through the action of polyamine oxidase. N^1-Acetylspermidine can be metabolized to acetylputrescine in transformed chick embryo fibroblasts [30], and acetylputrescine also can be deacetylated in the cytosol [31]. A recent report from Matsui and Pegg [32] indicates that carcinogens, such as dimethylnitrosamine, induce N^1-acetyltransferase, the formation of N^1-acetylspermidine, and ultimately, large increases in the concentration of putrescine, presumably by a conversion of spermidine to putrescine.

The concentration of acetylated polyamines excreted in urine will depend on the rate of formation and breakdown of these compounds in tissues. The treatment of rats with agents which cause considerable cell damage, such as epidermal UV irradiation or cyclophosphamide injection causes both N^1- and N^8-acetylspermidine excretion to increase [22]. Studies of urinary acetylspermidine excretion in hepatoma-bearing rats [21] indicated that urinary N^1-acetylspermidine excretion increased exponentially during the time of linear increase in tumor mass whereas the excretion of N^8-acetylspermidine increased only when the tumor mass was 35 g, shortly before the period of observed necrosis.

Because of the importance of polyamines as markers of growth kinetics, both normal and neoplastic, it is important now to generate information related to the regulation of the formation and excretion of acetylputrescine, N^1-acetylspermidine, and N^8-acetylspermidine. These studies may add to the usefulness of polyamines as markers of tumor cell kinetics as well as to our understanding of possible differences in acetylation patterns of normal and neoplastic cells.

ACKNOWLEDGEMENT

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DETERMINATION OF HISTAMINE, METHYLHISTAMINES AND HISTAMINE—*o*-PHTHALDIALDEHYDE COMPLEXES BY TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURES

APPLICATION TO BIOLOGICAL SAMPLES

J.C. ROBERT*, J. VATIER, B.K. NGUYEN PHUOC and S. BONFILS

Inserm U. 10, Unité de Recherche de Gastroentérologie, Hôpital Bichat, 170, Boulevard Ney, F-75877 Paris Cedex 18 (France)

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SUMMARY

Two high-performance liquid chromatographic procedures were proposed to measure histamine. The first, with UV detection and a strong acid cation exchanger (Partisil 10, SCX Whatman), made it possible to isolate histamine and some methylated derivatives. The second, with a C_{18} sorbent (μBondapak, Waters, 10 μm particle size) eluted with ion-pairing phases, made it possible to isolate the histamine—*o*-phthaldialdehyde complexes. This last procedure allied with a chromatographic purification step gave lower or identical amounts of histamine than those described in human urine (16 ± 7 μg per 24 h), canine whole blood (1.5 ± 1 ng/ml) and human gastric juice (2.3 ± 1.4 ng/ml). The two procedures gave the concentration of a histamine-like compound isolated from the antral mucosa.

INTRODUCTION

The technical difficulties in measuring histamine in biological samples have led scientists to use bioassay [1, 2], radioenzymatic assay [3] or fluorometric assay [4, 5]. For several years, the addition of chromatographic separations such as thin-layer chromatography [6], gas—liquid chromatography [7, 8], low-pressure liquid chromatography [9, 10], and high-performance liquid chromatography (HPLC) [11—21] has allowed the separation of histamine, its methylated derivatives and biogenic amines.

The isolation of a histamine-like compound from the antral mucosa [22—24] which possesses some biological differences from synthetic histamine made it necessary for our laboratory to have a specific analysis for this amine. We propose two HPLC procedures: the first allows us to analyse histamine and some methylated derivatives in UV light at 210 nm with a strongly acid

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cation-exchange column (Partisil 10, SCX Whatman); the second procedure analyses only histamine—o-phthaldialdehyde (OPT) complexes with ion-pairing reversed phases (on a μBondapak C_{18} column, particle size 10 μm). The addition of 1-octanesulfonic acid, sodium salt, was more effective than that of 1-heptanesulfonic acid, sodium salt.

In addition, we describe a rapid and sensitive assay of histamine in three biological fluids. It consists of a chromatographic purification step without the tedious two liquid—liquid extractions. The fluorescent complexes are then analysed with ion-pairing reversed-phase HPLC.

The antral histamine-like compound was measured by the two procedures; values for normal histamine excretion in human urine, human gastric juice concentration and canine whole blood concentration are presented and compared with those described in the literature.

EXPERIMENTAL

Chemical reagents

Sodium hydroxide, acetone, perchloric acid, sulfuric acid, acetic acid, hydrochloric acid, sodium acetate, sodium dihydrogen phosphate, potassium dihydrogen phosphate (Prolabo, Paris, France) were analytical grade; methanol RPE-ACS was obtained from Carlo Erba, Milan, Italy; the 1-octanesulfonic acid, sodium salt, its heptane and pentane analogues were products of Fisons, distributed by Interchim (Montluçon, France); OPT was a Fluka product, distributed by Interchim; histamine dihydrochloride and tris(hydroxymethyl)-aminomethane (Tris) were products of Merck (Darmstadt, G.F.R.). 3-Methylhistamine, 1-methylhistamine, N^{α}-methylhistamine were purchased from Calbiochem, distributed by Eurobio (Paris, France).

HPLC apparatus

The liquid chromatographic system consisted of a Model 848 pump module (Dupont, Orsay, France) equipped with a Rheodyne 7021 injection valve with a 100-μl loop (Touzart et Matignon, Vitry sur Seine, France), an absorbance monitor Isco Model 1840 (Roucaire, Velizy, France) with an 8-μl flow-cell unit, a JY3 spectrofluorometer (Jobin Yvon, Longjumeau, France) with a 20-μl flow-cell unit, and an Ifelec IF 3802 recorder (Jobin Yvon). The system was operated at room temperature.

Histamine analysis with UV detection

HPLC procedure. Chromatography was performed on a Partisil 10 SCX Whatman column, 25 × 0.4 cm I.D., particle size 10 μm, with a mobile phase of 0.4 *M* potassium dihydrogen phosphate aqueous solution (pH 4.5). The histamine compounds were detected at 210 nm. The flow-rate was 2 ml/min (61 bar).

Sample preparation. Antral histamine was isolated from the mucosa as described earlier [22]. After the last step of purification on a carboxymethyl-cellulose column (CM-52; Whatman, Orléans, France) with an ammonium formate gradient of 0.1 to 0.3 *M*, the antral histamine solution was divided into batches and kept at −30°C. Each batch contained an equivalent part of

10 g of fresh antral tissue per ml. From 10 to 50 μl could be injected into the loop.

Histamine analysis with fluorometric detection

HPLC procedure. Chromatography was performed on a μBondapak C_{18} column, particle size 10 μm, 30 × 0.4 cm I.D. (Waters, Paris, France) with a mobile phase of methanol, 0.02 *M* sodium acetate in distilled water, acetic acid (55:43:2, v/v) and 0.15 × 10^{-3} *M* of 1-octanesulfonic acid, sodium salt, as the ion-pairing agent. The flow-rate was 1.5 ml/min (136 bar). Fluorometric intensity was monitored at the emission wavelength of 450 nm with the excitation wavelength set at 360 nm.

Sample preparation. Antral histamine solution (10 μl) was diluted 30-fold with 0.25 *N* hydrochloric acid to obtain an approximately 10^{-7} *M* histamine concentration; the fluorometric assay was developed according to the general procedure (see below).

A 1-ml volume of human urine was adjusted to pH 7.5 with either 0.5 *M* Tris or 10% acetic acid and was diluted to 2 ml with distilled water. A 2-ml volume of canine whole blood or 1 ml of human gastric juice were adjusted to the same pH. Before purification clear gastric juice was obtained by filtration on XAD-2 adsorbent; Servachrom XAD-2, 100—125 μm particle size (distributed by Tebu, Versailles, France), was prepared as previously described [25] and 1 ml was poured into a 10-ml Econo Column (Biorad, Touzart & Matignon). The gastric juice was filtered through the XAD-2 column and 1 ml of 0.050 *M* Tris buffer (pH 7.5) was also added to separate all histamine from the adsorbent.

Sample purification of biological fluids. The histamine was extracted according to the chromatographic procedure described by Oates et al. [26] with some changes in technical conditions; Amberlite CG-50 type I (Prolabo) was prepared as described [26] and purification was carried out as follows. A 0.4-ml volume of Amberlite was poured into a 10-ml Econo Column, rinsed with 2.5 ml of 0.5 *M* sodium phosphate, pH 7.5, and 2.5 ml of distilled water. The sample was gently deposited on the resin, then cleaned with 2.5 ml of distilled water, 3 × 2.5 ml of 0.5 *M* sodium acetate pH 6.5, 2 × 2.5 ml of distilled water and 0.5 ml of 0.5 *N* hydrochloric acid. Histamine was eluted with 2 × 1 ml of 0.5 *N* hydrochloric acid.

Two other extraction procedures were studied on a urine sample. The liquid—liquid extraction [26] was added to the above-mentioned chromatographic purification. Histamine was also extracted as described by Huff et al. [27]; they eluted histamine from Amberlite CG-50 with 0.5 *N* sodium hydroxide instead of hydrochloric acid.

Fluorometric procedure. Samples of 1 ml were made alkaline (pH 12.1) by the addition of 0.2 ml of 2 *N* sodium hydroxide and the derivatisation was performed by mixing 0.1 ml of 0.1% OPT dissolved in methanol.

The fluorometric reaction was developed for 40 min at 0°C in darkness as described by Håkanson et al. [28] and then stopped by acidification (pH 2.2) with 0.2 ml of 1 *N* sulfuric acid. Fluorescence was stable for 2 h.

Standardisation and quantitation

All the histamine standard solutions were prepared daily from stock solutions. With UV absorption, 50 μl of standard solutions (2×10^{-5} to 16×10^{-5} *M*) were injected into the loop; with fluorometric detection, 15 μl of the histamine—OPT standard solutions (2.5×10^{-8} to 80×10^{-8} *M*) were injected. One blank was always run with the histamine range and a second one was prepared during the purification by substituting distilled water for the biological fluids.

There was a linear relationship between the fluorometric intensity, UV absorbance (peak height) and the concentration of histamines; the reproducibility of the two methods was tested by ten repeated injections of the same histamine solutions (see Analytical results). The recoveries of histamine (%) and the relative standard deviations (R.S.D. %) of ten assays on pooled samples were checked by adding known amounts of histamine: 100 pmol/ml of urine or gastric juice, 50 pmol/ml of whole blood, 100 pmol/ml or 300 nmol/ml of antral histamine solution (see Biological results). A 75-μl volume of biological histamine—OPT complexes injected corresponded to 25 μl of human urine or human gastric juice, to 50 μl of canine whole blood, and to 3.3 mg of fresh tissue. The results were expressed as histamine base.

Nature and collection of the samples

Pig antral mucosae were obtained from a slaughterhouse and stored at —30°C before histamine extraction. Biological fluids were obtained from normal humans or animals; human urine was collected in plastic containers with sodium metabisulfite [15], human gastric juice and canine whole blood as described by Lorenz et al. [5, 29].

ANALYTICAL RESULTS

HPLC procedure with UV absorption of histamine

Histamine (H), N^{α}-methylhistamine (N^{α}-MH), 1-methylhistamine (1-MH) and 3-methylhistamine (3-MH) were well separated at 210 nm in 0.4 *M* potassium dihydrogen phosphate aqueous solution (see Fig. 1).

Table I gives the capacity ratio (k') values of histamines expressed in terms of dihydrogen phosphate concentration; pH had no effect on the values in the pH range 2.5—5.5. When the buffer concentration was lower than 0.3 *M*, methylated histamines began to be retained on the column. With a 0.1 absorbance unit full-scale (a.u.f.s.), the limits of detection were: 0.2 nmol for H, N^{α}-MH and 3-MH, and 1 nmol for 1-MH, per 50 μl of injected sample at a signal-to-noise ratio of 3:1.

The reproducibility of the assays was tested by ten repeated injections of H, N^{α}-MH, 3-MH (10 nmol of each) and 1-MH (50 nmol); the means of the calculated peak heights showed a relative standard deviation of 1%, 1.3%, 1.3% and 1.5%, respectively.

HPLC procedure for the histamine—OPT complexes

In order to measure histamine in biological fluids with high sensitivity, it was necessary to form fluorescent compounds. Table II shows the values for

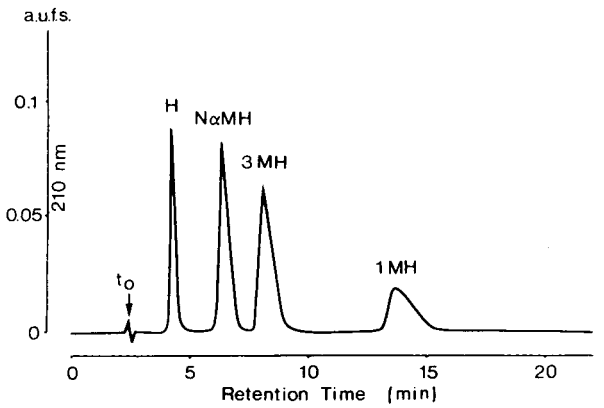

Fig. 1. Chromatogram of standards on Partisil 10, SCX Whatman: 8 nmol of each; H = histamine, N^{α}-MH = N^{α}-methylhistamine, 3MH = 3-methylhistamine, 1MH = 1-methylhistamine. Injection volume = 50 µl. The mobile phase consisted of 0.4 *M* potassium dihydrogen phosphate. Flow-rate: 2 ml/min; t_0 corresponded to $k' = 0$.

TABLE I

k' VALUES OF THE HISTAMINES IN TERMS OF POTASSIUM DIHYDROGEN PHOSPHATE CONCENTRATION

— = Methylated histamines were retained on the column.

	Potassium dihydrogen phosphate concentration (mol/l)								
	0.5	0.4	0.3	0.25	0.2	0.15	0.125	0.1	0.075
H	0.55	0.68	0.83	1.04	1.20	1.80	2.36	3.75	5.2
N^{α}-MH	1.32	1.55	1.96	2.96	4.21	5.96	—	—	—
3-MH	1.96	2.24	3.35	5.98	—	—	—	—	—
1-MH	3.60	4.43	6.17	—	—	—	—	—	—

the capacity ratio (k') of the complexes with the addition of ion-pairing agents.

The use of 0.15×10^{-3} *M* 1-octanesulfonic acid, sodium salt, with methanol—0.020 *M* sodium acetate—acetic acid (55:43:2, v/v) gave a k' value of 1, a peak without shoulder and chromatographic separation within 4 min. The apparent pH was 3.8 and in the range 3—4.5; pH had no effect on k' values or on fluorescence intensity.

The limit of detection was 0.1 pmol per 15 µl of injected sample at a signal-to-noise ratio of 3:1. The reproducibility of the assay was tested by ten repeated injections of 6 pmol of histamine—OPT complexes; the mean of the calculated peak heights showed a relative standard deviation of 2.2%.

TABLE II

EFFECT OF THE ION-PAIRING AGENT ON k' VALUES OF HISTAMINE—OPT COMPLEXES WITH TWO MOBILE PHASES OF METHANOL—0.020 *M* SODIUM ACETATE—ACETIC ACID

1-Pentanesulfonic acid, sodium salt, had no effect on k' values of histamine—OPT complexes ($k' = 0$).

Mobile phase ratio	1-Heptane sulfonic acid ($\times 10^{-3}$ mol/l)	k'	1-Octane-sulfonic acid ($\times 10^{-3}$ mol/l)	k'
50:48:2 (v/v)	0	0	0	0
	0.025	0.5	0.050	0.9*
	0.050	0.65	0.100	1.4, 1.6**
	0.075	0.75*	0.150	1.7, 1.9**
	0.100	0.85*		
	0.150	0.91*		
55:43:2 (v/v)	0	0	0	0
	0.025	0.2	0.050	0.5
	0.050	0.35	0.100	0.8
	0.075	0.45	0.150	1.0
	0.100	0.5	0.200	1.15
	0.150	0.55		
	0.200	0.6		

*A shoulder appeared.
**Two peaks appeared in the ratio 6:1.

BIOLOGICAL RESULTS

Chromatographic purification of the biological samples and their histamine content

Antrum. The concentration of antral histamine was determined as follows: by the total fluorometric assay [22], mean ± S.D. ($n = 5$) 30 ± 1.5 μg per 10 g; by the HPLC fluorometric assay, mean ± S.D. ($n = 5$) 29.5 ± 0.9 μg per 10 g; by the HPLC UV assay, mean ± S.D. ($n = 5$) 30 ± 0.6 μg per 10 g; the means of the recovery (%) ($n = 5$) and the relative standard deviation (RSD %) were 98 ± 4%, 99 ± 2.5% and 101 ± 1.5%, respectively.

Analysis of the chromatogram did not indicate the presence of methylated histamines.

Urine. The histamine urinary excretion of three normal subjects was studied during non-consecutive days (number of samples: $n = 15$). The results were: mean ± S.D. = 16 ± 7 μg per 24 h, range 7—47 μg per 24 h.

The recovery and the relative standard deviation were tested on pooled urine ($n = 5$): mean ± S.D. = 14.5 ± 0.8 ng/ml; recovery ± RSD = 79.6 ± 5.4%; the limit of detection was 0.5 ng/ml of urine.

No histamine was seen in the blanks. Fig. 2 shows a chromatogram of urinary histamine—OPT. There was no interference with other amine compounds such as spermidine ($k' = 4$); an unidentified peak appeared before that of histamine—OPT.

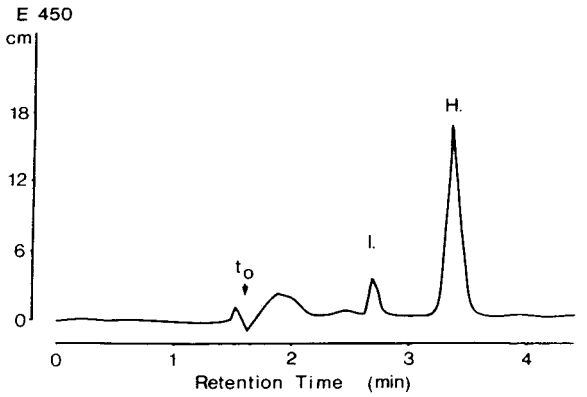

Fig. 2. Chromatogram of a urinary sample on μBondapak C_{18} (Waters), 10 μm particle size: I = unidentified peak; H = histamine—OPT complexes. Injection volume = 75 μl. The mobile phase consisted of methanol—0.020 *M* sodium acetate—acetic acid (55:43:2, v/v) and 0.15×10^{-3} *M* 1-octanesulfonic acid, sodium salt. Flow-rate: 1.5 ml/min; t_0 corresponded to $k' = 0$; emission wavelength 450 nm, excitation wavelength 360 nm; 100 fluorescence units corresponded to 30 cm.

Canine whole blood. The histaminemia of three dogs was measured six times. The mean ± S.D. was 1.5 ± 1 ng/ml, range 0.4—3 ng/ml.

The recovery (%) and the RSD (%) were tested on pooled canine whole blood (n = 10): mean ± S.D. = 0.9 ± 0.08 ng/ml; recovery ± RSD = 64 ± 8%; the limit of detection was 0.3 ng/ml of canine whole blood.

Human gastric juice. Amounts of 200 nmol of H, N^{α}-MH, 3-MH and 1 μmol of 1-MH dissolved in 0.1 ml of water were deposited on XAD-2 adsorbent which was then cleaned with 1.9 ml of 0.050 *M* Tris, pH 7.5. The HPLC UV assay indicated good recoveries: 99%, 98%, 101% and 98%, with RSD of 2%, 3%, 3.5%, and 4%, respectively.

The histamine concentrations of eight gastric juices were measured; the mean ± S.D. was 2.3 ± 1.4 ng/ml, range 0.9—4.2 ng/ml.

The recovery and the RSD were tested on pooled gastric juice (n = 10): mean ± S.D. = 2.1 ± 0.2 ng/ml; recovery ± RSD = 83 ± 9%; the limit of detection was 0.6 ng/ml of gastric juice.

DISCUSSION

HPLC procedure with UV detection

Gaetani and Laurei [13] were the first scientists to use a high-performance carboxylic resin to evaluate histamine compounds at 208 nm (they prepared their stationary phase themselves). At present, the use of the SCX Whatman column is known [20]. Both resins gave comparable results in the elution order of histamine and methyl derivatives. Even if UV detection was less sensitive than fluorometric detection, it had the great advantage of measuring histamine and its derivatives in tissues without the usual derivatisation with OPT [10, 30] or dansyl chloride [9].

HPLC procedure with fluorescence detection

Our goal was to obtain a rapid and specific separation of the histamine—OPT adducts. Furthermore, the use of a mobile phase in the low pH range permitted good stabilisation of the complexes [18, 19, 28].

Reversed phases containing hydrochloric acid, sodium chloride, or basic buffers are known [11, 12, 16, 18] but they were recommended neither by the manifacturers nor by Rabel [31]. Meil et al. [32] described a reversed-phase system where histamine—OPT was eluted near the solvent front, and they preferred to use a CN column [14]. Similarly, Davis et al. [15] analysed the OPT complexes on a phenyl column. The main disadvantage of these two procedures was the time necessary to analyse the purified histamine sample; analyses with an amino acid analyser [33] were also very time-consuming.

Under these conditions, the nature of the mobile phase was studied on the basis of the ratio buffer/methanol described in the literature [11, 12, 14, 18].

Phosphate was replaced by sodium acetate and acetic acid to obtain an apparent pH of 3.8; only the addition of 1-heptane- or 1-octanesulfonic acid, sodium salt, increased capacity ratios. The formation of two main products forced us to find a compromise between the resolution and the capacity ratio. We demonstrated that the addition of 0.15×10^{-3} *M* 1-octanesulfonic acid, sodium salt, to the mobile phase methanol—0.020 *M* sodium acetate—acetic acid (55:43:2, v/v), gave the best solution with $k' = 1$ and a "single peak".

The advantage of ion-pairing agent was demonstrated because histamine—OPT adducts could not be isolated from the SCX Whatman column with either phosphate buffers or water—methanol mobile phases.

Biological samples

Antrum. With UV absorption, the antral histamine solution showed a single histamine peak. 1-MH and 3-MH were not present although they do exist in the antral mucosa [34]. Both chromatographic analyses gave comparable antral histamine concentrations and demonstrated the close relationship between the fluorometric intensity and the UV absorption (peak heights).

Urine. Several methods might be used to purify the sample. If 2-mercapto-ethanol and OPT were added to crude urine, purification of the fluorescent complexes could be performed by the liquid—liquid extractions alone, but HPLC analyses were very time-consuming [14, 15] because the fluorogenic reagents reacted with primary amines. In order to develop a simple, simultaneous and shorter determination of histamine and 1-methylhistamine by HPLC, Tsuruta et al. [12] added a second purification step on a phosphocellulose resin.

When the fluorescence reaction was run without 2-mercaptoethanol, ammonia had to be eliminated from the urine with a cation-exchange resin; Oates et al. [26], Gilbert et al. [35] added the liquid—liquid extraction, Myers et al. [36] added a diamine oxidase reaction step and Endo [37] added a second chromatographic purification.

At the present time, HPLC analysis could replace the total fluorometric determination and the second purification step. Table III shows a comparative determination of histamine on pooled urine. Three purification methods were

TABLE III

COMPARATIVE VALUES OF HISTAMINE CONCENTRATION FOR POOLED URINE

Method	ng/ml ±	S.D.	Recovery (%)	R.S.D. (%)	n §
Huff's method [27] + total detection	47 ±	6.3	84	13.5	5
Huff's method [27] + HPLC analysis	*	*	*	*	5
CG-50** + L*** [26] + total detection	29 ±	2.7	54	9.5	5
CG-50** + L [26] + HPLC analysis	15 ±	1.2	55	7.8	5
CG-50** + total detection	27 ±	2.5	80	8.7	5
CG-50** + HPLC analysis	14.5 ±	0.8	79.6	6.2	5

*The HPLC chromatogram showed several peaks and a shoulder appeared in front of the histamine—OPT peak.
**The sample purification is described in the experimental part. CG-50 is a weakly acid cation exchanger.
***L = Liquid—liquid extractions.
§ n = No. of samples.

allied to either a total fluorometric determination or an HPLC analysis. The total fluorometric determination always gave higher concentrations than those obtained with HPLC. Huff's [27] method gave the highest concentration with a shoulder in front of the histamine—OPT peak. We obtained the lowest concentration with a single chromatographic purification step allied with a selective and rapid HPLC analysis and the tedious liquid—liquid extractions could be eliminated.

The amounts of histamine we found in 24-h urine of normal persons (16 ± 7 μg per 24 h) were lower than those described with the fluorometric method by total detection [26, 34, 36, 37] and even with the help of HPLC [12], but were comparable to those obtained with Myers' method [36] and the enzymatic assay [39—41]. The results are shown in Table IV.

Canine whole blood. Purification and histamine analysis for canine whole blood were run as for human urine; the liquid—liquid extractions were eliminated. Our mean, 1.5 ng/ml, was close to the result obtained by Lorenz et al. [42], 2 ng/ml (see Table V).

Human gastric juice. The lack of fluidity of human gastric juices and, probably, the formation of histamine biliary salt complexes such as those formed in plasma with metals [43], proteins [44] and interactions with ascorbic acid [45], made it difficult to extract histamine.

XAD-2 adsorbent, which retains steroids [46] and biliary salts [25] at neutral pH, completely eluted histamine and its methylated derivatives; the XAD-2 filtrate was then treated like human urine and canine whole blood. Our mean, 2.3 ng/ml, was lower than that obtained by Lorenz et al. [42], 6 ng/ml, probably because the HPLC analysis was specific (see Table V).

CONCLUSION

Two HPLC methods were proposed to determine histamine. The first, with UV detection could be used particularly well for tissues; the second, allied with a simple purification step, could be used for three biological fluids.

TABLE IV

URINARY HISTAMINE LEVELS IN NORMAL SUBJECTS AS DETERMINED BY DIFFERENT ASSAYS

Authors [ref.]	Procedure*	Histamine level (μg per 24 h)
Oates et al. [26]	S → CG-50 → L → OPT → Total detection	45
Gilbert et al. [35]	S → CG-50 → L → OPT → Total detection	42
Huff et al. [27]	S → CG-50 → OPT → Total detection	45
Beall [38]	S → CM-50 → L → OPT → Total detection	46
Endo [37]	S → Dowex 50 → CM-cell. → OPT → Total detection	170—1100 (total hydrolysis)
Myers et al. [36]	S → Dowex 50 → OPT → DAO → Total detection	14
Mell et al. [14]	S → OPT + ME → L → HPLC	16—60 ng/ml
Tsuruta et al. [12]	S → L → P-cell. → OPT + ME → HPLC	
Beaven et al. [3]	S → Enzymatic method	16
Horakova et al. [39]	S → Enzymatic method	19
Rosenthal et al. [40]	S → Enzymatic method	17
Bruce et al. [41]	S → Enzymatic method	25
Present study	S → CG-50 → OPT → HPLC	16

*S = sample; L = liquid—liquid extractions; Dowex 50 = strongly acid cation exchanger; CG-50, P-cell., CM-cell. = weakly acid cation exchangers; ME = 2 mercaptoethanol; DAO = diamine oxidase.

TABLE V

HISTAMINE CONCENTRATION IN CANINE WHOLE BLOOD AND HUMAN GASTRIC JUICE

Biological fluid	Authors [ref.]	Procedure*	Histamine concentration (ng/ml)
Canine whole blood	Lorenz [42]	S → AG-50 → L ——→ OPT → Total detection	2
	Present study	S → CG-50 ——→ OPT → HPLC	1.5
Human gastric juice	Lorenz [42]	S → AG-50 → L ——→ OPT → Total detection	6
	Present study	S → XAD-2 → CG-50 → OPT → HPLC	2.3

*S = sample; L = liquid–liquid extractions; AG-50 = strongly acid cation exchanger; CG-50 = weakly acid cation exchanger; XAD-2 = polystyrene adsorbent.

The difficulty of measuring histamine in biological fluids has been pointed out by Gleish et al. [47]. Our intention in this work was to improve the conditions of histamine measurement.

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POSSIBILITÉS D'ÉTUDE DES VARIANTES DE LA CHOLINESTÉRASE PLASMATIQUE HUMAINE PAR ÉLECTROPHORÈSE D'AFFINITÉ

PATRICK MASSON* et PHILIPPE VALLIN

Centre de Recherches du Service de Santé des Armées, Division de Chimie et Pharmacologie, 108 boulevard Pinel, 69275 Lyon Cedex 03 (France)

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SUMMARY

Possibilities of study of the human plasma cholinesterase variants by affinity electrophoresis

Affinity electrophoresis has been applied to the study of the multiple molecular forms of three human plasma cholinesterase phenotypes (usual enzyme U, atypical enzyme A and intermediate UA). Electrophoreses were carried out in polyacrylamide gels containing a water-soluble macromolecular derivative of *m*-amino-(substituted)-phenyltrimethylammonium immobilized within the gel network.

Apparent dissociation constants ($K_{D\ app}$) were estimated from the mobilities of the enzymes versus ligand concentration.

The ratio of $K_{D\ app}$ values of the molecular forms of phenotypes A and U which is approximately 2 is consistent with the hypothesis that the anionic site is altered in atypical enzyme.

INTRODUCTION

La biosynthèse de la cholinestérase (E.C. 3.1.1.8) du plasma humain est contrôlée par plusieurs loci indépendants, Deux d'entre eux, E_1 et E_2 ont été identifiés. Le premier présente un polymorphisme très étendu et comporte au moins 8 allèles, le plus fréquent étant le gène "usuel" E_1^u (96%) [1]. Le second s'exprime avec une fréquence de l'ordre de 10% dans les populations caucasoïdes et produit une enzyme de faible mobilité électrophorétique, le composé C_5 [2]. Parmi les allèles de E_1, le gène E_1^a code pour l'enzyme "atypique" impliquée dans la majorité des accidents d'hypersensibilité à la succinylcholine, myorelaxant d'action brève. Environ un individu sur 2000 est homozygote pour E_1^a et développera une apnée prolongée après injection de ce curarisant [3].

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La nature des différences structurales entre le phénotype U ($E_1^uE_1^u$) et le phénotype A ($E_1^aE_1^a$) n'est pas encore connue de façon précise. Cependant, sur la base d'arguments cinétiques [4—8], de la chromatographie d'affinité [9] et de l'électrophorèse des fragments de digestion trypsique des deux enzymes marquées au [3-^{14}C]diisopropylfluorophosphate [10], il apparaît que la différence se situe au niveau du sous-site anionique du centre actif. Selon l'hypothèse de Kalow et Davies [4], la mutation ne concernerait qu'un seul aminoacide de ce site et correspondrait à la substitution d'un aminoacide dicarboxylique par un aminoacide neutre ou basique.

Nous avons émis l'hypothèse qu'il devait être possible d'étudier le site anionique des allozymes du locus E_1 par électrophorèse d'affinité en opérant avec des ligands spécifiques immobilisés dans un support de polyacrylamide.

Un travail préliminaire nous a permis d'analyser le comportement des formes moléculaires C_1 (monomère), C_3 (dimère) et C_4 (tétramère) de l'allozyme U et de déterminer leur domaine d'affinité pour un ligand, copolymère linéaire de l'acrylamide et d'un dérivé du *p*-aminophényltriméthylammonium (p-APTMA), emprisonné dans les mailles du gel [11].

Le présent article expose les premiers résultats que nous avons obtenus avec les phénotypes U, A et UA ($E_1^uE_1^a$) et l'isomère *méta* du ligand cationique précédent.

MATÉRIEL ET MÉTHODES

Origine des plasmas et de la cholinestérase purifiée

Les plasmas sur lesquels nous avons pratiqué nos essais provenaient d'une collection, conservée à —30°C, constituée lors d'une étude portant sur la population des donneurs de sang de la région lyonnaise [12] et de sujets $E_1^aE_1^a$ ayant développé une paralysie des muscles respiratoires à la suite d'une injection de succinylcholine.

L'allozyme U ($E_1^uE_1^u$) a été purifié à partir de sang périmé fourni par le Centre Régional de Transfusion Sanguine de Lyon-Beynost selon la méthode antérieurement décrite [13].

L'identité des phénotypes a été reconfirmée par la détermination des nombres de dibucaïne [14] et de fluorure [15].

Synthèse du ligand et formation du copolymère

À 11.82 mmoles de chlorhydrate de *m*-N,N-diméthyl-phénylènediamine (Merck) en solution dans 40 ml d'hexaméthylphosphotriamide, nous avons ajouté 2 ml de pyridine, puis lentement 12.3 mmoles de chlorure d'acide acrylique sous agitation. Après 12 h de réaction, le *m*-N-acryl-(N,N-diméthyl)-phénylènediamine a été isolé par précipitation dans la soude à 5%, puis méthylé sous reflux d'acétone avec un excès d'iodure de méthyle pendant 45 min. L'iodure de *m*-N'-acryl-(N,N,N-triméthylammonium)phénylènediamine précipitant pendant la réaction a été récupéré par filtration puis lavé à l'acétone froid (rendement: 46%). Sa structure a été confirmée par spectrographie infrarouge et de NMR. Son spectre ultraviolet en solution aqueuse présente un maximum d'absorption à 262 nm (ϵ = 12,000 $M^{-1} \cdot cm^{-1}$).

La copolymérisation de cette molécule avec l'acylamide a été effectuée

par voie radicalaire en milieu aqueux saturé d'azote dans les conditions opératoires définies antérieurement pour l'isomère *para* [11]. Sa concentration analytique dans le copolymère dialysé déterminée par spectrographie (λ_{max} = 244 nm, ϵ = 12,000 $M^{-1} \cdot cm^{-1}$) était de 7 mM.

Électrophorèse d'affinité

Les électrophorèses d'affinité ont été effectuées en tube (8.5 cm × 0.5 cm I.D.) avec des gels de polyacrylamide de séparation (T = 6%, C = 3.44%; pour une définition de T et C voir réf. 30) dans lesquels le copolymère linéaire ligand—acrylamide a été incorporé en concentrations différentes au moment de la polymérisation [11]. Nous avons opéré à 4°C dans une cuve Uniscil UE4 avec des galeries de 12 tubes dans lesquels la concentration analytique en ligand variait entre 0.0175 mM et 1.40 mM. Les migrations se sont déroulées dans un tampon de circulation 0.041 M Tris—glycine (pH 8.4) sous une intensité de 3 mA/gel et ont été poursuivies pendant 90 min.

Les zones d'activité cholinestérasique correspondant aux formes moléculaires C_1, C_2, C_3 et C_4 de l'enzyme ont été révélées selon la méthode de Juul [16] arrêtée à la formation de complexes blancs insolubles entre la thiocholine, produit d'hydrolyse de l'iodure de butyrylthiocholine et les ions cuivriques présents dans le tampon. Les distances de migrations ont été mesurées par densitométrie des gels sur un appareil Vernon PHI 4 (filtre Wratten No. 47 B) après 5 min d'incubation à 37°C pour la forme C_4 et après 90 min pour les trois autres formes d'activité plus faible. Les mobilités relatives (R_{mi}) ont été déterminées par rapport au colorant traceur (Bleu de Bromophénol); portées en fonction de la concentration en ligand (L), elles nous ont permis d'estimer les constantes de dissociation apparentes ($K_{D\ app}$) des complexes enzyme—ligand à partir de l'équation 1 [17].

$$\frac{1}{R_{mi}} = \frac{1}{R_{m\,0}} \cdot \left(1 + \frac{[L]}{K_{D\ app}}\right) \qquad (1)$$

dans laquelle $R_{m\,0}$ représente la mobilité relative en l'absence de ligand. Les estérases ont été détectées selon la méthode de Markert et Hunter [18] en utilisant le β-naphtylacétate comme substrat et le Fast Violet B Salt comme diazonium de couplage. Après fixation de la coloration par immersion pendant 30 min dans un mélange éthanol—acide acétique (3:2), les gels ont été transférés dans une solution d'acide acétique à 10%. Les distances de migration et les intensités relatives de ces enzymes ainsi que celles des protéines visibles (zones opalescentes) après précipitation par le mélange dénaturant précédent ont été mesurées par densitométrie (filtre Wratten No. 47 B). Les protéines ont, par ailleurs, été révélées par coloration au Bleu de Coomassie.

Électrophorèse en l'absence de ligand

Les électrophorèses en l'absence de ligand (L = 0) ont été effectuées dans des gels de séparation de concentration T = 6% dans des conditions identiques aux précédentes. Des pré-électrophorèses de ce type nous ont permis d'isoler séparément les quatre formes moléculaires de la cholinestérase après tronçonnage des zones de gel les renfermant [11].

RÉSULTATS ET DISCUSSION

Allozyme U purifié

Le comportement des trois formes moléculaires C_1, C_3 et C_4 présentes dans la préparation de cholinestérase (allozyme U) vis-à-vis du ligand immobilisé {poly[*m*-A(N-acryl)-PTMA—acrylamide]} est identique à celui que nous avons observé avec l'isomère *para* de ce ligand [11]. Pour chaque espèce moléculaire, dans le domaine d'affinité qui lui est propre, au-delà d'une concentration en ligand nommée concentration de transition, il apparaît une seconde zone, B, qui s'intensifie rapidement aux dépens de la première, A, lorsque L continue de croître. Nous avons interprété ce phénomène comme le résultat d'une isomérisation des sites anioniques de l'enzyme induite par le ligand entraînant une augmentation de leur affinité ($K_{D,B} < K_{D,A}$) [11]. Par ailleurs, les valeurs des constantes de dissociation apparentes (Tableau I) sont de l'ordre de grandeur de celles qui ont été obtenues avec le précédent ligand (ref. 11, Tableau I). L'affinité pour le dérivé *méta* apparaît cependant légèrement plus forte, confirmant ainsi les données cinétiques et chromatographiques de Picard [19] avec la même enzyme ainsi que celles de plusieurs auteurs [20—22] avec des acétylcholinestérases de différentes sources.

TABLEAU I

VALEURS DES CONSTANTES DE DISSOCIATION APPARENTES ($K_{D\,app} \cdot 10^4\ M$) DES COMPLEXES FORMÉS ENTRE LE POLY [*m*-A(N-ACRYL)PTMA—ACRYLAMIDE] ET LES FORMES MOLÉCULAIRES DES PHÉNOTYPES U, UA ET A DE LA CHOLINESTÉRASE DU PLASMA HUMAIN

		Allozyme U purifiée par chromatographie	Formes moléculaires non purifiées (plasma)			Formes moléculaires purifiées par pré-électrophorèse		
			U	UA	A	U	UA	A
C_1	A	1.28 ± 0.32	1.32	1.48	2.33	1.00	1.39	1.51
	B	0.31 ± 0.10	0.29	0.41	0.56			
C_2	A	*	***	***	***	0.118	§§	0.214
	B							
C_3	A	0.189 ± 0.072				0.308	§§	0.687
	B	**	***	***	***			
C_4	A	0.098 ± 0.015	§	§	§	0.108	§§	0.192
	B	0.023 ± 0.003						

* La forme C_2, dimère constitué d'une sous-unité C_1 associée à une sous-unité non enzymatique de plus petite taille [24] n'est pas copurifiée avec les trois isomères de taille.
** Confondue avec C_4.
*** Déterminations impossibles en raison du chevauchement de C_2 et C_3.
§ Résultats aléatoires par suite de la compétition de protéines parasites.
§§ Valeurs fluctuant entre U et A.

Étude directe des formes moléculaires des trois phénotypes dans les échantillons de plasma

L'étude de la mobilité des quatre formes moléculaires de la cholinestérase

dans des plasmas de phénotype U, UA ou A, en fonction de la concentration en ligand, n'a fourni des données immédiatement interprétables que pour les formes C_1. Pour les phénotypes U et A, le rapport des constantes de dissociation apparentes ($K_{D\ app,\ A}$) des complexes C_1—ligand est voisin de 2. Dans le cas du phénotype UA, en théorie, deux zones C_1, l'une de type U, l'autre de type A, devraient se séparer lorsque L augmente. Cependant, la faible différence d'affinité apparente entre les deux enzymes, objectivée par le rapport des K_D, ne permet pas leur résolution dans nos conditions expérimentales. Aussi, la forme C_1 de ce phénotype migre avec une mobilité intermédiaire entre U et A (Tableau I).

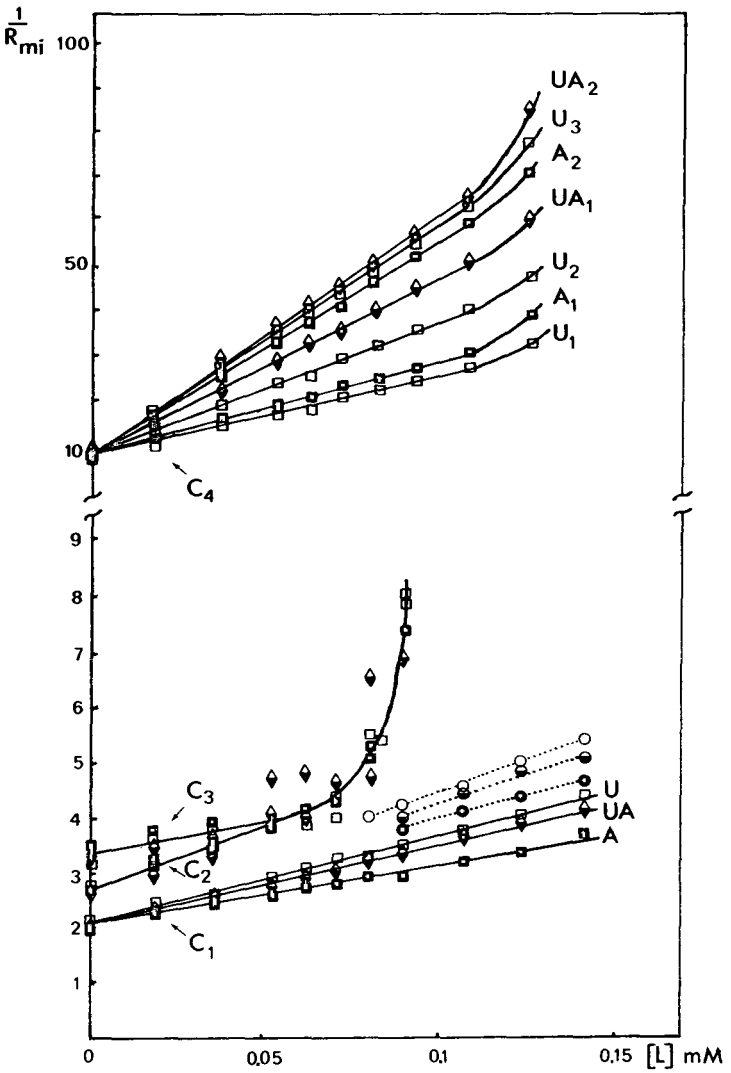

Fig. 1. Variation de R_{mi}^{-1} en fonction de L pour les quatre formes moléculaires (C_1, C_2, C_3, C_4) des phénotypes U, UA et A dans différents plasmas. ▪, phénotype A; ◆, phénotype UA; □, phenotype U. En pointillé, les formes C_1 dans l'état B (●, phénotype A; ◐, phénotype UA; ○, phénotype U).

Les valeurs des $K_{D\,app}$ pour les formes C_2 et C_3 n'ont pu être calculées. En effet, l'affinité de C_2 pour le ligand, supérieure à celle de C_3, entraîne rapidement, lorsque L croît, le chevauchement de ces deux formes (Fig. 1).

Les résultats concernant les formes C_4 n'ont, par ailleurs, pu être pris en considération, en raison de leur caractère aléatoire (Fig. 1). Une analyse de variance pratiquée sur les pentes des droites représentant la variation de R_{mi}^{-1} en fonction de L a confirmé que ces pentes ne diffèrent pas de façon significative dans leur ensemble. Leur variabilité à l'intérieur d'un même phénotype est en effet supérieure à la dispersion factorielle liée aux différences d'affinité entre les trois phénotypes. Nous avons alors émis l'hypothèse que cette variabilité était inhérente à la nature de la technique et résultait de la fixation aspécifique de protéines sur le ligand immobilisé. Cette hypothèse a été vérifiée en deux temps.

Nous avons tout d'abord déterminé l'influence de la quantité de protéines dans l'échantillon sur la mobilité de la forme C_4 d'un plasma quelconque à concentration nulle en ligand et en parallèle à concentration élevée ($L = 1.80 \cdot 10^{-4}$). Dans les gels témoins ($L = 0$), l'inverse de la mobilité de C_4 ($R_{m\,0}^{-1}$) est indépendante de la quantité de protéines déposées, par contre, dans les gels d'affinité, elle décroît rapidement lorsque $[P]$ augmente et tend vers $R_{m\,0}^{-1}$ (Fig. 2). Corrélativement, on note après coloration au Bleu de Coomassie ou

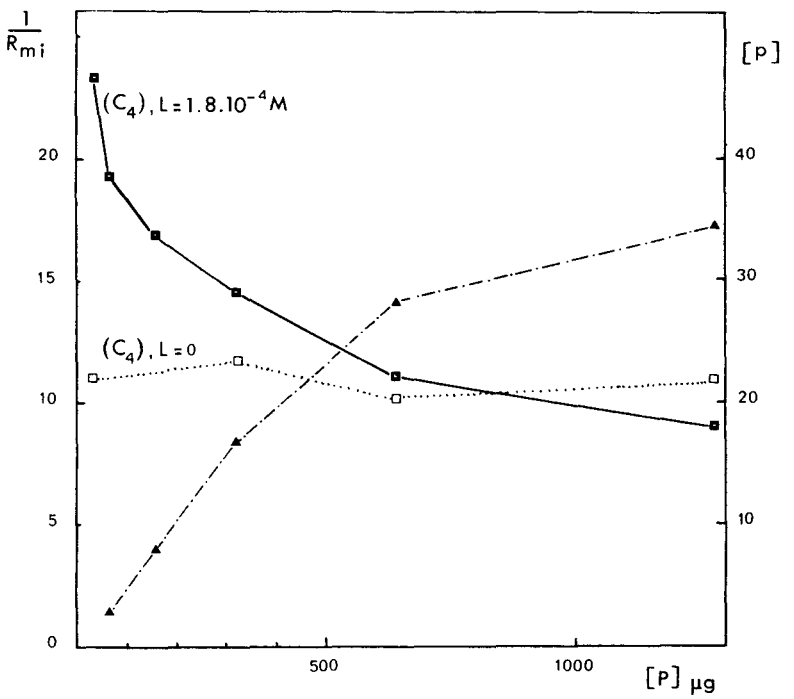

Fig. 2. Étude de la variation de R_{mi}^{-1} de la forme C_4 en fonction de la quantité de protéines plasmatiques $[P]$ dans l'échantillon. En trait plein ($L = 1.80 \cdot 10^{-4}$ M) et en pointillé ($L = 0$). La courbe en tirets (▲) représente la quantité de protéines $[p]$ arrêtées au sommet des gels de séparation en fonction de $[P]$ lorsque $L = 1.80 \cdot 10^{-4}$ M (les concentrations $[p]$ déterminées par densitométrie des gels sont exprimées en unités arbitraires).

précipitation par le mélange éthanol—acide acétique, la présence en quantité progressivement croissante de protéines [p] arrêtées à l'entrée des gels de séparation. Aucune activité arylestérasique n'est détectée à ce niveau.

Dans un second temps, nous avons cherché à apprécier l'importance des variations qualitatives entre les plasmas. Nous avons opéré avec des plasmas de phénotype U, UA ou A ayant la même concentration en protéines. Pour chacun d'eux, des dépôts de 10 μl/gel (environ 600 μg) ont été effectués et les électrophorèses à T = 8% pratiquées respectivement en l'absence et en présence de ligand ($L = 1.80 \cdot 10^{-4}$). Après migration, les gels de séparation ont été sectionnés à leur partie supérieure sur une hauteur de 2 mm. Les tronçons isolés ont été ensuite soumis à des électrophorèses à T = 6% et L = 0. Pour les tronçons issus des gels contenant le ligand, les zones protéiques révélées après coloration au Bleu de Coomassie se répartissent à tous les niveaux des gels; leur nombre, leur position (R_m) et leur intensité variant d'un échantillon à l'autre.

Donc, ces protéines dont la concentration est toujours très supérieure à celle de la cholinestérase (5—10 mg/l), non détectée par la coloration au Bleu de Coomassie, varient qualitativement et quantitativement d'un plasma à l'autre. En raison du manque de spécificité du ligand et/ou du fonctionnement de la matrice comme un échangeur d'anions, elles se fixent au sommet des gels d'affinité et occupent les résidus ligands disponibles sur des distances plus ou moins grandes selon leur concentration dans les échantillons. Or, la forme C_4, peu mobile du fait de sa taille, traverse les gels d'affinité sur des distances $d_i < d_0$ [d_0 (T = 6%, L = 0) = 8—10 mm] ; durant son parcours, elle est donc en compétition avec ces protéines et sa mobilité relative ne reflète plus son affinité spécifique pour le ligand.

Étude des formes moléculaires isolées par électrophorèse

En travaillant avec les formes moléculaires isolées par pré-électrophorèse à T = 6%, nous avons pu estimer les constantes de dissociation apparentes des formes C_2, C_3 et C_4 inaccessibles dans les conditions précédentes, et confirmer les valeurs obtenues pour les formes C_1 des trois phénotypes (Fig. 3).

Pour le phénotype U, nos résultats (Tableau I) sont comparables en ce qui concerne C_1, C_3 et C_4 à ceux auxquels nous avons eu accès avec l'enzyme purifiée. L'affinité relativement élevée de la forme C_2 est, à première vue, paradoxale. En effet, cette enzyme est constituée d'un monomère C_1 lié par un pont disulfure [23] à une sous-unité plus petite, dépourvue d'activité. Cependant, C_2 possède des propriétés électriques particulières [24] apparaissant essentiellement liées à la nature de cette seconde sousunité dont la surface pourrait comporter un nombre relativement élevé de résidus aspartates et/ou glutamates. La présence de ces groupements pourrait donc expliquer la haute affinité de C_2 vis-à-vis du présent ligand cationique.

Le rapport des constantes de dissociation pour les quatre formes moléculaires des phénotypes U et A est voisin de 2 (Tableau I). L'affinité réduite de l'enzyme "atypique" par rapport à l'enzyme "usuelle" traduit l'altération du sous-site anionique du centre actif et apporte un argument supplémentaire en faveur de l'hypothèse de Kalow et Davies [4]. Cependant, la différence d'affinité apparente entre les formes moléculaires des deux allozymes apparaît moins

importante que ne le laissait prévoir les écarts entre les volumes d'élution des formes C_4 des homozygotes (A_4 et U_4) et de l'hétérozygote UA (A_4, A_3U, A_2U_2, AU_3 et U_4) lors de leur séparation par chromatographie d'affinité sur diaminocaproyl-phényltriméthylammonium-Sépharose 4B [9]. Un calcul approché, tenant compte des observations d'O'Carra et al. [25], effectué à partir des données chromatographiques de La Du et Choi [9], permet d'attribuer au rapport de $K_{D\ app}$ (C_4) des phénotypes A et U une valeur minimale de 4.5.

Ce meilleur pouvoir séparateur de la chromatographie d'affinité tient à plusieurs facteurs. La présence du bras espaceur ϵ-aminocaproyle éloignant le ligand du support diminue les effets de matrice non spécifiques et les contraintes stériques, et favorise l'accessibilité de l'enzyme à l'affinant. En électro-

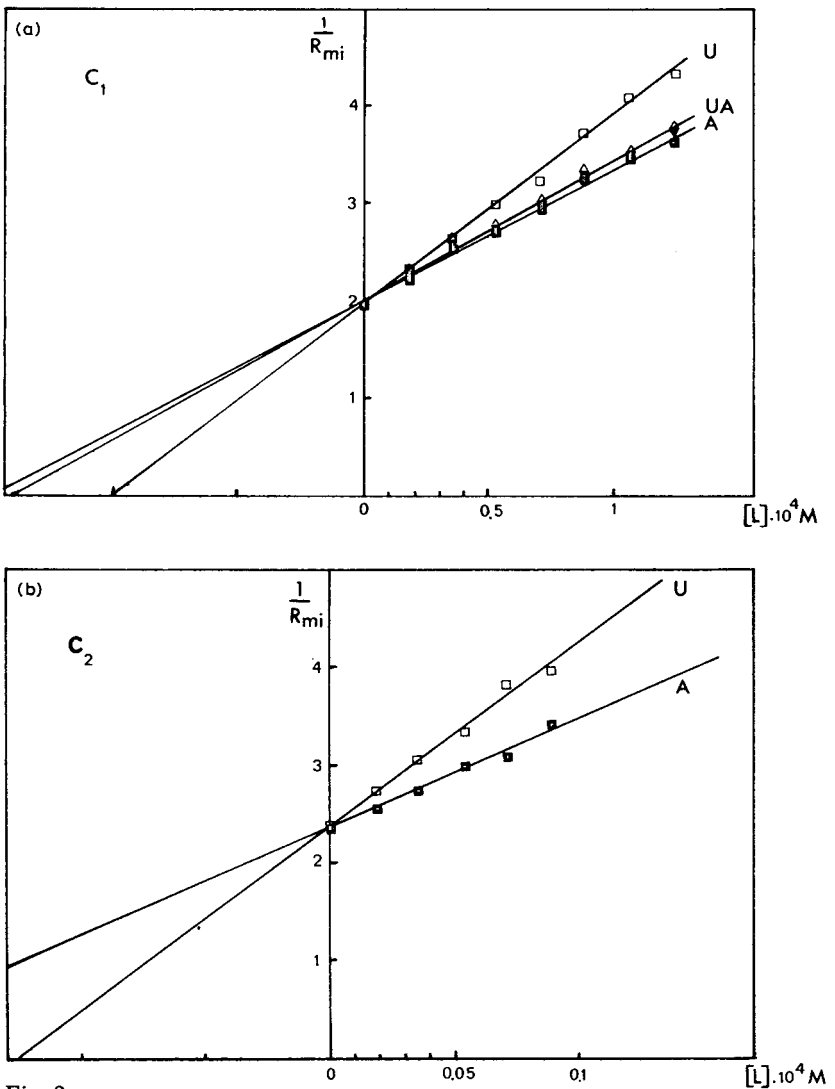

Fig. 3.

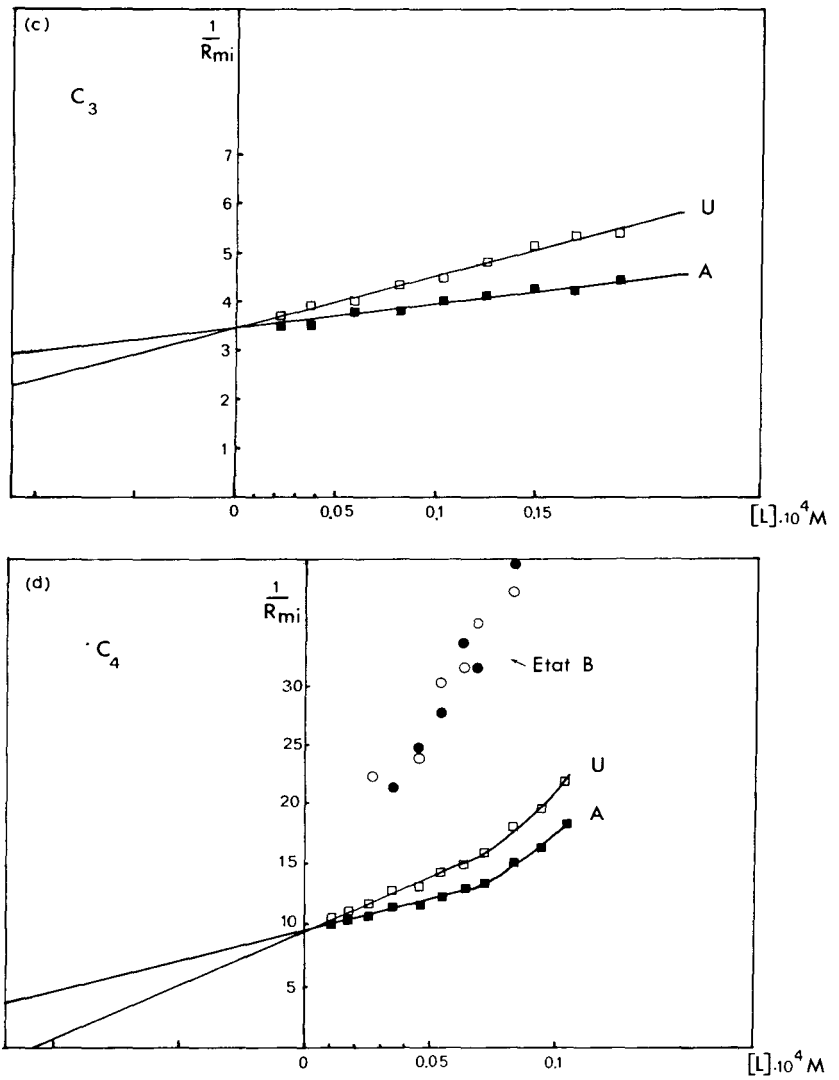

Fig. 3. Courbes représentatives de la variation de R_{mi}^{-1} en fonction de L pour les quatre formes moléculaires des phénotypes U, A et UA isolées après pré-électrophorèse des plasmas (T = 6%, L = 0). (a) Forme C_1, (b) forme C_2, (c) forme C_3, (d) forme C_4.

phorèse d'affinité, la situation est plus complexe [26]. Dans notre cas, le ligand est directement couplé à une chaîne de polyacrylamide emprisonnée dans les mailles d'un gel de faible porosité. Une microdistribution en îlots du ligand peut provoquer au sein de ce gel des perturbations locales de la force ionique et du champ électrique. Par ailleurs, sous la pression du champ électrique, des transitions de phase du gel, induites par le ligand chargé, ne sont pas à exclure [27]. Ainsi, la conjonction d'effets stériques et électriques doit favoriser des interactions coulombiennes non spécifiques, entre la surface de l'enzyme et le ligand, et entraîner une diminution du pouvoir discriminant de ce dernier vis-à-vis des allozymes.

Concernant le phénotype UA, la chromatographie d'affinité ne peut séparer nettement les cinq tétramères de ce phénotype. Dans nos conditions expérimentales, leur séparation a été totalement impossible et les valeurs des $K_{D\ app}$ pour les formes C_2, C_3 et C_4, fluctuant entre celles des formes correspondantes U et A, n'ont pu être déterminées. Pour la forme C_1, nos résultats sont comparables à ceux obtenus directement sur le plasma (Tableau I, Fig. 1 et 3a): l'affinité de C_1 (UA) apparaît intermédiaire entre celle de C_1 (U) et celle de C_1 (A). Toutefois, à partir de $L = 0.2 \cdot 10^{-4}$ M, sur le côté anodique de la zone C_1, un épaulement apparaît de façon inconstante. Pour des concentrations plus fortes en ligand, une seconde zone, peu intense mais plus mobile, correspondant vraisemblablement à C_1 de type A, émerge peu à peu du pic principal (C_1 de type U).

La synthèse de ligands au pouvoir discriminant élevé permettant de séparer par électrophorèse d'affinité les différents hybrides de chaque forme moléculaire du phénotype UA constitue l'un de nos objectifs. Sa réalisation est la condition nécessaire au développement de cette technique pour le phénotypage des variantes de la cholinestérase du plasma humain.

CONCLUSION

La technique d'électrophorèse d'affinité que nous développons, bien que permettant de mettre en évidence des différences fonctionnelles minimes entre des allozymes, ne peut, dans son état actuel, être appliquée au phénotypage des variantes de la cholinestérase du plasma humain. Cet aspect pratique ne pourra être abordé qu'après levée de l'obstacle que constitue la présence, dans le plasma, d'autres protéines compétitives de la cholinestérase. La résolution de ce problème passe par la recherche de nouveaux ligands de spécificité plus étroite et plus discriminants et/ou par la mise en oeuvre de techniques d'électrophorèse d'affinité bidimensionnelle.

Au plan général, une telle démarche devrait permettre d'appliquer l'électrophorèse d'affinité, d'utilisation encore peu répandue en biochimie génétique [28, 29], à l'étude des iso- et allozymes dans des milieux biologiques complexes et entraîner un élargissement du champ du polymorphisme biochimique au-delà de la variabilité électrophorétique mise en évidence par les techniques habituelles d'électrophorèse et d'isofocalisation sur gel.

RÉSUMÉ

L'électrophorèse d'affinité a été appliquée à l'étude des formes moléculaires de trois phénotypes de la cholinestérase du plasma humain, l'enzyme usuelle U, l'enzyme atypique A et l'intermédiaire UA. Les électrophorèses ont été effectuées dans des gels de polyacrylamide contenant un dérivé macromoléculaire hydrosoluble du *m*-amino(substitué)phényltriméthylammonium immobilisé dans la matrice.

Les constantes de dissociation apparentes ($K_{D\ app}$) ont été estimées à partir de la mesure des mobilités en fonction de la concentration en ligand.

Le rapport des $K_{D\ app}$ des formes moléculaires des phénotypes A et U, approximativement égal à 2, est en accord avec l'hypothèse selon laquelle le site anionique de l'enzyme atypique est modifié.

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CHROMBIO. 1557

EVALUATION OF REDUCTIVE AMPEROMETRIC DETECTION IN THE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTINEOPLASTIC PLATINUM COMPLEXES

STEVE J. BANNISTER*, LARRY A. STERNSON* and A.J. REPTA

Pharmaceutical Chemistry Department, The University of Kansas, Lawrence, KS 66045 (U.S.A.)

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SUMMARY

The usefulness of reductive electrochemical detection at mercury drop electrodes has been determined for platinum complexes separated by solvent-generated anion-exchange high-performance liquid chromatography. Both current-sampled dropping mercury and hanging mercury drop electrodes (DME and HMDE) provide significant advantages over UV absorbance and off-line non-flame atomic absorption detection. The effects of chromatographic and polarographic parameters on analytical system performance have been investigated. By raising the detector cell temperature, the detector response to *cis*-dichlorodiammineplatinum(II) (DDP) can be shifted anodically to 0.0 V vs. Ag/AgCl, thereby increasing detector selectivity for this compound. The noise-limited minimum detectable quantities of DDP with DME and HMDE are 1.8 ng and 70 pg injected, respectively. DDP can be determined in untreated urine at levels below 100 ng/ml.

INTRODUCTION

Cisplatin [*cis*-dichlorodiammineplatinum(II); DDP, Fig. 1] is a square planar complex of divalent platinum that is quite active against a variety of solid tumors and is widely used clinically in the treatment of testicular and ovarian cancers [1]. Work in these laboratories has resulted in the development of chemically bonded [2] and solvent-generated anion-exchange [3, 4] (SGAX) chromatographic systems for the separation of platinum complexes differing in ligand composition found in biological samples following cisplatin administration.

Because of the absence of significant molecular electronic absorptivity (λ_{max} = 301 nm; ϵ_{301} = 150) [5], the central platinum ion is the analyte characteristic

*Present address: Laboratory Data Control Division, Milton Roy Co., Riviera Beach, FL 33404, U.S.A.

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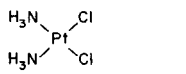
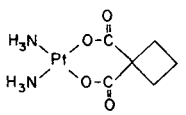
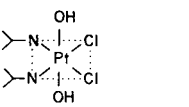


Cisplatin
cis- dichlorodiammineplatinum(II)
DDP



(cyclobutanedicarboxylato)diammine-
platinum(II)
CBDCA



cis - dichloro - trans - dihydroxo -
bis(isopropylamine)platinum(IV)
CHIP

Fig. 1. Antineoplastic platinum complexes investigated.

most readily exploited for the detection of cisplatin and other complexes. Post-column analysis of eluent fractions by non-flame atomic absorption spectrophotometry (NFAA) has been combined with the chromatographic selectivity of column switching in a method capable of determining DDP at levels of 2 μg/ml in urine [6].

The feasibility of electrochemical platinum(II) detection for chromatographic systems has now been investigated to meet the need for a detector at least as sensitive as the described application of NFAA but capable of continuously monitoring the column effluent. With both oxidation and reduction of platinum(II) possible [7], electrochemistry provides numerous electrode and reaction possibilities. Reduction at mercury was chosen for study since, as explained in this paper, the nature of the platinum(II) reduction and the presence of surfactant in the mobile phase makes solid electrodes unattractive.

Platinum(II) complexes are poorly behaved depolarizers, interacting strongly with the electrode surface [7, 8]. The reduction of these compounds involves a two electron transfer to the central atom of an adsorbed molecule [8, 9]. Polarograms of chloro- and chloroammine-platinum(II) complexes include maxima and minima [7, 10] resulting from these surface effects. Platinum metal, adsorbed on the electrode surface as growing crystal nuclei, is produced in the irreversible reduction [11]. The electro-deposited metal alters the advantageous hydrogen overpotential of mercury causing catalytic hydrogen currents at potentials as anodic as -600 mV (saturated calomel electrode) [12]. Electro-oxidations of platinum(II) complexes occur through bridging halides and are quite slow in the absence of free halide [13].

Because of these analytically unsuitable electrode reactions, voltammetric methods have been infrequently used for the determination of platinum. In one reported electrochemical method [14], the catalytic hydrogen current resulting from platinum metal deposition is used quantitatively in a sensitive differential pulse polarographic determination of platinum at mercury.

Electrochemical detectors for high-performance liquid chromatography (HPLC) have been reviewed recently [15—17]. While oxidative amperometry

and coulometry at glassy carbon or carbon paste surfaces are most commonly used, reduction at mercury or solid electrodes has also been frequently applied. Mercury electrodes have been configured as either mercury immobilized on a solid support [18] or as mercury drops [19–21]. This paper describes the application of polarographic HPLC column monitoring to the clinical analysis of cisplatin in urine.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC columns used were obtained from Technicon (Tarrytown, NY, U.S.A.) and were 150 × 4.6 mm packed with C_8-bonded 5-μm spherical porous silica. The column temperature was maintained with a water jacket (Alltech, Arlington Heights, IL, U.S.A.) and thermostated using a circulating pump, type FJ (Haake, Saddle Brook, NJ, U.S.A.). Columns were prepared for use by washing with 200 ml of water followed by 150 ml of 0.5% (w/v) hexadecyltrimethylammonium bromide (HTAB)—water. Deoxygenated mobile phase (500 ml) was pumped through the column prior to use. The mobile phase was not removed from the column for interim storage.

UV detection was with a Model 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.) set at 301 nm. A 2-sec low-pass filter time constant was used. A Model 210 loop injector (Altex, Berkeley, CA, U.S.A.) with loops of various capacities was used for sample introduction. Mobile phases were pumped with either a Model 6000A reciprocating piston pump (Waters) or a Model 4100 screw-driven syringe pump (Varian, Walnut Creek, CA, U.S.A.). A syringe infusion pump Model 341 (Sage Instrument, Cambridge, MA, U.S.A.) fitted with a 35-ml syringe was used to pump solutions directly onto the electrode and for post-column electrolyte addition. Chromatograms were recorded on an Omniscribe dual-pen recorder (Houston Instruments, Houston, TX, U.S.A.) while polarograms were recorded on a Houston Omnigraphic X-Y recorder.

Electrochemical measurements

Cell potential control and current measurements were performed with a Model 174A Polarographic Analyzer (Princeton Applied Research, Princeton, NJ, U.S.A.). A Model 303 Static Mercury Drop Electrode was used to record quiet solution polarograms and was modified with a Model 310 flow adaptor, both from Princeton Applied Research, for flowing stream measurements. Elevated detector flow cell temperatures were achieved by wrapping half of a 1-m, 140-W heating tape around the cell. An inlet thermostat was added to the flow cell to insure that the stream temperature was that measured in the cell. One meter of stainless-steel tubing (1.6 mm I.D. × 0.23 mm I.D.) was coiled inside the wall of the cell and fitted into the electrode flow adaptor in place of the PTFE capillary tubing originally installed. The opposite end was attached to the column outlet. The temperature was monitored with a calibrated thermistor immersed in the cell and was adjusted by changing the heater voltage.

Oxygen in the inert sparge gas was reduced with a vanadous chloride scrubbing system [22]. Eight grams of ammonium metavanadate (Aldrich, Milwau-

kee, WI, U.S.A.) were boiled in 100 ml of concentrated hydrochloric acid (Fisher, Fairlawn, NJ, U.S.A.) for 10 min. After cooling and dilution to 1 l with water, the solution was added to three gas scrubbing vessels, each containing 15 g of amalgamated zinc granules (prepared from 20-mesh zinc, Fisher). The deoxygenating scrubbers were connected in series and were followed by two similar vessels containing water. Oxygen permeation through the PTFE feed line on a Waters pump was eliminated by enclosing this line in 7.9 mm I.D. PVC tubing through which inert gas was flowing. Configured in this manner, the entire fluid path of the HPLC instrument is sequestered from oxygen, without the use of a cumbersome glove box.

Urine analysis for cisplatin

Urine samples were frozen in dry ice immediately after collection and stored in dry ice until analysis. Sample preparation prior to chromatographic analysis [6] included thawing in a 50°C water bath with constant swirling followed by 30 sec of ultrasonication and filtration through a 3-μm Millipore filter. An aliquot (50 μl) of this material was injected onto a chromatographic system comprised of a dynamic anion exchange column (described above) thermostated at 60°C, a mobile phase of 5 m*M* citrate (pH 6.5) containing 0.1 m*M* HTAB and an electrochemical detector consisting of hanging mercury drop cathode (area = 0.026 cm^2; polarized at 0.00 V vs. Ag/AgCl) in a cell thermostated at 60°C.

Non-flame atomic absorption

Total platinum content of urine samples and of HPLC effluent fractions was determined as previously described [6] using a 175 B atomic absorption spectrophotometer fitted with a CRA-90 electrothermal atomizer (both from Varian).

Materials

Cisplatin was obtained in crystalline form from the National Cancer Institute (Bethesda, MD, U.S.A.) and its solutions were prepared in 0.1 *M* potassium chloride. Citric acid, sodium citrate and potassium chloride were AR grade (Mallinckrodt, St. Louis, MO, U.S.A.). HTAB was from Aldrich. Mercury was triple-distilled instrument grade (D.F. Goldsmith, Evanston, IL, U.S.A.). All water used was distilled in glass after mixed-bed deionization.

RESULTS AND DISCUSSION

Polarographic detectors used in conjunction with HPLC require a mobile phase optimized for both analyte resolution and analyte response, since the column effluent serves as the electrode reaction medium. In addition, potentiostat and electrode parameters must be optimized for the analyte(s) of interest. The development and evaluation of a clinically applicable assay for cisplatin has included these interrelated aspects.

Mobile phase composition

Although DDP and other platinum complexes differing in ligand composition can be resolved by SGAX using a simple aqueous HTAB mobile phase, this

system was found to be incompatible with electrochemical detection because of inadequate supporting electrolyte. Both the HTA cation and bromide are specifically adsorbed to the mercury electrode [23], altering double-layer capacitance [23] and diminishing electrode reactivity [24].

Addition of citrate to the mobile phase offered greater manipulation of DDP retention [4], and also served as a non-reactive supporting electrolyte. Citrate concentrations as low as 3 m*M* are adequate to allow the recording of polarographically detected chromatograms. A mobile phase containing 5 m*M* citrate was used for all subsequent studies.

DDP retention in HTAB—citrate mobile phases is pH dependent. As pH rises and the balance of citrate is shifted toward polyanions, retention increases [4]. Detector signal and noise were not affected by mobile phase pH. Final pH selection was made in conjunction with column temperature selection as described below.

In all instances, samples of cisplatin contained chloride ions. Standards were prepared in 0.1 *M* potassium chloride solutions to avoid chemical degradation of DDP [5] and biological samples similarly contained high levels of endogenous chloride [25]. Chloride causes a baseline disturbance in the polarographic chromatogram just after DDP elution (Fig. 2). Mobile phase citrate concentrations

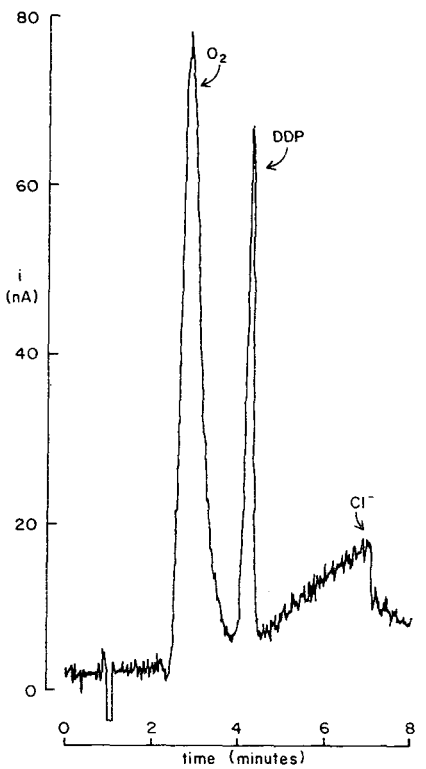


Fig. 2. Effect of injected chloride on polarographic chromatogram. Column: 150 × 4.6 mm, 7 μm C_8; sample: 150 μl of 2.0 μg DDP per ml 0.1 *M* potassium chloride; mobile phase: 10 m*M* citrate—0.1 m*M* HTAB, pH 7.3; flow-rate: 2.0 ml/min; column temperature: 24°C; detector: 0.5 sec DME, 0.026 cm^2 drop, −100 mV (Ag/AgCl); cell temperature: 24°C.

below 10 m*M* resolve the chloride wave from DDP so that analyte peak measurements are not compromised. Other anions (iodide, bromide and nitrate) did not produce the anomalous wave attributed to chloride.

Effect of column temperature

Column temperature affects the DDP—SGAX system in three ways. As temperature increases: (a) chromatographic efficiency increases; (b) the stability of both eluting DDP and the column itself decrease; and (c) column selectivity, measured for eluite pairs having nonidentical retention mechanisms [26], is altered.

Column temperature was raised to increase column efficiency, and therefore resolution and peak height improve. DDP retention decreased relative to oxygen, a significant interference in polarographically detected HPLC [18]. The k'_{DDP} value decreased from 7.8 to 1.8 (a 333% change) when column temperature was increased from 28°C to 78°C, while this increase in column temperature only resulted in a slight increase in k'_{O_2} from 2.9 to 3.2 (a 10% change).

To compensate for the diminished retention of DDP at higher temperatures, mobile phase pH was raised to maintain DDP elution at $k' = 2\text{-}3$, just prior to oxygen, but resolved from the unretained and early-eluting material. Column stability, however, limited the extent to which temperature and pH could be simultaneously raised. Twelve hours at 78°C with citrate mobile phase (pH 7.3, measured at 24°C) was sufficient to destroy column performance. Decreased retention, band broadening and asymmetry, and voiding at the head of the column were observed, indicating hydrolysis of both the C_8-anchoring silyl ether and the silica support [27]. Data defining the time course of this degradation were not collected. Subsequent work at temperatures below 65°C and pH values (measured at 24°C) $\leq$6.5 yielded column lifetimes of ca. 250 h of system operation. Losses in efficiency and retention, degradation of peak symmetry and peak shouldering under these conditions were again symptomatic of hydrolysis of the bonded phase. Repeated urine injections probably contributed to column deterioration. This was confirmed by backpressure elevations and darkening of the packing at the top of the column.

Elevated column temperatures accelerated DDP degradation. Riley et al. [3] estimated that at 25°C, <5% loss of DDP occurs on-column and concluded that the predominant reaction in the mobile phase is aquation. Monitoring the change in reduction current in a 64°C incubation of DDP (0.44 m*M*) in mobile phase (0.1 m*M* HTAB—5 m*M* citrate, pH = 6.5 at 24°C) with an immersed dropping mercury electrode (DME) (0 mV vs Ag/AgCl) revealed apparent first-order kinetics for DDP degradation. The rate constant for the process was 0.054 min^{-1}. The current diminished from 5.2 μA initially, to 0.09 μA, consistent with previous reports of the polarographic inactivity of aquated species derived from DDP [28, 29].

DDP degradation during its 4.5-min residence on column corresponds to a calculated loss of 22% of parent drug. This degradation will have a significant effect on sensitivity (units of peak area per unit concentration) but of greater analytical significance is the effect of this loss on precision. The increased rate of DDP aquation with temperature ($\Delta H^{\neq}$ = 20 kcal/mol [5]) is only partially compensated by the decreased reaction time which results from decreased re-

tention. Careful control of column temperature is, therefore, essential for analytical precision.

The selectivity of the polarographic detector for DDP relative to the aquated species formed on-column prevents the loss of peak shape integrity seen in similar systems with UV detection [3]. The lack of polarographic response to the aquated species (at the potential used) and the first-order kinetic behavior prevent on-column degradation from compromising calibration linearity of response to cisplatin.

Effect of flow-rate

When air-saturated mobile phase was pumped across the electrode the oxygen reduction current was distinctly flow dependent. DDP reduction current, measured in a deoxygenated flowing stream, however, was insensitive to flow-rate above 0.5 ml/min (Fig. 3). An apparent inverse relationship between flow and peak-height response was seen under chromatographic conditions (Fig. 4), that did not originate in the detector. The polarographic peak-height trend parallels that of the UV detector above ca. 0.5 ml/min, and arises from increasing column efficiency with decreasing flow-rate. The flow independence of the polarographic DDP response is caused by the slow adsorption step in the DDP electrode process. A flow-rate of 1.0 ml/min was chosen for analyses, taking advantage of the increase in efficiency and sensitivity without making the retention of DDP inconveniently long.

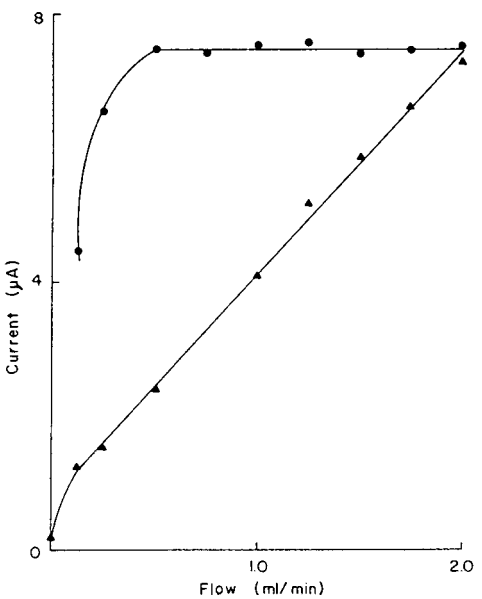


Fig. 3. Effect of flow-rate on DDP and oxygen reduction currents, measured in solution pumped directly into detector cell. O_2 (▲): air equilibrated mobile phase (5 m*M* citrate—0.1 m*M* HTAB, pH 6.5) at −700 mV (Ag/AgCl). DDP (●): 132 μg DDP per ml deoxygenated mobile phase at −410 mV (Ag/AgCl). Detector: 0.5 sec DME, 0.026 cm^2 drop; cell temperature: 24°C.

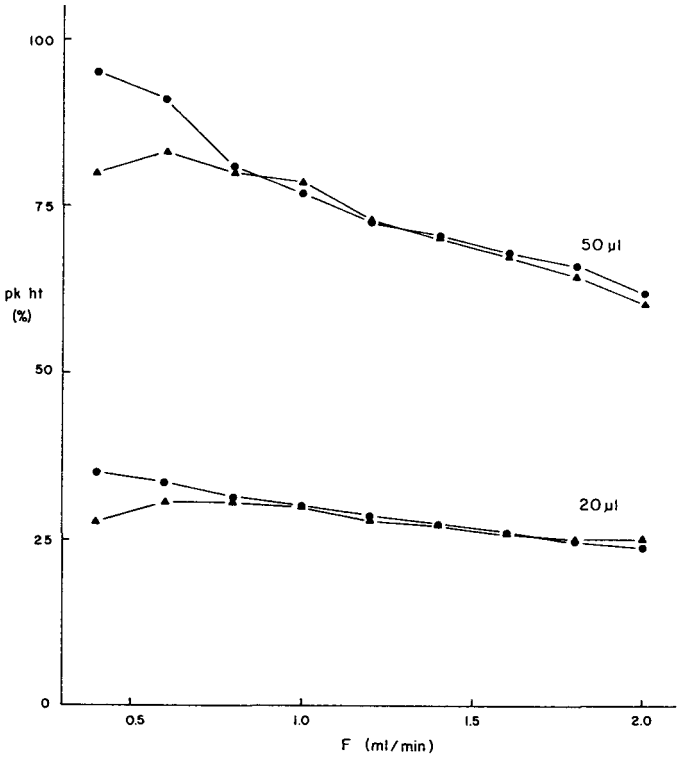


Fig. 4. Effect of flow-rate on chromatographic peak height. Column as in Fig. 2; sample: 20 and 50 µl of 100 µg DDP per ml; mobile phase as in Fig. 3; column temperature: 60°C; detector (DME): 1.0 sec DME, 0.026 cm^2 drop, 0.0 V (Ag/AgCl), 100% = 6.9 µA; detector (UV) 301 nm absorbance, 100% = 0.031 a.u. (•) UV, (▲) DME.

Effect of detector mode

Detection at a dropping mercury electrode was performed in sampled d.c. and normal pulse modes [30]. Because the hanging mercury drop electrode (HMDE) has a constant surface area, continuously monitored d.c. could be used with this electrode configuration, in addition to the other modes. Differential pulse, while offering additional selectivity [18], was not used because instrumental deficiencies in the potentiostat (time constant 5 sec at 1 sec drop time [31]) distorted the chromatographic bands (width at half-height 5—20 sec) [32].

When peak-height sensitivity to DDP (50 µl of 1 µg/ml injected; 55°C column; 5 m*M* citrate—0.1 m*M* HTAB, pH 6.5, at a flow-rate of 1 ml/min) was determined with a DME (24°C cell temperature, 1 sec drop time), sampled d.c. (−120 mV, Ag/AgCl) and normal pulse (0—−120 mV, Ag/AgCl) both yielded a sensitivity of 0.14 µA/µg injected. The sensitivity advantage for pulse, seen in diffusion-limited systems [18], is not seen in this system, consistent with an adsorption-limited reduction of DDP.

Effect of drop size

The electrode used quickly produces a capillary-suspended drop that is static during most of its lifetime. The nominal masses of the three drop sizes available

are 1.2, 2.5 and 5.4 mg corresponding to surface areas of 0.0096, 0.0156 and 0.0261 cm^2 [33]. Actual drop size is dependent on mercury head pressure (limited to about 5% variation) and capillary and mercury flow-valve condition.

DDP solutions were prepared at 25, 50 and 100 μg/ml and injected with the detector set at each of the drop size selections. The observed increase in peak height response was not directly proportional to the increase in electrode surface area which is probably attributable to adsorption of DDP and HTAB to the drop surface. Baseline noise, however, was independent of drop size, so "large" (0.026 cm^2) drops were used in all subsequent measurements.

Drop time

Drop time effects three components of the analytical signal: (a) faradaic current; (b) capacitive current; and (c) peak representation (since it is recorded as a series of discrete current measurements at a DME).

Detector sensitivity decreased slightly as the drop time increased from 0.5 sec (DME) to several minutes (HMDE) (Fig. 5). Fresh drops were hung with each injection when a HMDE was used but sensitivity to DDP did not change if the drop formation was not synchronized with injection.

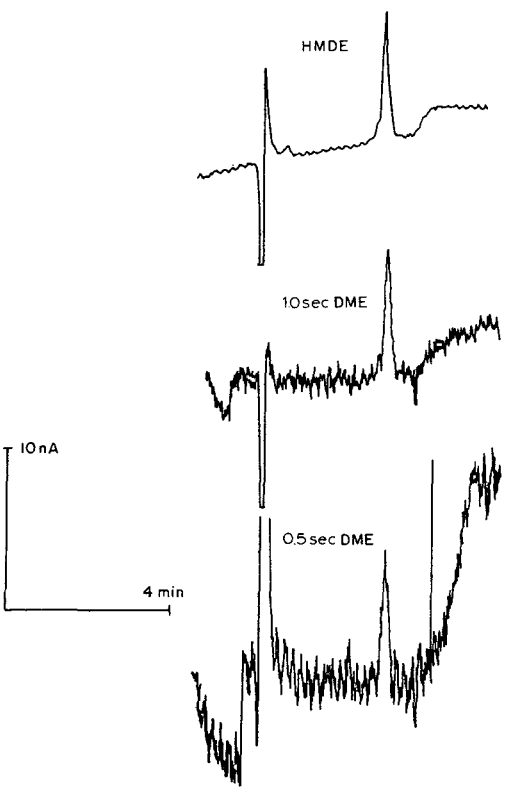


Fig. 5. Effect of drop time on noise and sensitivity. Column as in Fig. 2; sample: 50 μl of 500 ng DDP per ml; mobile phase as in Fig. 3; flow-rate: 1.0 ml/min; column temperature: 60°C; detector: 0.026 cm^2 drop, 0.0 V (Ag/AgCl); cell temperature: 60°C; 0.3 sec low-pass filter; HPLC pump: reciprocating piston.

The slight loss in sensitivity with increasing drop time may be due to loss of reactive surface area due to HTAB adsorption [23, 24]. Adsorption of inactive organic [10] and inorganic [8] substances has been shown to inhibit the reduction of those platinum complexes requiring such intimate electrode contact. Since HTAB is adsorbed at the mercury surface and is not itself reduced (accumulating on the surface until a potential-dependent coverage is reached), the available reactive electrode surface at the time the current is sampled is diminished as the drop time is increased, resulting in a decrease in the reduction current measured. Limiting coverage is apparently complete throughout peak detection in the HMDE mode and sensitivity is decreased from the 0.5- and 1.0-sec DME recordings.

HTAB adsorption on a time scale comparable to drop life (1—10 sec to reach limiting coverage) is consistent with reported surfactant adsorption kinetics [24, 34] and accounts for the drop-time dependence of detector noise (Fig. 5). In the absence of depolarizer, drop growth is accompanied by capacitive current as the charged double layer is expanded. Without complicating adsorption this capacitive current decays exponentially after drop growth ends [33]. When drops were formed and then expanded stepwise during continuous fixed-potential polarization, the decay of charging current was found to be greatly potential dependent. At potentials anodic to −100 mV (Ag/AgCl) the decay was slow. Long-chain alkyltrimethylammonium cations adsorb to the electrode surface in complete monolayers at potentials cathodic to the potential of zero charge (PZC) [23]. Since the detector was always operated at potentials anodic to the PZC (i.e., positive charge on the mercury surface side of the double layer), electrostatic repulsion inhibited the rate of adsorbate approach [35] and decreased the limiting coverage [23]. Slow adsorption of HTAB (limited by mass transfer) alters the double layer and results in current flow in this dynamic capacitor. This prolongation of charging current is responsible for the increase in baseline noise seen with decreasing drop time. Drop-to-drop decay variations cause fluctuations in the current sample taken at the end of each drop. Continuous amperometric monitoring of a hanging mercury drop eliminates drop-variable capacitive noise. An additional contribution to noise at a 0.5-sec DME may be a failure to re-establish drop-sheathing laminar flow after mechanical drop dislodgement [31].

While long drop times reduce noise they also degrade peak representation fidelity. Drop times greater than 1 sec result in signal sampling which is too infrequent for precise peak height measurement (stepped signal). Continuous detection at a hanging drop, in addition to reducing capacitive noise, improves the signal quality by eliminating discrete data sampling entirely.

Sparge gas

Removing oxygen from the mobile phase and preventing its redissolution was most conveniently accomplished by continuous sparging with an inert gas [36]. The mole fraction solubilities (in water at 25°C) of helium, nitrogen and argon are: $0.7 \cdot 10^{-5}$, $1.4 \cdot 10^{-5}$ and $2.5 \cdot 10^{-5}$, respectively [36]. A helium atmosphere, therefore, leaves less dissolved gas in the mobile phase than either nitrogen or argon and was used throughout. Helium lowers the high-frequency noise, seen when the polarographic detector cell is heated. Nitrogen and argon

sparging cause significant bubble formation in the heated cell. Growing on the reference electrode frit, these bubbles produce signal noise apparently by destabilizing the liquid junction. Small bubbles formed at the heated, atmospheric pressure outlet of the column, impinge on the electrode surface causing local flow disturbances and noise.

Effect of cell temperature

Fortuitous, but incompletely understood, is the observation of a change in detector response with an increase in cell temperature (Fig. 6). Two not completely separate components to this change are seen: an increase in current and an anodic shift of the potential of maximum response. Miner [37] has reported HPLC detector current–temperature coefficients of 0.5–5.0%/°C for organic eluites detected amperometrically at a glassy carbon anode. Although the calculation of a single coefficient is inappropriate in the present work, due to the shift in the hydrodynamic polarogram, the large increase in current is consistent with the non-diffusion controlled DDP electrode reaction [37].

The observed shift in peak potential in flowing stream was not expected. The reference electrode contribution is insignificant; the Ag/AgCl reference electrode is relatively temperature insensitive. From 25 to 50°C its potential

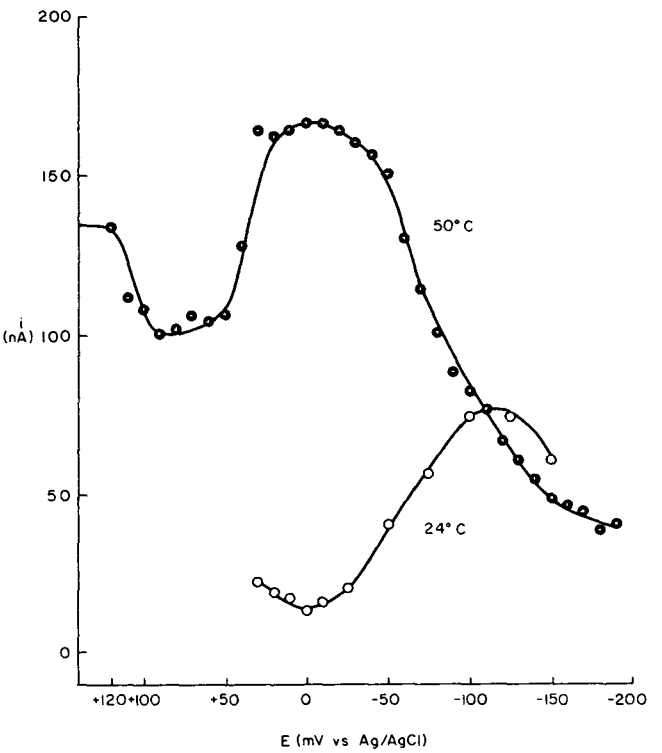


Fig. 6. Effect of cell temperature on the hydrodynamic polarogram. Column as in Fig. 2; sample: 50 μl of 10.5 μg DDP per ml; mobile phase as in Fig. 3; flow-rate: 2 ml/min; column temperature: 50°C; detector: 1.0 sec DME, 0.026 cm^2 drop.

changes only 18 mV, shifting cathodically relative to the hydrogen electrode [38]. The specific adsorption of DDP to the working electrode and its reduction to platinum metal with liberation of all ligands might suggest an entropic explanation [39] for the potential shift. However, the absence of this effect in quiet solution eliminates this simple explanation. The shift in the chromatographically determined DDP polarogram may be kinetic in nature, resulting from altered relative rates of HTAB and DDP adsorption producing a potential-dependent change in the reactive electrode surface area. Alternatively, it may be due to a catalytic component of the measured current (chemical oxidation of mercury by DDP [40]) increasing with temperature.

The temperature-induced detector response changes have great practical significance. The combined cell temperature and potential changes (−120 mV at 24°C to 0 mV at 50°C) increase the DDP response by a factor of 2.1 while increasing the baseline noise by a factor of only 1.1, thereby yielding significant enhancement in DDP detectability.

In addition to the signal-to-noise ratio improvement due to the current increase, there is an improvement in detector selectivity for DDP brought about by the anodic potential shift. Changing the cell potential from −120 mV to 0 mV (Ag/AgCl) virtually eliminates the detector response to oxygen, since 0 mV is anodic to the foot of the first oxygen wave, $E_{1/2} = -50$ mV (Ag/AgCl). The baseline disturbance caused by the injection of chloride is diminished by a factor of 10 by the potential change from −120 to 0 mV. Many of the interferences in urine chromatograms arising from material reduced by the detector are also decreased by the anodic potential shift. Detector selectivity cannot, however, be increased indefinitely by anodic adjustments. Urine contains many easily oxidizable substances (e.g., catecholamines and ascorbic acid) [25] and such materials will generate increasingly intense negative (oxidation) peaks as the potential is made more positive. In addition thiols present in urine (e.g., glutathione and cysteine) [25] will promote the oxidation of the mercury electrode itself at 0 mV (Ag/AgCl) [41], also producing negative peaks.

Determination of DDP in urine

DDP is not extractable from aqueous media and the physical properties of urine (particularly its lack of macromolecular solutes) make this medium more suitable for direct HPLC analysis than other biological fluids. Absorbance detection at 301 nm allows the quantitation of DDP in urine only at levels $\geqslant 100$ μg/ml [6].

The described method has been successfully used to determine DDP in urine samples collected during DDP therapy (Figs. 7 and 8). Correlation with a previously applied clinical method [6] was high and is described by the equation ElCD = 0.95 × NFAA +0.31, $r = 0.998$ (Table I). DDP can be quantitated in urine at levels below 100 ng/ml (Fig. 8) with an interassay precision of ±4%. Replicate injections of individual urine samples resulted in determination of cisplatin with precision of ±2%. Polarographic detector selectivity for DDP obviates the need for dissimilar-phase column switching which was previously used [6] to provide chromatographic selectivity.

The actual minimum detectable quantity is dependent on sample composition and is greater than the detection limit imposed by the system itself (Table

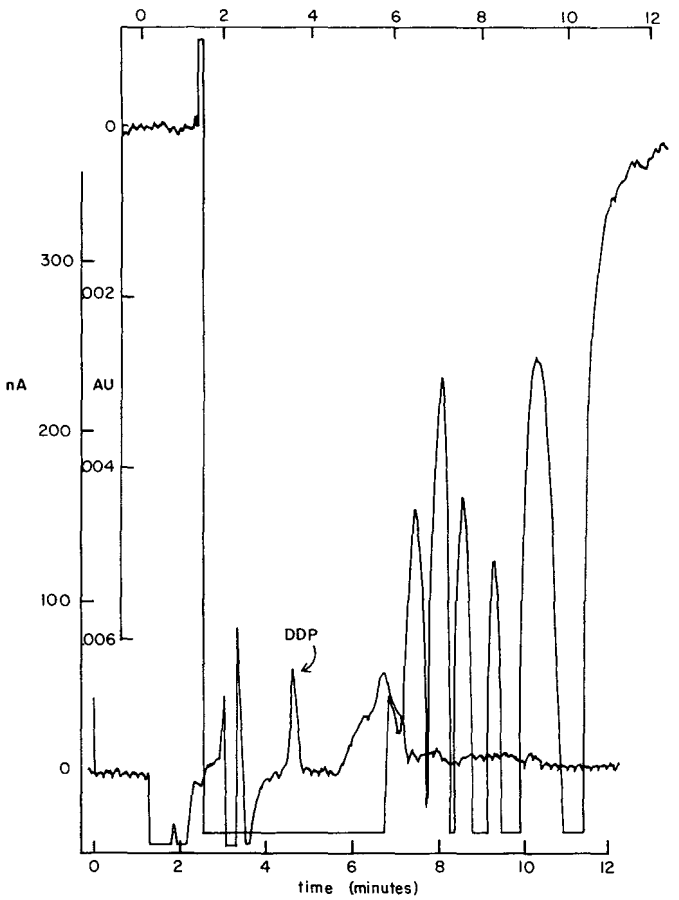


Fig. 7. ElCD and UV chromatograms of patient urine taken 4 min after DDP administration (patient 1). Column as in Fig. 2; sample: 50 μl urine estimated to contain 1.9 μg DDP per ml; mobile phase as in Fig. 3; flow-rate: 1.0 ml/min; column temperature: 60°C; detector (ElCD): 1.0 sec DME, 0.026 cm^2 drop, 0.0 V (Ag/AgCl); cell temperature: 60°C; detector (UV): 301 nm absorbance; HPLC pump: reciprocating piston.

II). Eluites, which are either reduced or oxidized, produce positive or negative recorder peaks, respectively, in the vicinity of DDP and cause the minimum quantity detectable in urine samples to be greater than the 1.4 ng/ml (50 μl injection) best-case detection limit. Thus, in Fig. 8 it can be seen that noise is insignificant relative to DDP peak height, but that the preceeding oxidation peaks would compromise DDP quantitation at much lower levels. It is not reasonable to state a detection limit in urine as a characteristic of system performance because of the variability of interferences. This variability applies to urine samples from a single patient during the course of therapy. Initial samples are clear and nearly colorless as the kidneys remove the fluid of pre-therapy hydration. Urine color and sediment increases within 2 h as the rate of urine production decreases.

Detector potential adjustments have been used to maximize response selectivity, compensating for increased chromatogram complexity in later sam-

ples (i.e., those taken 6 h after dosing). Fig. 8 was recorded at −40 mV (Ag/AgCl) to minimize interfering oxidation peaks. The change in detector sensitivity that accompanies the reinjection of the sample at a different potential is corrected by interpolated two-point recalibration, above and below the apparent sample concentration.

TABLE I

DETERMINATION OF CISPLATIN AND TOTAL PLATINUM IN URINE OF PATIENTS DURING THERAPY

150 mg cisplatin per m^2 body surface, 1 mg/ml in 0.5% dextrose—half-normal saline, intravenous injection over 15 min.

Time*	Urine volume (ml)	Cisplatin** (μg/ml)	Cisplatin*** (μg/ml)	Total platinum § (μg platinum per ml)
Patient 1				
0 h 0 min	525	44.2	45.6	32.7
0 h 30 min	200	44.6	46.9	31.2
2 h 0 min	15	15.1	12.6	13.5
4 h 0 min	30	1.9	2.6	1.1
8 h 0 min	50	1.5	1.5	6.6
25 h 30 min	125	1.5	1.5	2.8
Patient 2				
§§	30	0	—	0.16
1 h 40 min	375	69.6	—	43.3
3 h 25 min	500	7.9	—	5.81
5 h 5 min	250	1.2	—	2.02
7 h 45 min	500	0.09	—	1.72

*Time after end of drug administration.
**LC-ElCD. Patient 1: first course of DDP; conditions as in Fig. 7; patient 2: second course of DDP; conditions as in Fig. 8.
***LC-NFAA (ref. 6).
§ Direct NFAA total platinum (i.e., platinum concentration irrespective of ligands coordinated to the metal or platinum valency).
§§ Sample before dose.

TABLE II

SUMMARY OF DETECTOR CHARACTERISTICS

Values determined with previously unused, equilibrated column.

	Sensitivity*	Noise**	Detection limit*** (ng)
0.5 sec DME	1.23 nA/ng	2 nA	3.2
1.0 sec DME	1.12 nA/ng	1 nA	1.8
HMDE	1.10 nA/ng	0.04 nA	0.07
301 nm absorbance	$5 \cdot 10^{-6}$ a.u./ng	$2 \cdot 10^{-4}$ a.u.	80

*Slope of peak height vs. mass of DDP injected. Chromatographic conditions as in Fig. 8; detector cell temperature: 60°C; 0.026 cm^2 drop; 0.0 V (Ag/AgCl).
**Peak-to-peak with 1-sec (ElCD) and 2-sec (UV) low-pass filter.
***Mass injected giving signal which is twice noise.

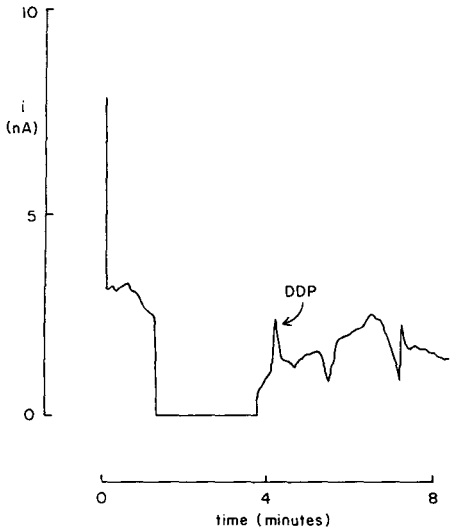


Fig. 8. Chromatogram of patient urine taken 7 h 45 min after DDP administration (patient 2). Column as in Fig. 2; sample: 50 μl urine estimated to contain 91 ng DDP per ml; mobile phase as in Fig. 3; flow-rate: 1 ml/min; column temperature: 60°C; detector: HMDE; 0.026 cm^2 drop, −40 mV (Ag/AgCl), cell temperature: 62°C; 1 sec low-pass filter; HPLC pump: syringe.

Application to the determination of other complexes

A variety of platinum complexes are currently being evaluated for antineoplastic activity and clinical utility [42]. Several have been successfully chromatographed [6] using the SGAX system with UV detection. CBDCA and CHIP (Fig. 1) are both eluted similarly to DDP. CHIP, a tetravalent platinum complex, can be detected polarographically with a sensitivity comparable to that of DDP (Fig. 9). However, no polarographic response to CBDCA (2 m*M*) was seen even in quiet solutions at potentials more cathodic than 1.0 V vs. Ag/AgCl.

The polarography of platinum complexes is highly dependent on the nature of the ligands [28, 29]. Half-wave potentials and electrode mechanisms are similarly changed by ligand substitutions. Detector selectivity will decrease with increasing cathodic potentials necessary for the detection of the less reducible complexes, and sensitivity to different complexes will change with electrode reaction mechanism.

Operating considerations

Operated as either a DME or HMDE, the detector produces a linear peak-height response to injected DDP. These calibration slopes, as well as noise and calculated interference-free detection limits are shown in Table II. Performance parameters determined for DDP detection by UV absorbance at 301 nm are also given for comparison. The sensitivities, and therefore the detection limits, are determined from recorded peak heights and so are a function of the chromatographic system. Because column condition and temperature, mobile phase composition and flow-rate will all affect peak height, these data should not be considered as absolute detector parameters. They are, however, an accurate

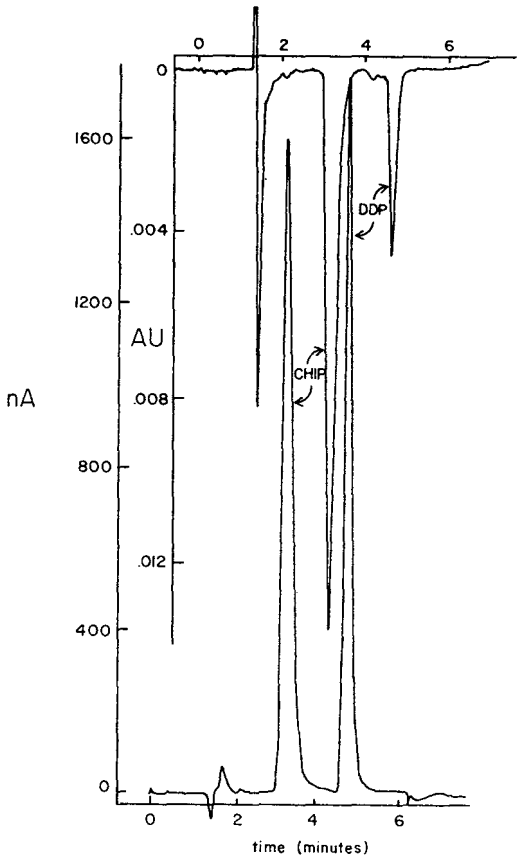


Fig. 9. ElCD and UV chromatograms of CHIP and DDP. Column as in Fig. 2; sample: 6.2 μg CHIP and 4.2 μg DDP in 50 μl; mobile phase as in Fig. 3; flow-rate: 1 ml/min; column temperature: 50°C; detector (ElCD) 1.0 sec DME, 0.026 cm^2 drop, 0.0 V (Ag/AgCl); cell temperature: 50°C; detector (UV): 301 nm absorbance.

comparison of the detectors under the conditions described. Since the sensitivities of the electrochemical detection modes are similar, noise determines the detection limits. The detection limit for the HMDE, with a 50-μl injection is 1.4 ng DDP per ml, comparable to direct total platinum determinations by NFAA in the absence of interferences.

While detection at a HMDE is useful for the determination of trace levels of DDP it is also slightly less convenient than a DME. Twenty to 24 min after initial drop formation, detector response is abruptly lost but is reproducibly restored with a new drop. In addition to hanging a fresh drop with each injection, attention must be given to unexplained but reproducible + 0.4 nA/min drift. At current ranges of 10 nA full scale and less, this drift is significant. The constantly regenerated DME operates at higher platinum levels with less user intervention.

CONCLUSIONS

Electrochemical detection provides the necessary sensitivity and selectivity for chromatographic monitoring of DDP in urine for at least 8 h after drug administration. In the absence of interferences its limit of detection is 1000-fold lower than that of UV absorbance. In addition, its selectivity allows the quantitation of DDP in untreated urine. The renewable mercury drop electrode is useful in the described system because of (a) requisite analyte adsorption prior to reduction, complicated by competing adsorption of mobile-phase surfactant, and (b) accumulation of the reduction product on the electrode. Such cumulative surface effects degrade signal quality and are difficult to overcome with solid electrodes.

Detector selectivity for DDP is improved by the anodic shift in potential of maximum response with increasing temperature. It is not, however, a platinum-specific detector, so that its application to the determination of other platinum complexes formed in vivo after DDP administration must be preceded by identification and electrochemical characterization of these species.

ACKNOWLEDGEMENTS

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CHROMBIO. 1551

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ANALYSIS OF A SYNTHETIC COPOLYMER WITH ANTITUMOR ACTIVITY (COPOVITHANE) AND METHYLAMINE IN HUMAN BLOOD PLASMA AND URINE*

W. WINGENDER

Institute for Pharmacokinetics, Bayer AG, D-5600 Wuppertal 1 (G.F.R.)

(First received May 4th, 1982; revised manuscript received October 20th, 1982)

SUMMARY

A method for the determination of a synthetic polymeric compound with antitumor activity (copovithane) and methylamine in blood plasma and urine is described. Copovithane is prepared by radical polymerisation of a diurethane with N-vinylpyrrolidone.

The method is based on high-performance liquid chromatography of the methylamine hydrochloride which arises during the hydrochloric acid hydrolysis of the parent substance. The methylamine hydrochloride is converted to the trinitrobenzenesulphonyl derivative for the purpose of chromatographic detection.

The limit of determination for copovithane in blood plasma is 1.2 mg/l and in urine 1.5 mg/day. The determination limit for methylamine in blood plasma is 0.2 mg/l and in urine 0.3 mg/day. The imprecision is dependent on the sample, and amounts to ± 6.8% for blood plasma and ± 6.4% for urine.

INTRODUCTION

A variety of synthetic polymeric compounds with antitumor activity have been discovered. The majority of them have toxic side-effects. Recently, a group of water-soluble homo- and copolymers which contain 2-methylene-1,3-propanediol as the essential chemical unit have been found [1]. These copolymers significantly inhibit the growth of experimental murine tumors in a wide therapeutic dose range.

The compounds are prepared by radical polymerisation of diurethanes or diesters of 2-methylene-1,3-propanediol alone, or with copolymerisable ionic or non-ionic monomers such as maleic acid, acrylic acid, acrylamide, and N-vinylpyrrolidone.

*Dedicated "in memoriam" to Prof. Dr. Otto Bayer.

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Comparative investigations revealed that a non-ionic copolymer consisting of 30% by weight of 2-methylene-1,3-propanediyl-bis(methylcarbamate) (Fig. 1, I) and 70% by weight of N-vinylpyrrolidone (Fig, 1, II) was prominent by its excellent tolerability.

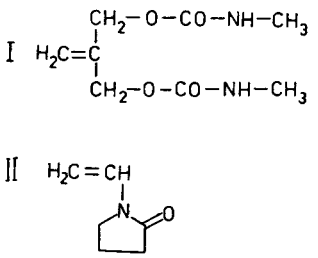


Fig. 1. Chemical structures of 2-methylene-1,3-propanediyl-bis(methylcarbamate) (I), and N-vinylpyrrolidone (II).

The copolymer with an average molecular mass of about 5000—6000 was selected for detailed investigations.

The assay method presented here is based on high-performance liquid chromatography (HPLC) of the methylamine which is generated by hydrolysis and on its detection by conversion to the trinitrobenzenesulphonyl derivative.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1084 B liquid chromatograph was used, equipped with a HP 79875 UV detector. Ready-to-use columns (250 × 4 mm I.D., Hibar; E. Merck, Darmstadt, G.F.R.) filled with LiChrosorb RP-18 (10 μm) were employed. UV monitoring was performed at 340 nm.

Reagents

All reagents used were of guaranteed reagent grade (Merck), and were used without further purification.

Copovithane (BAY i 7433) standards were obtained by preparative gel permeation chromatography on Fractogel PVA 20,000 (E. Merck) with methanol as the eluent*. Determination by membrane osmosis of the average molecular weight proved that the preparation has a narrow distribution of its molecular weight and that the preparation contains less than 0.5% by weight of molecules with a molecular weight greater than 25,000.

Methylamine hydrochloride standards were purchased from EGA-Chemie (Steinheim, G.F.R.).

Method

The method consists of four principal steps: protein precipitation, acid hydrolysis, derivatisation and HPLC analysis. To blood plasma samples (2 ml each) are added drop-wise 8 ml of acetone over a period of 2 min while under

*Copovithane standards were prepared by Dr. B. Boemer, Bayer AG, Leverkusen, G.F.R.

vigorous homogenisation with an Ultra-Turrax in order to precipitate the proteins. After centrifugation (2000 *g*, 5 min) the supernatant is then shaken for 15 min with 12 ml of dichloroethane to remove the acetone, and then centrifuged again. The aqueous phase (1 ml) is diluted with 1 ml of water and, by means of a 2-ml disposable syringe, layered onto a Sep-Pak C_{18} cartridge (Waters No. 51910; Waters Assoc., Milford, MA, U.S.A.) which has been pre-conditioned by washing with 20 ml of water and 20 ml of methanol. A further 2 ml of water are added to the column and then it is eluted with 10 ml of 80% methanol. The eluate is reduced to dryness at 70°C under a stream of nitrogen. The residue is taken up in 1 ml of 5 *N* hydrochloric acid and hydrolysed for 16 h at 160°C. The hydrolysate is cooled in an ice-bath and adjusted to pH 8.00 ± 0.01 with 2 ml of standard buffer pH 8.00 (E. Merck) and 0.5 ml of 10 *N* sodium hydroxide. The hydrolysate is then derivatized by the addition of 0.75 ml of 0.5% of 2,4,6-trinitrobenzenesulphonic acid solution (TNBS). The reaction is allowed to proceed for 2.5 h at room temperature in complete darkness and then the reaction product is extracted by shaking (15 min) with 6 ml of toluene. The phases are allowed to separate (2 min) and 3 ml of the toluene phase are reduced to dryness by heating at 50°C under a stream of nitrogen. The residue is taken up in 250 µl of the mobile phase acetonitrile–water (45:55) and transferred to a micro sample vessel.

The analysis of the urine samples follows the same procedure. The protein precipitation step is not required.

Chromatographic conditions

The chromatographic system consists of a mobile phase of acetonitrile–water (45:55) percolating at a flow-rate of 2 ml/min through a ready-to-use column packed with LiChrosorb RP-18 (10 µm). The detector wavelength was set at 340 nm, which corresponded to the largest difference between the methylamine-TNBS derivative and mobile phase absorptions. The column temperature was maintained at 35°C. In all analytical studies constant volumes of 50 µl were injected onto the column at each operation.

RESULTS

Concentrations of copovithane in biological material

Copovithane (BAY i 7433) is poorly soluble in organic solvents that are immiscible with water. This excludes the possibility of direct extraction of the substance from blood plasma and urine.

In order to concentrate the substance present in the blood into a smaller volume, the plasma proteins are precipitated with acetone. The acetone is then removed by shaking with dichloroethane and the aqueous phase is used for further analysis.

Quantitative analysis of copovithane in urine does not require the protein precipitation step.

Removal of interfering substance

The methylamine that arises from the hydrochloric acid hydrolysis of control plasma or control urine is mostly derived from creatine and creatinine.

A considerable reduction in the blank value is achieved by passing the sample over a conditioned Sep-Pak C_{18} cartridge. This results in a reduction of more than 90% in the amount of methylamine formed by hydrolysis.

Optimal hydrolysis conditions

On average a 100% yield of methylamine hydrochloride is achieved by a 16-h hydrolysis of copovithane in 5 *N* hydrochloric acid at 160° C.

Sham hydrolysis, carried out with methylamine hydrochloride under the above-mentioned conditions, did not cause any reduction in yield.

Derivatisation with 2,4,6-trinitrobenzenesulphonic acid

Methylamine hydrochloride does not display a characteristic absorption spectrum in the UV range, so it is necessary to introduce a chromophore for detection purposes.

2,4,6-Trinitrobenzenesulphonic acid was found to be optimal for the derivatisation of methylamine hydrochloride. Maximal formation of the derivative occurred during a period of 2.5 h at pH 8.0 at room temperature in the absence of light. The methylamine-TNBS derivative can easily be separated from excess TNBS by extraction with toluene.

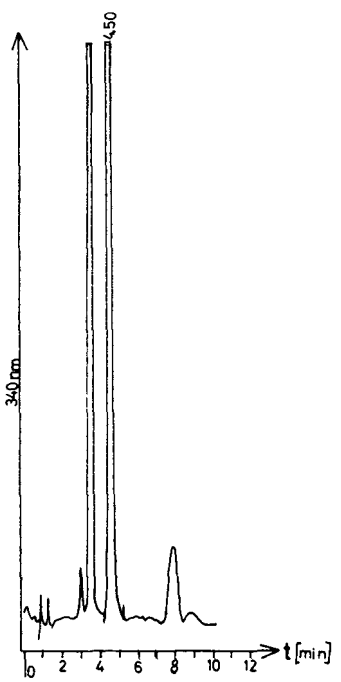
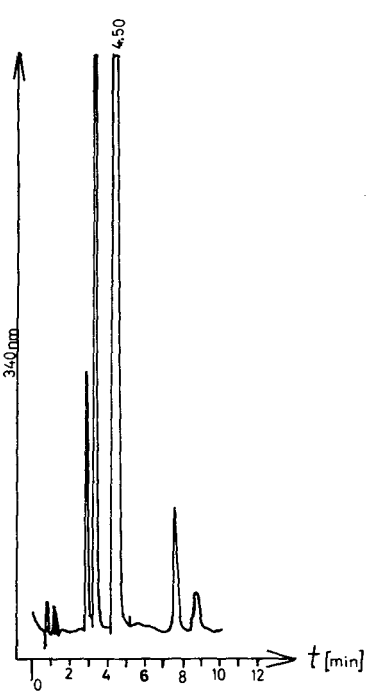


Fig. 2. Chromatogram of methylamine-TNBS derivative standard, monitored with a UV detector (340 nm). RP-18 column; mobile phase acetonitrile—water (45:55); retention time 4.50 ± 0.01 min.

High-performance liquid chromatography

The methylamine-TNBS derivative is chromatographed on an RP-18 column using the solvent system acetonitrile—water (45:55). The retention time is 4.50 ± 0.01 min. A typical chromatogram is shown in Fig. 2. Chromatograms of samples collected from blood plasma and urine are demonstrated in Figs. 3 and 4, respectively.



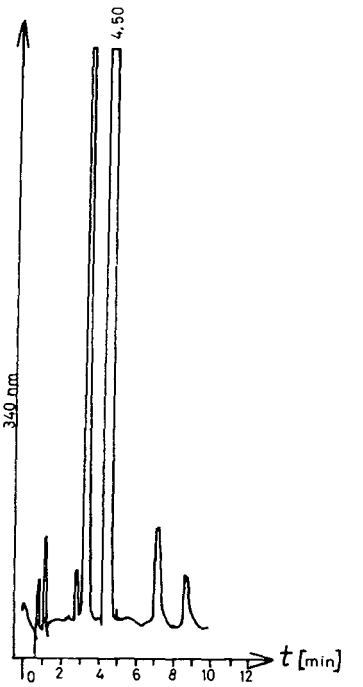


Fig. 3. Chromatogram of methylamine-TNBS derivative after HCl hydrolysis of copovithane in blood plasma; chromatographic conditions as in Fig. 2.

Fig. 4. Chromatogram of methylamine-TNBS derivative after HCl hydrolysis of copovithane in urine; chromatographic conditions as in Fig. 2.

Standard curve

The peak areas derived from HPLC of the methylamine-TNBS derivative were proportional to its concentration within the range 10 ng/ml to 100 μg/ml.

Reagent blank

The average reagent blank has a peak area associated with the retention time of 4.50 min, which corresponds to an apparent copovithane concentration of 0.8 ± 0.06 μg/ml of blood plasma and 1.47 ± 0.49 μg/ml of urine.

The corresponding values for methylamine hydrochloride are 0.2 ± 0.015 μg/ml of blood plasma and 0.47 ± 0.16 μg/ml of urine.

Biological blank

Samples of blood plasma from nineteen untreated volunteers were processed as described in the Method section. The analysis resulted in a mean apparent biological content of copovithane (after subtraction of the reagent blank) of 0.3 ± 0.53 μg/ml of blood plasma.

Processing the urine collected over a period of 24 h from ten untreated volunteers resulted in a mean biological blank value of 1.7 ± 0.65 mg/day.

The biological blank for methylamine was 0.05 ± 0.09 μg/ml of blood plasma and 0.3 ± 0.115 mg/day for urine.

Limit of determination

The limit of determination is calculated from the standard deviation of the biological blank values with 95% confidence limits. The resulting values for copovithane are: 1.2 μg/ml of blood plasma and 1.5 mg/day for urine; and for methylamine, 0.2 μg/ml of blood plasma and 0.3 mg/day for urine.

Yield

The yield of the procedure was determined by adding 10, 25, 50 and 100 μg of copovithane to each millilitre of ten blood plasma samples obtained from ten untreated volunteers and then processing the samples as described in the Method section. The mean overall yield was found to be 64.6 ± 4.5%. The correction factor for the yield is therefore 1.55.

The yield in urine was determined by adding various amounts of copovithane to each of ten urine mixtures. The final concentrations of copovithane were set at 10, 25, 50 and 100 μg/ml, and then all samples were processed as described in the Method section. The mean yield obtained from these assays was 55.9 ± 3.5%. The yield correction factor for urine is therefore 1.78.

Imprecision

The imprecision of the determination is a correlate of the relative standard deviation of the yield. This amounts to ± 6.8% for the blood plasma determination and ± 6.4% for the urine determination.

Standardisation

Each analysis series is quantified by chromatography of three external standards (methylamine-TNBS derivatives). A standard curve is then constructed by means of linear regression analysis of the peak areas of these standards.

Calculation

$$\frac{(A_T - A_B) \cdot S \cdot 5.621 \cdot f}{A_S} = \mu g \text{ copovithane per ml of plasma or urine}$$

where A_T = area test sample, A_B = area blank, A_S = area standard, S = methylamine hydrochloride standard, 5.621 = conversion factor and f = yield correction factor.

DISCUSSION

The instrumental chromatographic analysis of polymeric compounds did not develop with the same speed as that of low-molecular-weight compounds. High-performance size-exclusion chromatography offers the versatility of providing information on both low- and high-molecular-weight components of polymers. However, this technique is not suitable to detect trace quantities. For this purpose chemical cleavage or degradation of various individual polymers has been described [2, 3]. The polymer reactants produced by cleavage frequently contain highly polar groups, i.e. carboxyl, hydroxyl, and amino groups, which exhibit poor chromatographic behaviour. Therefore, simple derivatisation must be carried out before chromatographic analysis.

Chemical cleavage has been applied to linear polyurethane materials using acidic or alkaline hydrolysis or by aminolysis [4]. It frequently requires prolonged reaction periods and becomes less satisfactory as the complexity or cross-linking is increased.

The amines generated after hydrolysis of polyurethanes cause special analytical problems because of the similar chemical nature of these compounds. The most sensitive techniques to analyze amines are spectrofluorimetry and gas chromatography. The use of HPLC for the analysis of trace quantities of amines becomes more important since amine derivatives can be detected by UV absorption. Wellons and Carey [5] described an HPLC method for amine analysis by forming the *m*-toluoyl derivatives. The method was modified by Chen and Farquharson [6]. The analytical method described in this paper proved also to be a valid procedure for determining trace amounts of methylamine in biological fluids.

The determination of copovithane in blood plasma and urine is a relatively time-consuming method, because the substance cannot be extracted from blood plasma and urine by means of organic solvents and because the acid hydrolysis takes 16 h.

Due to the chromatographic background signal of methylamine, which generates creatinine and creatine during sample work-up by acid hydrolysis, also in untreated volunteers, the method presented is not specific for copovithane.

Nevertheless, the imprecision of measurement of copovithane in cancer patients during patient monitoring will not be further complicated because the creatinine and creatine levels in blood plasma and urine do not vary significantly between cancer patients and healthy persons [7—9].

The relative coefficient of variation of this method does not exceed the normal physiological variation of creatinine content in blood plasma and urine [10].

Furthermore, the assay method possibly is not specific for unchanged copovithane since potential metabolites that yield methylamine hydrochloride on hydrolysis with hydrochloric acid will also be detected.

The sensitivity of the method is good. Preliminary studies have shown that the maximal blood plasma concentration following intravenous administration of 10 mg of copovithane per kg body mass (probably the lowest therapeutic

dose) reached values of ca. 100 mg/l. These values are approximately 100 times greater than the limit of detection. The blood plasma level of copovithane could be easily followed over six half-lives.

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CHROMBIO. 1565

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE NEW HYPOXIC CELL RADIOSENSITISER, Ro 03-8799, IN BIOLOGICAL SAMPLES

S.L. MALCOLM*, A. LEE and J.K. GROVES

Roche Products Ltd., Broadwater Road, Welwyn Garden City, Hertfordshire, AL7 3AY (Great Britain)

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SUMMARY

A high-performance liquid chromatographic method of analysis with UV detection has been developed to measure levels of a new radiosensitiser, Ro 03-8799 and its N-oxide metabolite, in biological fluids and tissues.

The accuracy and precision of the method have been determined in both plasma and urine, where the limits of quantitation are 100 and 500 ng/ml, respectively. Typical results are presented from a human volunteer study where samples were analysed by this method.

Important aspects of the method, involving both sample handling techniques and chromatographic conditions are discussed.

INTRODUCTION

Ro 03-8799, 2-nitro-α-(piperidino-methyl)-1-imidazole ethanol, is one of a series of 2-nitroimidazoles which are hypoxic cell radiosensitisers. These are compounds which enhance the effects of ionising radiation during radiotherapy of various tumours. During radiation the hypoxic cells in tumours are resistant to X-rays [1, 2] and only the oxic cells are killed. Nitroimidazoles form radical anions which render the hypoxic cells susceptible to the radiation [3, 4].

Because large doses are needed to achieve sufficiently high tumour concentrations, the effectiveness of radiosensitisers is limited by their toxicity. Misonidazole is one of the more effective radiosensitisers, and has been used in clinical trials for a number of years, but the doses that can be administered are limited by peripheral neuropathy [5, 6]. Ro 03-8799 is currently being evaluated in the clinic as a more potent and less toxic successor to misonidazole.

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A rapid and sensitive method to measure Ro 03-8799 (Fig. 1) in biological samples has therefore been developed for use in these trials. Since metabolic studies have shown (unpublished work) that the N-oxide (Ro 31-0313) is formed as a metabolite in six species studied, including man, we have developed a method which is capable of measuring both unchanged drug and N-oxide. Also, because the effectiveness of radiosensitisers depends on their penetration into tissues and therefore tumours, it was felt that the analytical method should be capable of quantifying these compounds in tissues and tumours as well as biological fluids.

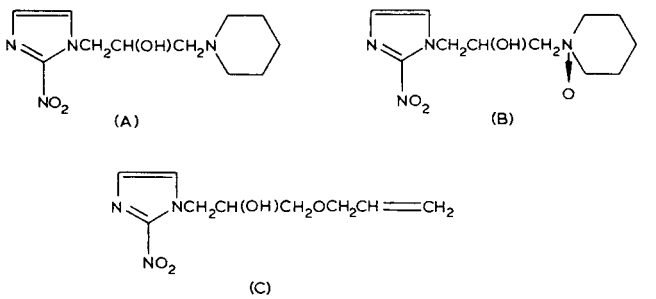

Fig. 1. Structures of Ro 03-8799 (A), Ro 31-0313 (B) and Ro 07-1902 (C).

MATERIALS AND METHODS

Chromatography

The samples are injected onto a Waters μBondapak ODS (10% coating) silica column (25 × 0.4 cm I.D., particle size 10 μm) by a Waters sample processor (WISP). The column is connected to an LDC Spectromonitor III, the wavelength being set at 326 nm. The UV absorbance is recorded by a Hewlett-Packard 3385 integrator and peak areas are used for quantitation. The eluent is 15% acetonitrile in glycine—hydrochloric acid buffer, pH 2.6, containing 3 m*M* heptanesulphonic acid. The flow-rate is kept constant at 1.3 ml/min using a Constametric Model III pump.

Biological samples

Plasma and erythrocytes. Blood samples are transferred into heparinised tubes, plasma and red cells separated by centrifugation and each stored frozen until analysed. Plasma (200 μl) and red cells (ca. 200 mg) are added to centrifuge tubes containing an appropriate amount of internal standard (Ro 07-1902, see Fig. 1) in aqueous solution, then acetonitrile (2 ml) is added to precipitate the proteins. After centrifuging, the supernatant is transferred to a clean tube and blown to dryness under a gentle stream of nitrogen. The residue is taken up in eluent (100 μl) and an aliquot injected (50 μl) onto the high-performance liquid chromatographic (HPLC) column.

Bile and urine. After collection, samples are stored frozen until analysed. Urine (500 μl) and bile (100 μl) are added to centrifuge tubes containing an appropriate amount of internal standard solution (10—50 μl), diluted with water to 600 μl (urine) or 200 μl (bile), centrifuged and injected (10—50 μl) onto the HPLC column.

Tumours and tissues. At the time of collection, all samples should be immersed in methanol, cooled in an acetone—dry ice mixture (ca. —75°C) and stored at this temperature until analysed. In dealing with small rodents, samples are collected by opening up the peritoneal cavity of anaesthetised animals (diethyl ether), freezing the whole animal in liquid nitrogen, then removing the required tissues/organs and storing them in cold methanol as above. For analysis the samples are thawed in ice, water is added to make the total volume equal to five times the weight of tissue, and then they are homogenised using a Silverson mixer-emulsifier. The homogenate (200 μl) is added to a centrifuge tube containing an appropriate amount of internal solution (10—50 μl) and the sample made up to 300 μl with water. It is spun at > 30,000 *g* for 60 min and an aliquot of supernatant (50 μl) is injected onto the HPLC column. Some tumour samples are particularly hard and resistant to normal homogenisation. In these cases we have used a Spex freezer mill, which crushes the tissue at the temperature of liquid nitrogen. Methanol is then added and the sample processed as described above.

Quantitation

For each analysis, a series of known amounts of Ro 03-8799 and Ro 31-0313 are added to a blank plasma, urine, etc., and worked up at the same time and in the same way as the samples. Quantitation of unknowns is by interpolation from the standard curve of the peak area ratio (compound/internal standard) vs. the concentration of the standards.

RESULTS

Choice of method

Both drug and N-oxide metabolite are 1-substituted 2-nitroimidazoles and have intrinsic UV absorbance at 326 nm. The extinction coefficients (Fig. 2) of Ro 03-8799 and Ro 31-0313 are sufficiently high to be able to detect 1 ng of pure compound applied to the column, using the system described.

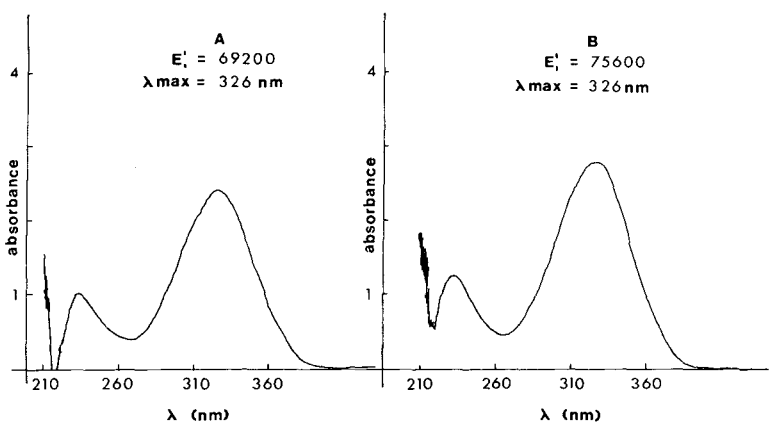

Fig. 2. UV absorbance of (A) Ro 03-8799, $E_1' = 69200$, $\lambda_{max} = 326$ nm and (B) Ro 31-0313, $E_1' = 75600$, $\lambda_{max} = 326$ nm, measured in eluent.

Ro 03-8799 is a lipophilic compound [7] (its octanol—water partition coefficient at pH 10.5 is 8.5) and is easily extracted from biological material at basic pH. This is not the case for Ro 31-0313 which, being an N-oxide, is a charged molecule. Therefore sample clean-up is restricted to removing the proteins. A reversed-phase HPLC column is used, because endogenous polar material remaining in the samples is eluted first, and therefore does not reduce column efficiency.

Both compounds have basic centres; the pK_a values of Ro 03-8799 and Ro 31-0313 are 8.7 and 4.7 [8], respectively. At eluent pH greater than 5 the peak shape of Ro 31-0313 is poor. Therefore we use a low pH with an ion-pair reagent (heptanesulphonic acid) to elute both basic compounds.

Plasma and red cells

Typical traces are shown in Fig. 3. To establish the accuracy and precision of the method, standard curves of parent drug and N-oxide covering the range 0.05—50 μg/ml were made up on four different occasions in blank human plasma. Results of these analyses showed that over the concentration range 0.1—50 μg/ml the precision of the method (mean ± S.D. of the calculated results) is < ± 5% and that the accuracy (comparison of the theoretical with the calculated concentration ± S.D.) is 100.2 ± 3%. Below 0.1 μg/ml these figures fall below acceptable limits. However, for this compound and its metabolite, therapeutic doses give concentrations well above this level.

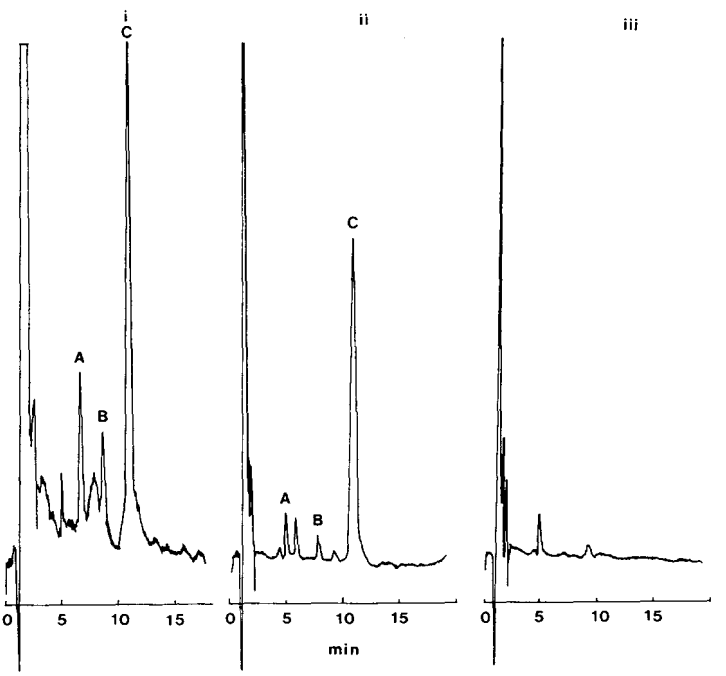

Fig. 3. Typical chromatograms from (i) volunteer patient 8-h plasma containing ca. 100 ng/ml Ro 03-8799 (A) and Ro 31-0313 (B) and internal standard (C), (ii) human plasma standard of 100 ng/ml A and B, with internal standard, C, and (iii) blank human plasma.

Using this method we have successfully analysed plasma samples from several species including man. A human volunteer study, in conjunction with Mount Vernon Hospital [9] was carried out, where volunteer patients were given intravenous infusions of [2-^{14}C]Ro 03-8799. Plasma, red cells, urine and tumour biopsies were all analysed for Ro 03-8799 and Ro 31-0313. Fig. 4 shows a typical concentration versus time plot of plasma and red cells. Tumour biopsies taken at the same time as three of the plasma samples all showed high levels of Ro 03-8799 (Table I). Although the biopsies were taken from the same tumour site, they were not identical samples [9] which could account for the variable tumour to plasma ratio.

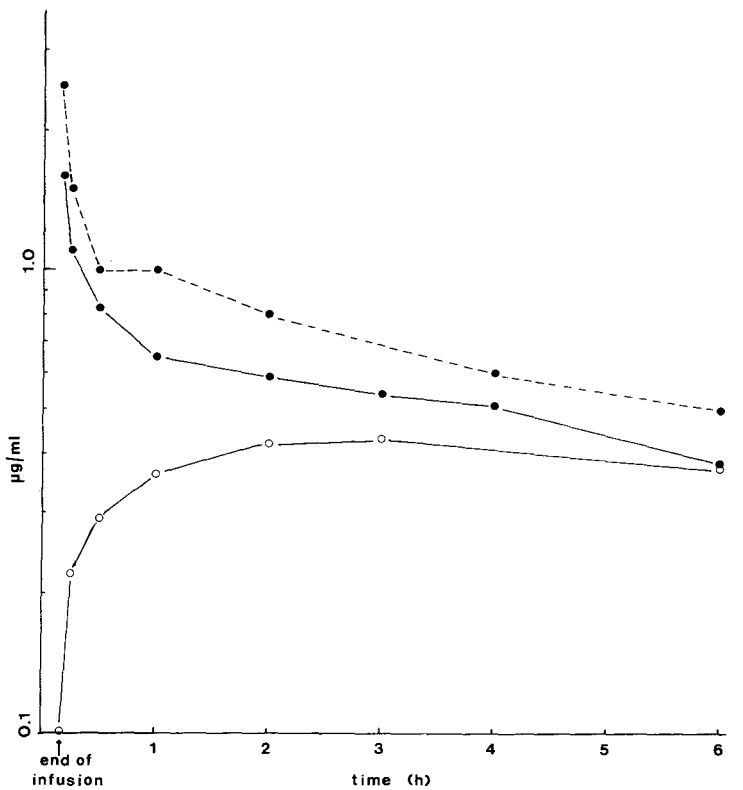

Fig. 4. Levels of Ro 03-8799 (•) and Ro 31-0313 (○) in plasma (——) and red cells (— — —) in a patient after an infusion of 100 mg of [2-^{14}C]Ro 03-8799.

Urine

Analysis of urine samples from animals fed on standard laboratory diets is straightforward. However, we have found considerable variation in human urine samples. There can be large and variable interfering peaks under both compounds depending on the source of the samples. This makes quantitation at low levels inaccurate. Standard samples of parent drug and N-oxide covering the range 0.4—50 µg/ml were made up on five different days. The precision and accuracy of the method were calculated as described for plasma. In the range 1—50 µg/ml the precision was $< \pm$ 10% for both compounds, and the accuracy

TABLE I

COMPARISON OF LEVELS (μg/g) OF TOTAL DRUG-RELATED MATERIAL (^{14}C) AND Ro 03-8799 IN TUMOUR AND PLASMA FROM A PATIENT GIVEN A 100-mg INFUSION OF [2-^{14}C]Ro 03-8799

Time (h)	Tumour		Plasma	
	^{14}C	Ro 03-8799	^{14}C	Ro 03-8799
0.5	3.9	1.6	1.0	0.83
1.0	1.8	1.0	1.0	0.65
2.0	3.8	2.0	1.0	0.59

100 ± 2% for parent drug and 98 ± 4% for N-oxide. For all practical purposes the quantitation limit is 0.5 μg/ml where the precision is ± 20%. For the quantitative analysis of human urine samples therefore, it is important to collect blank samples corresponding to those being analysed.

Tissues

A tissue distribution study was carried out in rats dosed with [2-^{14}C]Ro 03-8799. Initially samples were removed from the animals and stored frozen until analysed. Although the levels of drug-related material, measured by radioactivity, were high (3—10 times plasma levels at 15—60 min) we were unable to account for any of this material as unchanged drug or N-oxide metabolite. By altering the conditions under which the tissues are removed from the animals, to that given in the Experimental section, we increased the amount recovered as Ro 03-8799 at 30 min after dosing to 60% in all tissues apart from liver. 35% of the radioactivity measured in the liver can be shown to be unchanged drug (30%) and N-oxide metabolite (5%).

DISCUSSION

The analytical method described has proved very successful in analysing Ro 03-8799 and its N-oxide. The procedure has been applied to various biological samples and there is no interference from other metabolites. To obtain reproducible results however, certain aspects of the method are critical.

The pH of the eluent is important when analysing plasma samples because at a pH of 7 or greater no N-oxide is detected. This is because it is not present as such in the general circulation, but is immediately formed under acidic conditions (pH < 7). Measurement of the authentic metabolite as the N-oxide is considered to be justified since this allows all the drug-related material in human plasma to be determined. Also the N-oxide is present in urine which is slightly acidic.

The precautions described for handling tissue samples are necessary to prevent ex vivo metabolism. Ro 03-8799 was designed as an electron-affinic compound and is therefore an ideal substrate for nitro-reductase enzyme systems [10, 11]. In the anoxic conditions prevailing during tissue removal, the nitroimidazole can be rapidly reduced to a complex mixture having no characteristic UV absorption.

Using the procedures described, samples from the preliminary study in humans have been successfully analysed. The simple clean-up coupled with the automated HPLC analysis makes it possible to process large numbers of samples easily and quickly. The method should therefore be suitable for use by various laboratories where clinical trials on this new radiosensitiser are being carried out.

ACKNOWLEDGEMENTS

Ro 03-8799, Ro 31-0313 and Ro 07-1902 were synthesised by Dr C.E. Smithen and [2-^{14}C]Ro 03-8799 by Mrs G.M. Smith of Roche Products Ltd., Welwyn Garden City. We are indebted to Dr. S. Dische and his colleagues at Mount Vernon Hospital for their co-operation with the preliminary human study of Ro 03-8799.

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COMPARISON OF TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF OXMETIDINE AND ITS METABOLITES IN PLASMA, BILE AND URINE SAMPLES

R.M. LEE* and R.D. McDOWALL

Department of Metabolic Biochemistry, Smith Kline and French Research Ltd., The Frythe, Welwyn, Hertfordshire (Great Britain)

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SUMMARY

Two high-performance liquid chromatographic methods for the assay of oxmetidine are described: both utilize the same liquid extraction from plasma, urine and bile samples. A normal-phase technique is considered most suitable for the analysis of plasma extracts and a reversed-phase method is preferred for the assay of excretory fluids such as urine and bile which will contain polar metabolites in detectable quantity as well as unchanged oxmetidine.

The methods are sensitive enough to follow the kinetic changes in concentration for up to 8 h after the administration of recommended therapeutic doses. Both methods can be automated in respect of the high-performance liquid chromatograph and the samples can be stored for several weeks at —20°C without prejudicing the accuracy of the analysis.

INTRODUCTION

Oxmeditine (SK&F 92994, Fig. 1) is a potent antagonist of the action of histamine at H_2-receptors [1]. It has been tested in animal models and in human volunteer studies [2] during which correlations between the pharmacodynamics and the plasma kinetics have been sought.

In order to follow the pharmacokinetics of absorption and elimination, high-performance liquid chromatographic (HPLC) methods of determining plasma concentrations of oxmetidine were investigated. The first was a simple modification of the normal-phase adsorption chromatography used extensively for cimetidine analysis [3], the second was a reversed-phase technique, which was developed with a view to carrying out the rapid assay of urine samples for oxmetidine and metabolites without the delay normally associated with a normal-phase technique. This delay is engendered by the need to allow polar materials, e.g. creatinine and the metabolites of oxmetidine which absorb at

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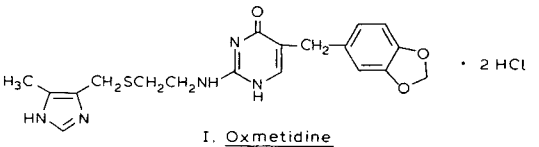
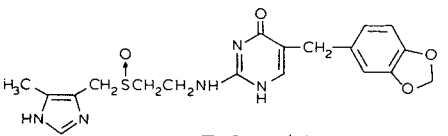


· 2 HCl

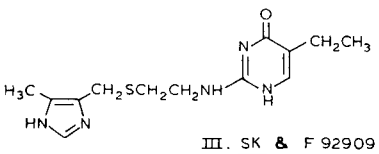
I. Oxmetidine

2-[2-(5-methyl-4-imidazolylmethythio)ethylamino]-5-(3,4-methylenedioxybenzyl)-4-pyrimidone diHCl



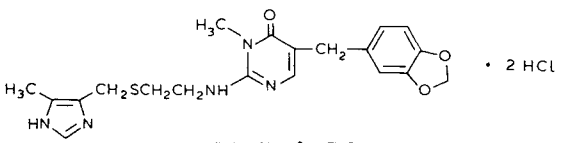
II. Oxmetidine sulphoxide

2-[2-(5-methyl-4-imidazolylmethylsulphinyl)ethylamino]-5-(3,4-methylenedioxybenzyl)-4-pyrimidone



III. SK & F 92909

2-[2-(5-methyl-4-imidazolylmethylthio)ethylamino]-5-ethyl-4-pyrimidone



· 2 HCl

IV. SK & F 93586

2-[2-(5-methyl-4-imidazolylmethylthio)-ethylamino]-1-methyl-5-(3,4-methylenedioxybenzyl)-6-pyrimidone diHCl

Fig. 1. Formulae of oxmetidine, its sulphoxide, and internal standards.

about 230 nm, to elute from a silica adsorption column. Such polar substances are not present in interfering amounts in plasma samples, but are a source of considerable delay in the assay of urine and bile, for which a reversed-phase column should be more suitable.

EXPERIMENTAL

All chemicals in this study were analytical grade with the following exceptions: 1-octanol (Koch-Light, Colnbrook, Great Britain, puriss), methanol and acetonitrile (Rathburn, Walkerburn, Great Britain, HPLC grade), 0.88 sp. gr. ammonium hydroxide solution (May and Baker, Dagenham, Great Britain, reagent grade), camphorsulphonic acid (Aldrich, Milwaukee, WI, U.S.A., reagent grade), β-D-glucuronidase EC 3.2.1.31 (Sigma, St. Louis, MO, U.S.A., type B3) from bovine liver. Samples of water were purified by deionisation, then distilled in an all-glass apparatus and stored in glass containers.

All solvents and solutions for HPLC were filtered through either 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A., type HA) for aqueous solutions or 0.5-μm membrane filters (Millipore type FH) for organic solvents.

A solution of carbonate buffer (1 mol/l; pH 9) was prepared by a modification of the method used by Delory and King [4]: to 5 l of sodium bicarbonate (1 mol/l) was added sufficient sodium carbonate (1 mol/l) to adjust the pH value of the solution to 9.0.

The solution of acetate buffer (0.1 mol/l; pH 5.0) was prepared by the addition of 322 ml sodium acetate solution to 678 ml acetic acid (0.1 mol/l).

A stock solution of oxmetidine was prepared by weighing 10 mg base equivalent of the dihydrochloride salt, dissolving it in approximately 0.5 ml water and making up to a 100-ml volume with ethanol. Solutions of oxmetidine sulphoxide (II, Fig. 1) and the two internal standards SK&F 92909 (III, Fig. 1) and SK&F 93586 (IV, Fig. 1) were similarly prepared by weighing 5, 40 and 100 mg base equivalent respectively, and dissolving each in separate aliquots of water and ethanol as above. All stock ethanolic solutions were stored at −20° C and found to be stable for at least three months under these conditions.

Working solutions of the respective internal standards were prepared fresh daily by diluting the stock solutions 100-fold with carbonate buffer (1.0 mol/l; pH 9).

Human plasma for preparation of standards was obtained by centrifugation (at 4° C) of the blood from volunteers and stored in 20-ml sterivials at −20° C until thawed for use. The polypropylene centrifuge tubes (12 ml) and stoppers used for sample extraction were obtained from Henleys Medical Supplies, London, Great Britain, (type 300 PP and 301 PT, respectively).

Preparation and storage of plasma samples

Blood from patients and volunteers, who had received oxmetidine, was taken by syringe into tubes containing lithium heparin as an anticoagulant. Each tube was mixed gently for a few minutes and then centrifuged to separate the plasma, which was transferred to plain tubes and stored at −20° C as soon as possible after the separation. Bile and urine samples were frozen in plain tubes as soon as possible after collection, and stored at −20° C until analysed.

Analysis of plasma

To a 2-ml plasma sample in a polypropylene centrifuge tube (12 ml) was added 1 ml of carbonate buffer (1 mol/l; pH 9), containing 4 μg base equivalent SK&F 92909 or 10 μg base equivalent of SK&F 93586 as an internal standard. If the volume of the sample was less than 2 ml then sufficient 0.9% sodium chloride solution was added to adjust the volume to the required amount.

A 5-ml volume of 1-octanol was added to the samples and the tubes were stoppered and rotated for 15 min on a blood-cell suspension mixer. The organic layer was separated from the aqueous phase by centrifugation at 1500 *g* (5 min) and 4—4.5 ml of the octanol layer were transferred to a second polypropylene centrifuge tube containing 3 ml 0.02 *M* hydrochloric acid.

The same rotary mixing and centrifugation technique was used to re-extract the compound into the acid and to separate the phases. The octanol layer was removed by aspiration and 2.5 ml of the acid was transferred to a clean polypropylene tube; 250 μl acetonitrile was added and mixed before saturating the whole with solid potassium carbonate (c5g). This had the effect of salting out the acetonitrile into a discrete layer, which was removed, after centrifuga-

tion (1500 *g* for 5 min at 4°C) and stored at −20°C to await HPLC separation and analysis.

In addition to the test samples, plasma samples containing known quantities of oxmetidine were carried through the procedure under the same conditions; 12 tubes containing 2 ml blank plasma to which had been added, in duplicate, 0, 1, 2, 4, 8 and 16 μg base equivalent oxmetidine were used, and from the assay of these a calibration curve was constructed.

Analysis of urine and bile

The procedure for the extraction of oxmetidine from urine and bile was exactly as described for the extraction from plasma. However, the urine of subjects receiving oxmetidine has been shown to contain the sulphoxide metabolite and glucuronide conjugates of both oxmetidine and its sulphoxide [5].

The unconjugated sulphoxide has been shown to be extracted under the conditions employed but the glucuronides were not; thus, an estimate of the concentrations of these conjugates in urine and bile was dependent upon their hydrolysis to the aglycones and re-assay of the samples to give a total for oxmetidine and sulphoxide which included the glucuronide-conjugated material.

This hydrolysis was achieved by incubating 1-ml samples of urine or bile with β-glucuronidase (2 mg) for 24 h at ambient temperature. The sample was then increased to 2 ml by the addition of 1 ml of 0.9% sodium chloride solution, and the internal standard was added in carbonate buffer as previously described. The rest of the extraction procedure was as given for plasma samples.

For urine and bile samples calibration curves were constructed for oxmetidine sulphoxide as well as for oxmetidine. To each 1 ml of urine or bile were added 0, 1, 5, 10 or 20 μg of oxmetidine and 0, 0.5, 2.5, 5 or 10 μg of oxmetidine sulphoxide; these spiked samples were extracted along with the test samples for each batch of samples assayed.

HPLC equipment

The chromatograph had the following components: the pump was a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A or equivalent, the injectors used were Rheodyne 7125 valve type (50 μl loop) or WISP (Waters Assoc.) automatic. The columns used were a normal-phase column, Ultrasphere-Si, particle size 5 μm, 250 × 4.6 mm I.D. and a reversed-phase column, Ultrasphere-ODS, particle size 5 μm, 150 × 4.6 mm I.D. The Perkin-Elmer LC100 column oven was set at 25°C for normal-phase working, and 75°C for reversed-phase working. The detectors, a Perkin-Elmer LC55B (226 nm) or a LC 75 (226 nm), were set to give 0.04 or 0.08 absorbance units full scale (a.u.f.s.).

HPLC solvent systems

Normal phase. For the normal-phase work with the Ultrasphere-Si 5-μm column the solvent was a mixture of acetonitrile—methanol—water—ammonium hydroxide (sp. gr. 0.88) (200:40:10:1.5, v/v).

The acetonitrile, methanol and water were mixed and degassed at reduced

pressure before the addition of the ammonium hydroxide. This solvent was then passed through the column for 1 h, with recycling, to equilibrate the system before commencing the assays. If an automatic injector is to be used, this solvent is usable for up to 48 h after equilibration provided that ammonia loss is reduced by capping the reservoir.

Reversed phase. The solvent for the reversed-phase system was a mixture of acetonitrile—0.02 *M* camphorsulphonic acid solution (30:70, v/v). The latter was prepared by dissolving 1.16 g of camphorsulphonic acid in 175 ml of distilled water and filtering the solution through a 0.45-μm Millipore HA filter.

The solvent mixture was degassed at reduced pressure and then pumped through the system for about 30 min in order to equilibrate the system at 75°C. During the preparation of this solvent, the column was back-flushed with filtered methanol at room temperature, since this was found to lengthen the working life of the column.

Flow-rates and retention times

With the Ultrasphere-Si 5-μm column, a flow-rate of 1.8 ml min^{-1} was used to give retention times of 3 min for oxmetidine and 4 min for the internal standard, SK&F 92909. In this system, oxmetidine sulphoxide was retained for about 10 min and creatinine for about 6 min. Using the Ultrasphere-ODS column and a flow-rate of 2.0 ml min^{-1}, oxmetidine sulphoxide was retained for 2.1 min, oxmetidine for 2.6 min and the internal standard (SK&F 93586) for 3.2 min; creatinine was not seen in a urine chromatogram, and, when deliberately added to an extract, creatinine enhanced the absorption associated with the solvent front.

Quantitation of chromatograms

The area under each peak was determined by an integrator (Perkin-Elmer Sigma 10, or Laboratory Data Control 308) connected to the UV detector. The ratio of the area of the unknowns to that of the internal standard was then used to calculate the concentration of the unknown by extrapolation from a two-variable linear regression, which was obtained from the corresponding ratios for known amounts of oxmetidine or oxmetidine sulphoxide added to pre-dose samples of plasma, bile or urine.

Studies with [^{14}C]*oxmetidine*

The efficiency of the extraction procedure was tested by spiking samples of plasma and urine with ^{14}C-labelled oxmetidine (17.1 Ci/mg; 99% radiochemically pure), and extracting these in the manner described.

The radioactivity in the recovered acetonitrile was compared to that in the spike by adding each of them to 5 ml of Picofluor scintillant and counting the resulting scintillations in a Denley Mark III spectrometer.

RESULTS AND DISCUSSION

Recovery of oxmetidine from plasma and urine

The recovery of ^{14}C added to plasma and urine samples as [^{14}C]oxmetidine was used as a measure of the availability of oxmetidine for assay, and the results are shown in Table I. For samples of plasma containing 1—7.5 μg of oxmetidine the average recovery was 61.8%; for samples of urine containing 1—20 μg (17—340 nCi of ^{14}C) the average recovery was 60.6%. With regard to the volumes of octanol and acid harvested the theoretical possible recovery was 75%.

TABLE I

RECOVERY OF RADIOACTIVITY FROM PLASMA AND URINE CONTAINING ADDED [^{14}C]OXMETIDINE

C.V. = coefficient of variation.

Fluid	Oxmetidine in sample ($mg\ l^{-1}$)	Original dpm in the spike, mean ± C.V. (%)	Recovered dpm in acetonitrile layer (n = 20), mean ± C.V. (%)	Mean recovery (%)
Plasma	1.0	42314 ± 1.7	25383 ± 10.9	60.0
	2.0	85623 ± 2.0	48081 ± 18	56.1
	5.0	200123 ± 7.0	121198 ± 11.4	60.6
	10.0	417410 ± 1.6	265899 ± 11.5	63.7
	15.0	612980 ± 1.7	420141 ± 8.0	68.5
Urine	1.0	50168 ± 6	29029 ± 16	57.9
	5.0	219907 ± 0.8	134954 ± 7.0	61.4
	10.0	429763 ± 1.0	252135 ± 1.5	58.7
	15.0	642319 ± 1.0	368336 ± 9.0	57.3

The variability in recovery between samples (expressed by the coefficient of variation in Table I) would be offset by the presence of an internal standard, which would be expected to undergo the same variability. Thus about 60% of the oxmetidine in biological fluids was available for the chromatographic separation and quantification by UV absorption.

Chromatographic separation

Normal phase. A typical normal-phase chromatogram of oxmetidine and the internal standard, SK&F 92909, extracted from a 2-ml plasma sample is shown in Fig. 2 together with the appropriate controls.

To test the precision and accuracy of the method ten spiked samples for each of six concentrations of oxmetidine were extracted on three separate occasions. The results of these assays are shown in Table II as concentrations calculated from average peak heights for each concentration together with the variability for precision (shown as coefficient of variation) and accuracy (as percentage error). At all concentrations between 0.1 and 7.5 mg/l the method can be seen to be accurate and precise enough for use in the assay of samples from clinical trials and other studies.

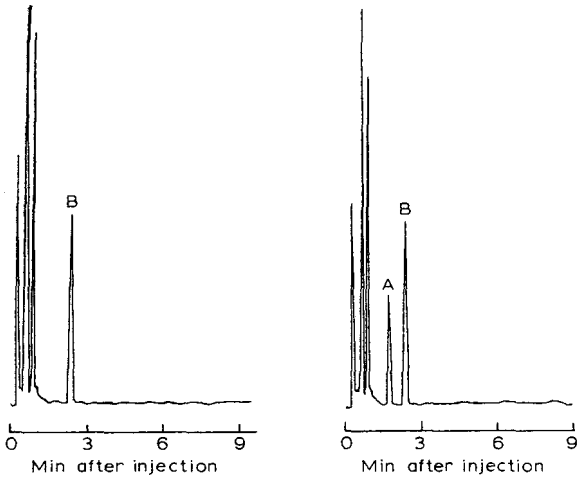


Fig. 2. Normal-phase chromatograms of extracted human plasma before (left) and after (right) the administration of oxmetidine. Peaks: A = oxmetidine, B = internal standard (SK&F 92909).

TABLE II

PRECISION AND ACCURACY OF THE NORMAL PHASE HPLC ASSAY OF OXMETIDINE IN PLASMA

Amount added to sample (μg per 2 ml)	Average concentrations (μg per 2 ml) calculated from peak heights		
	Mean ± S.D.	C.V. (%)	Error (%)
0.2	0.20 ± 0.02	10.0	Nil
1.0	1.02 ± 0.08	7.8	2.0
2.0	1.98 ± 0.06	3.1	2.0
5.0	4.94 ± 0.14	2.8	1.0
10.0	9.96 ± 0.30	3.0	0.4
15.0	15.08 ± 0.60	3.9	0.5

An example of such an assay is shown in Fig. 2 and indicates that extracted plasma samples contain only oxmetidine in detectable quantities, there being no peaks with longer retention times than the internal standard (4 min), which would have been observed if polar metabolites such as the sulphoxide had been present.

However, when urine samples were extracted and assayed by this technique (Fig. 3) two peaks with relatively long retention times were seen, corresponding to peaks obtained when authentic oxmetidine sulphoxide and creatinine were injected into the chromatograph. Because of the time required to clear these substances from the column, a reversed-phase HPLC technique was considered to be more suitable for urine sample assay.

Reversed phase. A typical reversed-phase chromatogram of oxmetidine, its sulphoxide and the internal standard, SK&F 93586, extracted from a spiked 2-ml urine sample is shown in Fig. 4, together with the appropriate controls.

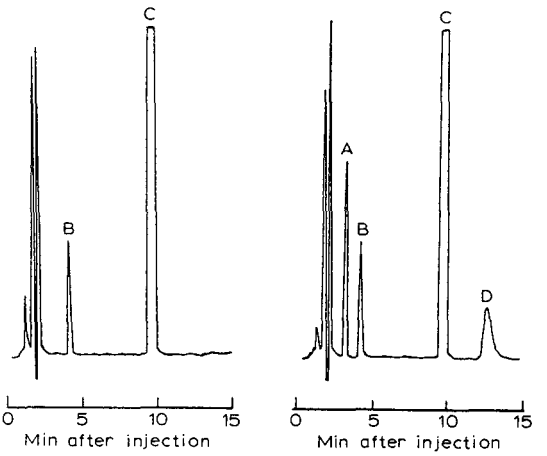


Fig. 3. Normal-phase chromatograms of extracted human urine before (left) and after (right) the administration of oxmetidine. Peaks: A = oxmetidine, B = internal standard (SK&F 92909), C = creatinine, D = oxmetidine sulphoxide.

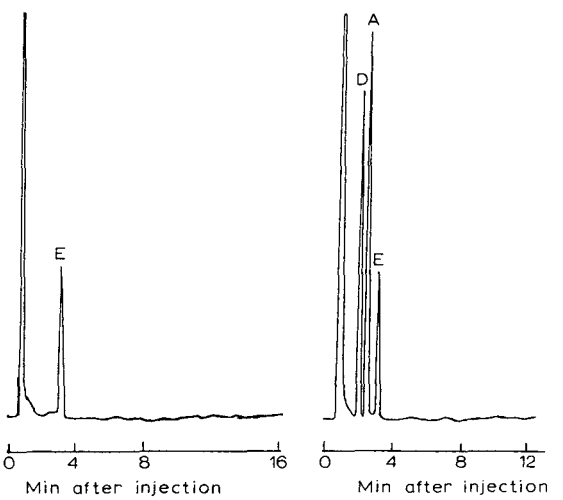


Fig. 4. Reversed-phase chromatograms of extracted human urine before (left) and after (right) the administration of oxmetidine. Peaks: A = oxmetidine, E = internal standard (SK&F 93586), D = oxmetidine sulphoxide.

The precision and accuracy of this technique was tested for four concentrations of oxmetidine and four concentrations of oxmetidine sulphoxide in urine. The results in Table III are concentrations calculated from the average peak areas for each concentration, together with the variation to be expected for precision and accuracy.

Precision for oxmetidine and for the sulphoxide in urine was good except at the lowest concentrations and accuracy was also less at low concentrations. Nevertheless, at concentrations likely to be encountered in urine samples from patients, the assay was sufficiently accurate and precise.

TABLE III

PRECISION AND ACCURACY OF THE DETERMINATION OF OXMETIDINE AND ITS SULPHOXIDE METABOLITE IN URINE BY REVERSED-PHASE HPLC

Concentration (mg/l)	Concentrations calculated from peak areas			
	Mean ± S.D.	n^*	C.V. (%)	Error (%)
Oxmetidine				
1.0	1.05 ± 0.13	30	12.4	4.8
5.0	4.84 ± 0.20	30	4.2	3.3
10.0	9.98 ± 0.38	29	3.8	0.2
20.0	20.93 ± 0.82	30	3.9	4.7
Oxmetidine sulphoxide				
0.5	0.62 ± 0.09	10	13.3	24.0
2.5	2.43 ± 0.19	30	7.9	3.0
5.0	5.03 ± 0.36	29	7.2	0.7
10.0	10.84 ± 1.00	30	9.2	8.4

*n = Number of samples assayed.

The typical chromatogram (Fig. 4), obtained during the assay of urine from volunteers receiving oxmetidine, indicates that extracted urine samples contain oxmetidine and oxmetidine sulphoxide in addition to the added internal standard. No other peaks were observed, so creatinine was added to the extract and the chromatography repeated, resulting in an enhancement of the absorption ascribed to the solvent front. The retention times for oxmetidine sulphoxide, oxmetidine and SK&F 93586 were 2.1, 2.6 and 3.2 min, respectively, and the assay was completed within 4 min, permitting an assay rate of 15 samples per hour manually or by automatic injector.

No peaks with long retention times (up to 20 min) were observed for extracts of urine and bile samples known to contain a glucuronide conjugate of oxmetidine, and it is concluded that this metabolite was not extracted from the samples. However, after treatment with glucuronidase, the concentration of oxmetidine in some urine and bile extracts increased, as also did the concentration of sulphoxide.

This reversed-phase method was also tried for the assay of plasma extracts, but was found to be unsuitable because of indigenous material which was extracted and co-chromatographed with the desired peaks. Thus, it was concluded that the reversed-phase technique should be reserved for urine and bile samples, and that plasma analysis of unchanged oxmetidine was best achieved by normal-phase chromatography. The stability of oxmetidine in the different biological fluids was, therefore, investigated using the technique appropriate to the sample.

Stability of oxmetidine in plasma

Short term stability at ambient temperatures is shown in Table IV and indicates that, at 0.5 and 2.5 mg l^{-1}, the concentration of oxmetidine in plasma apparently increased by 16—20% over 6 h. Although the samples were in

stoppered tubes, this increase may have been due to concentration by evaporation, so exposure to ambient temperature should be avoided as much as possible. Longer term stability at −20°C is also shown in Table IV and indicates that plasma samples may be stored at this temperature for 12 weeks without significant change in the value of the oxmetidine concentrations, although at 12 weeks the variation in the assay had increased.

TABLE IV

ASSAY OF OXMETIDINE IN PLASMA AFTER STORAGE AT AMBIENT TEMPERATURE OR AT LOW TEMPERATURE

Time after addition of oxmetidine to plasma	Storage temperature	Assay	
		0.5 mg l^{-1}	2.5 mg $^{-1}$
0 h	Ambient	0.49 ± 0.03	2.47 ± 0.07
2 h	Ambient	0.51 ± 0.01	2.68 ± 0.08
4 h	Ambient	0.53 ± 0.03	2.62 ± 0.04
6 h	Ambient	0.59 ± 0.02	2.90 ± 0.04
1 week	−20°C	0.51 ± 0.02	2.54 ± 0.03
2 weeks	−20°C	0.56 ± 0.03	2.58 ± 0.06
3 weeks	−20°C	0.48 ± 0.02	2.57 ± 0.08
5 weeks	−20°C	0.53 ± 0.02	2.59 ± 0.08
6 weeks	−20°C	0.53 ± 0.02	2.52 ± 0.06
12 weeks	−20°C	0.49 ± 0.05	2.62 ± 0.16

TABLE V

ASSAY OF OXMETIDINE IN URINE AFTER STORAGE AT AMBIENT TEMPERATURE OR AT LOW TEMPERATURE

Time after addition of oxmetidine to urine	Storage temperature	Assay	
		10 mg l^{-1}	20 mg l^{-1}
0 h	Ambient	10.04 ± 0.40	19.84 ± 0.41
6 h	Ambient	9.69 ± 0.21	19.81 ± 0.53
18 h	Ambient	9.44 ± 0.28	19.42 ± 0.22
24 h	Ambient	9.29 ± 0.27	18.52 ± 0.70
1 week	−20°C	9.59 ± 0.26	18.97 ± 0.53
3 weeks	−20°C	9.79 ± 0.19	19.28 ± 0.54
5 weeks	−20°C	9.75 ± 0.17	19.94 ± 0.27
6 weeks	−20°C	9.58 ± 0.23	18.97 ± 0.30
8 weeks	−20°C	9.49 ± 0.70	19.43 ± 1.20
14 weeks	−20°C	9.05 ± 0.24	17.51 ± 0.27

Stability of oxmetidine in urine

Urine samples did not lose significant amounts of oxmetidine when stored at room temperature for 6 h. However, by the following day a reduction in concentration of about 8% had occurred (Table V). At concentrations generally found in urine (10—20 mg l^{-1}) oxmetidine was stable for up to eight weeks

after the addition of the drug, when the samples were kept at −20° C. However at fourteen weeks of storage at low temperature, about 10% of the oxmetidine was no longer available, so that urine samples should be assayed within eight weeks of collection (Table V).

Oxmetidine has been assayed in plasma samples taken from studies designed to investigate the kinetics of the compound in man and experimental animals. In these studies, plasma concentrations ranging from 50 ng/ml to 50 μg/ml have been encountered.

Extraction and analysis of urine and bile samples have demonstrated that both of these fluids may contain unchanged oxmetidine and/or oxmetidine conjugated with glucuronic acid, and that the major unconjugated metabolite is the sulphoxide shown in Fig. 1.

ACKNOWLEDGEMENTS

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CHROMBIO. 1559

DETERMINATION OF CIBENZOLINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MARTIN R. HACKMAN*, TEH LO LEE and MARVIN A. BROOKS

Department of Pharmacokinetics and Biopharmaceutics, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of cibenzoline (Cipralan™) in human plasma and urine. The assay involves the extraction of the compound into benzene from plasma or urine buffered to pH 11 and HPLC analysis of the residue dissolved in acetonitrile—phosphate buffer (0.015 mol/l, pH 6.0) (80:20). A 10-μm ion-exchange (sulfonate) column was used with acetonitrile—phosphate buffer (0.015 mol/l, pH 6.0) (80:20) as the mobile phase. UV detection at 214 nm was used for quantitation with the di-*p*-methyl analogue of cibenzoline as the internal standard.

The recovery of cibenzoline in the assay ranged from 60 to 70% and was validated in human plasma and urine in the concentration range of 10—1000 ng/ml and 50—5000 ng/ml, respectively. A normal-phase HPLC assay was developed for the determination of the imidazole metabolite of cibenzoline. The assays were applied to the determination of plasma and urine concentrations of cibenzoline and trace amounts of its imidazole metabolite following oral administration of cibenzoline succinate to two human subjects.

INTRODUCTION

Cibenzoline [I] (Cipralan™), 4,5-dihydro-2-(2,2-diphenylcyclopropyl)-1H-imidazole (Fig. 1) is a member of a new class of antiarrhythmics [1].

An electron-capture gas—liquid chromatographic (EC—GLC) procedure for the determination of [I] in plasma and urine was reported [2]. It requires the extraction of the compound into diethyl ether from plasma or urine buffered to pH 10.6, back-extraction into 0.1 *N* hydrochloric acid, readjustment of the sample pH to 10.6 and re-extraction into benzene, the residue of which is reacted with trifluoroacetic anhydride (TFAA) to yield the N-trifluoroacetyl (TFA) derivative for EC—GLC analysis. The internal standard for the assay is

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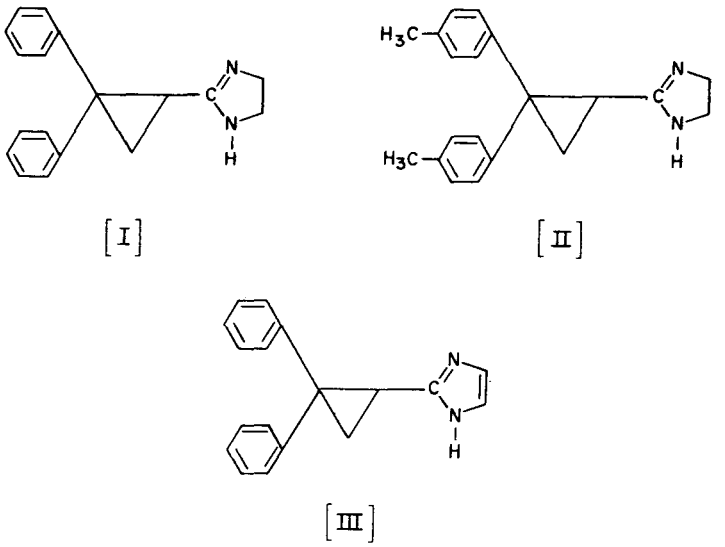

Fig. 1. Chemical structures of cibenzoline [I], di-*p*-methylcibenzoline (internal standard) [II] and imidazole metabolite [III].

the di-*p*-methyl analogue of cibenzoline 2-[2,2-bis(4-methylphenyl)-1-cyclopropyl]-4,5-dihydro-1H-imidazole [II]. The assay has a sensitivity limit of 10 ng [I] per ml of plasma, and was used in clinical pharmacokinetic studies for the analysis of plasma and urine following single intravenous and oral doses of [I] · succinate (Cipralan) [2].

The present work describes a high-performance liquid chromatographic (HPLC) assay for [I] as the intact compound in plasma and urine utilizing ion-exchange chromatography on a sulfonate column [3] with UV detection at 214 nm. The HPLC procedure is simpler and more expeditious for high sample throughput than the reported EC—GLC assay for [I] due to the elimination of the back-extraction and derivatization steps. The sensitivity limit (10 ng/ml) and precision of the HPLC and EC—GLC assays are equivalent. The HPLC assay also utilizes the di-*p*-methyl analogue of cibenzoline [II] as the internal standard.

The assay was applied to the determination of plasma and urine concentrations of [I] in two human subjects following oral administration of [I] · succinate.

A normal-phase HPLC assay is also described for the determination of the imidazole metabolite [III] of cibenzoline [4] in plasma and urine.

EXPERIMENTAL

Column

The column used for ion-exchange HPLC analysis was a 25 cm × 4.6 mm I.D. stainless-steel column prepacked with 7—8 μm Zorbax SCX (Dupont Instruments, Wilmington, DE, U.S.A.), which was jacketed in a styrofoam block.

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model 710B automatic sample injector (WISP) and a Model 441 UV detector equipped with a 214-nm zinc source and wavelength kit (Waters Assoc., Milford, MA, U.S.A.). For the plasma assay the UV detector was operated at 0.05 a.u.f.s. Two low-pass filtered (3 sec time constant) output responses were monitored using a four-channel Model 3314 recorder (Soltec Corporation, Sun Valley, CA, U.S.A.) at 2 and 10 mV/full scale deflection (equivalent to 0.01 and 0.05 a.u.f.s.). For the urine assay the detector was operated at 0.2 a.u.f.s. Three low-pass filtered output responses were monitored at 1, 5 and 10 mV/full scale deflection (equivalent to 0.02, 0.1 and 0.2 a.u.f.s.).

All reservoir-to-pump tubing was changed from Teflon FEP to polyethylene-lined ethylvinyl acetate tubing (Bev-A-Line V-HT, Thermoplastic Scientific, Warren, NJ, U.S.A.) (see Results and discussion).

Analytical standards

Compound [I], cibenzoline; 4,5-dihydro-2-(2,2-diphenylcyclopropyl)-1H-imidazole, $C_{18}H_{18}N_2$, M.W. = 262.33, compound [II], 2-[2,2-bis(4-methylphenyl)-1-cyclopropyl]-4,5-dihydro-1H-imidazole, $C_{20}H_{22}N_2$, M.W. = 290.39 and compound [III], 2,2-diphenylcyclopropyl-1H-imidazole, $C_{18}H_{16}N_2$, MW = 260.32, m.p. = 218—220°C, of pharmaceutical grade purity (>99%) are used as analytical standards (Fig. 1).

Prepare stock solution A, 1.00 mg of [I] per ml by dissolving 50.0 mg of [I] in 50 ml acetonitrile. Dilute stock solution A 1:10 with acetonitrile to prepare intermediate solution B containing 100 μg [I] per ml. Working standards (Numbers 1 to 10) are prepared in 10 ml of acetonitrile by taking aliquots of A or B indicated below:

Solution No.	Amount of Solution A or B (μl)	Final concentration (ng per 50 μl)
1	20 B	10
2	50 B	25
3	100 B	50
4	200 B	100
5	500 B	250
6	1000 B	500
7	150 A	750
8	200 A	1000
9	400 A	2000
10	1000 A	5000

Prepare stock solution C equivalent to 1.00 mg of [II] per ml by dissolving 50.0 mg of [II] in 50 ml acetonitrile. A working standard D of 100 ng per 50 μl [II] is prepared by diluting 100 μl stock solution C to 50 ml with acetonitrile. Working standards are routinely prepared every two weeks from the stock solution, and a fresh stock solution prepared once a month.

Reagents

Reagent grade chemicals are used to prepare: 1 mol/l phosphate buffer, pH 6.0 (prepared by mixing 430 ml of 1 mol/l orthophosphoric acid with 570 ml of 1 mol/l dipotassium dihydrogen orthophosphate and adjusting to pH 6.0), and 1 mol/l phosphate buffer, pH 11.0 [prepared by mixing 530 ml of 1 mol/l dipotassium hydrogen phosphate with 470 ml of saturated (0.7 mol/l) trisodium phosphate and adjusting the pH to 11.0]. Other reagents include acetonitrile (UV grade) and benzene (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

The mobile phase for ion-exchange HPLC is composed of acetonitrile—0.015 mol/l phosphate buffer, pH 6.0 (80:20), prepared by mixing 6.0 ml of a 1 mol/l phosphate buffer, pH 6.0, with 394.0 ml distilled water and diluting to 2 l with acetonitrile. All mobile phases are vacuum degassed prior to use and kept under constant sparging with helium (99.995%). A solution of acetonitrile—water (80:20) is used to flush the phosphate buffer from the ion-exchange column at the completion of a day's analysis to prevent salt build up.

Chromatographic parameters

The flow-rate for the ion-exchange HPLC assay is 1.5 ml/min with a resulting pressure of 4.8 MPa (700 psi), and retention times for [I] and [II] of 5.4 min ($k' = 5.0$) and 4.4 min ($k' = 3.9$), respectively.

Assay sample preparation

Into a 13 × 100 mm disposable borosilicate culture tube (Cat. No. 14-962-10C, Fisher Scientific, Springfield, NJ, U.S.A.) place 50 µl of solution D (100 ng of [II]), 1.0 ml of plasma or urine and 2.0 ml of 1 mol/l phosphate buffer, pH 11.0. (Because of the high concentrations of [I] in urine during the 0—24 h excretion interval, only 0.1 ml is assayed for these samples). Mix well on a Vortex mixer and add 2.5 ml of benzene using a repipet (Model No. 3020-GR, Lab Industries, Berkeley, CA, U.S.A.), stopper using a polyethylene stopper (Cat. No. 127-0019-000, Elkay Products, Shrewsbury, MA, U.S.A.) and then shake on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80 to 100 strokes per min for 10 min, followed by centrifugation at 1460 *g* for 10 min at 10°C (Model PR-J rotor No. 253, Damon/IEC Corp., Needham, MA, U.S.A.). Transfer 2.0 ml of the supernatant to another 13 × 100 mm culture tube and evaporate to dryness in a 65° C water bath under a stream of nitrogen (N-EVAP evaporator, Organomation Assoc., Worchester, MA, U.S.A.). Reconstitute the residue in 400 µl of mobile phase and transfer to a 1.5-ml polypropylene micro test tube (Cat. No. 696, W. Sarstedt, Princeton, NJ, U.S.A.) and cap (Cat. No. A-123, Kew Scientific, Columbus, OH, U.S.A.). Insert the capped micro test-tube into a glass shell vial (Cat. No. 60930-L, Kimble Glass, Toledo, OH, U.S.A.) making sure that the flange of the test-tube is inside the vial not resting on the lip of the vial. Place all vials in the auto-injector carousel.

HPLC analysis of plasma

Inject a 50-µl aliquot of the contents of the auto-sampler vial by automatic injection using pre-programmed parameters. Along with the samples process a

1.0-ml specimen of control plasma and eight 1.0-ml specimens of the same control plasma to which 50 μl of solutions No. 1—8 and 50 μl of solution D (internal standard) have been added (equivalent to 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 750.0 and 1000.0 ng [I] per ml). These standards are used to establish a least-squares regression calibration curve using a power function for the quantitation of the unknowns using peak height ratio of [I]/[II] vs. concentration of [I] (ng/ml). Appropriate corrections for any changes in sample aliquot or dilutions must be made.

A set of external standards of [I] prepared by adding and mixing 50 μl of solutions No. 1—8, 50 μl of solution D (100 ng of [II]) and 300 μl of mobile phase are also assayed. These samples are used to standardize the chromatographic system and to calculate percent recovery. Typical chromatograms following automatic sample injection are shown in Fig. 2 for (A) control plasma extracts, (B) an extract from a subject following the administration of [I] · succinate, and (C) control plus added standards.

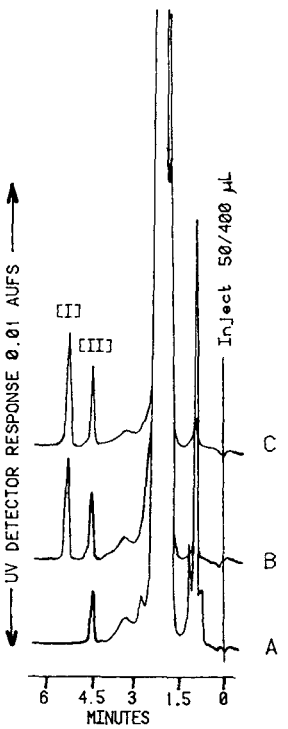

Fig. 2. Ion-exchange chromatograms of human plasma: (A) control containing 100 ng [II] per ml; (B) subject, 18 hours, day 12 following multiple oral dosing of [I] · succinate (see text); and (C) control containing 100 ng [I] per ml and 100 ng [II] per ml (internal standard).

HPLC analysis of urine

The procedure for the HPLC analysis in urine is the same as for the plasma assay except for the concentration range of the standards used to establish a least-squares regression calibration curve. These are prepared by adding 50 μl

of solutions No. 3—10 (equivalent to 50.0, 100.0, 250.0, 500.0, 750.0, 1000.0, 2000.0, and 5000,0 ng [II] per ml) and 50 μl of solution D to 1.0 ml of control urine. Solutions No. 3—9 are also used in a similar manner for the preparation of the external standards.

RESULTS AND DISCUSSION

Analytical parameters

Due to the absorbance of oxygen at 214 nm it was necessary to include precautions against oxygen diffusing into the aqueous mobile phase. The mobile phase was rendered free of oxygen by constant sparging with helium and the replacement of the standard Teflon FEP tubing with non-oxygen permeable polyethylene-lined ethylvinyl acetate tubing.

The efficiency of the ion-exchange HPLC column decreased with continuous use resulting in a diminution of the peak height response and resolution of compounds [I] and [II]. This loss in sensitivity and resolution limited the useful lifespan of the column to approximately 2—3 months. It is therefore essential that column performance be monitored routinely using external calibration standards. The column was jacketed in a styrofoam block to maintain better column temperature (25°C) regulation, which provided a relatively noise-free baseline.

The precision and accuracy of the plasma and urine assays were determined by the construction of least-squares regression calibration curves (power function) from the responses of the recovered standards. Mean intra- and inter-assay precision for the plasma assay and intra-assay precision for the urine assay are typically 4—5% (Table I). Definitive inter-assay data are presently unavailable for the urine assay; however, preliminary results indicate comparable precision and accuracy. The validated sensitivity limits for [I] were 10.0 ng/ml and 50.0 ng/ml of plasma or urine, respectively, using a 1.0-ml sample for analysis.

TABLE I

STATISTICAL VALIDATION OF THE ION-EXCHANGE HPLC ASSAY FOR [I] IN PLASMA AND URINE

Intra-assay — plasma				Inter-assay — plasma			Intra-assay — urine		
Amount added (ng/ml)	Amount found ± S.D. (ng/ml)	C.V. (%)	*n*	Amount found ± S.D. (ng/ml)	C.V. (%)	*n*	Amount found ± S.D. (ng/ml)	C.V. (%)	*n*
10.0	11.6 ± 1.0	8.5	3	10.1 ± 0.4	4.1	4	—		
25.0	24.0 ± 1.9	7.9	3	25.7 ± 0.8	2.9	4	—		
50.0	44.2 ± 0.7	1.5	3	47.7 ± 1.9	3.9	4	51.1 ± 0.4	8.5	3
100.0	93.3 ± 0.8	0.9	3	98.9 ± 1.0	1.1	4	103.3 ± 6.1	5.9	3
250.0	251.8 ± 5.2	2.1	2	252.8 ± 20.5	8.1	4	—		
500.0	513.9 ± 35.3	6.9	3	496.8 ± 23.3	4.7	4	472.1 ± 5.5	1.2	3
750.0	774.7 ± 19.9	2.7	3	747.2 ± 25.2	3.4	4	—		
1000.0	1032 ± 60	5.8	3	1022 ± 37	3.6	4	1024 ± 27	2.6	2
5000.0	—			—			5295 ± 350	6.6	3
	Average =	4.5%		Average =	4.0%		Average =	5.0%	
	$y = 0.06899\ x^{0.9474}$						$y = 1.315\ x^{0.997}$		
	$r = 0.999$						$r = 0.999$		

The recovery of [I] from human plasma and urine using the described assay was determined in the concentration range of 10 to 1000 ng/ml of plasma and 50 to 5000 ng/ml urine by substitution of the peak height obtained for these samples into the equation describing the calibration curve of the external (non-recovered) standards (see above); with the appropriate correction for the sample aliquot injected (50/400 μl) and for the extraction aliquot factor (2.0/2.5). The recovery of compound [I] in plasma and urine ranged from 60 to 70%.

Selectivity

Metabolite investigation has shown that [I] is biotransformed to [III] [4]. A preliminary method for cibenzoline utilized a reversed-phase HPLC assay using a 10-μm C-18 column with phosphate buffer, pH 3.5—methanol as the mobile phase. This assay showed that compounds [I] and [III] could not be chromatographically resolved. The reversed-phase assay was replaced by the ion-exchange assay to permit the specific quantitation of [I], in which metabolite [III] elutes in the solvent front. Attempts to quantitate [I] and [III] simultaneously by either reversed-phase, normal-phase or ion-exchange HPLC were not successful, hence a separate normal-phase HPLC assay was used for the quantitation of [III] in the benzene extract (see below).

The normal-phase assay for [III] differs from the ion-exchange assay for [I] mainly by the use of a 30 cm × 3.9 mm I.D. stainless-steel column prepacked with 10 μm Porasil (Waters Assoc.) [mobile phase: heptane—ethanol—concentrated ammonium hydroxide (90:10:1)], a SpectroMonitor III variable-wavelength UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) operated at 229 nm at 0.01 a.u.f.s. and a Hewlett-Packard (Paramus, NJ, U.S.A.) Model 7132 dual-channel recorder with 10 mV output. The residue of the benzene extract is reconstituted in 200 μl of heptane—ethanol (90:10), 20 μl of which are injected for analysis. Using these chromatographic parameters, the retention time for [III] was 6.02 min ($k' = 2.1$). The precision and accuracy of the normal-phase methods for plasma and urine were determined in a similar manner to that described for the ion-exchange methods except for the use of linear (instead of power function) least squares regression calibration curves:

$y = 29.16x + 0.046$, $r = 0.999$ (plasma)

$y = 62.52x + 0.45$, $r = 0.999$ (urine)

where y = peak height and x = concentration of [III] per ml of plasma or urine.

The recovery of [III] from human plasma and urine using the normal-phase HPLC assay was 88.5 ± 8.7% (S.D.) ($n = 11$) in plasma and 82.1 ± 7.3% (S.D.) ($n = 5$) in urine.

The sensitivity limit was 10.0 ng/ml of [III] using a 1.0-ml sample of human plasma and 50.0 ng/ml of [III] using a 1.0-ml sample of human urine. The intra-assay coefficient of variation (C.V.) for the analysis of [III] over the concentration ranges 0.01—0.5 and 0.05—2.5 μg/ml for plasma and urine, respectively, was approximately 5%. Typical chromatograms following automatic sample injection are shown in Fig. 3 for (A) control plasma extracts, (B)

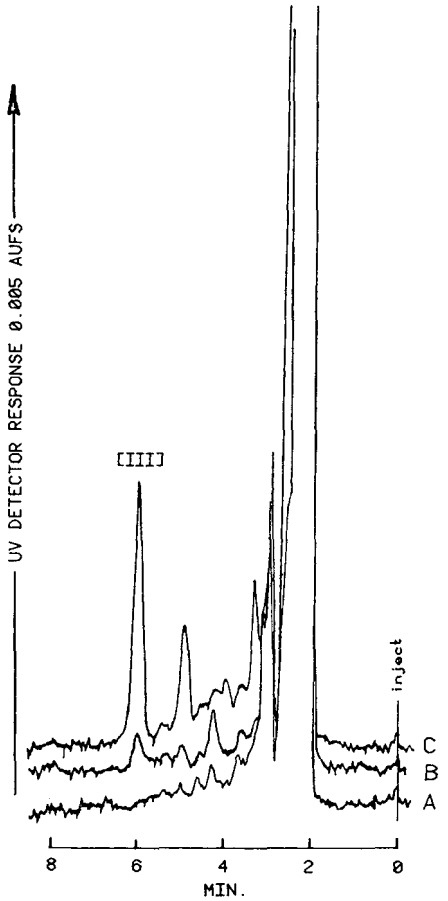

Fig. 3. Normal-phase chromatograms of human plasma: (A) control (20/200 μl injection aliquot); (B) subject, 2-hours, day 12, following multiple oral dosing of [I] · succinate (20/200 μl injection aliquot) (see text); and (C) control containing 250 ng [III] per ml (10/200 μl injection aliquot).

an extract from a subject following the administration of [I] · succinate, and (C) control plus added standards.

Application in biological samples

Plasma samples were collected immediately prior to and at 2, 6, 12, 18, 24, 48, and 72 h after the last 81.25-mg oral dose from two patients receiving cibenzoline · succinate for three days at each dose of 32.5 mg, 65.0 mg, and 81.25 mg q.i.d. The concentrations of [I] were measurable up to 72 h in subject 1 and up to 48 h in subject 2, with maximum observed plasma concentrations of 741 and 650 ng/ml (Fig. 4).

The ratio of metabolite [III]/cibenzoline [I], determined in subject 2 using the normal-phase HPLC assay showed only trace concentrations, i.e., 2—4% of [I] after chronic oral administration.

Concentrations of [I] and [III] in urine were also determined in a subject

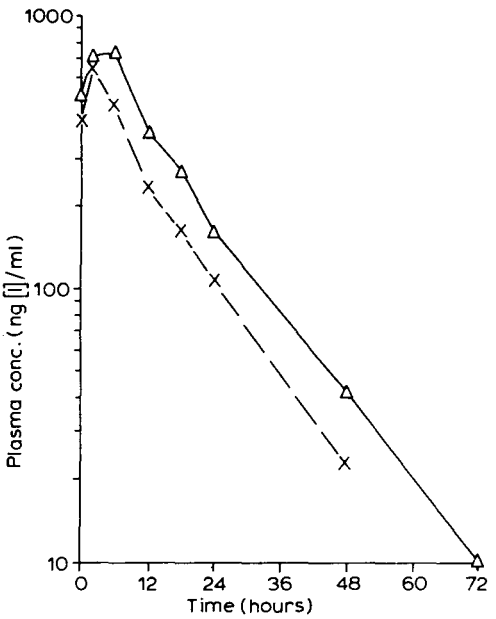

Fig. 4. Plasma concentration—time curves from two subjects following multiple oral dosing of [I] · succinate (see text).

TABLE II

URINARY EXCRETION PROFILE OF [I] AND [III] IN A SUBJECT FOLLOWING A SINGLE 65-mg ORAL DOSE OF [I]·SUCCINATE

Time interval (h)	Conc. [I] (μg/ml)	Conc. [III] (μg/ml)	Total mg [I]	Total mg [III]	Percent of dose as	
					[I]	[III]
0—2	35.2	1.11	5.28	0.169	11.3	0.38
2—4	17.0	0.52	1.70	0.052	3.80	0.12
4—8	19.6	0.52	4.40	0.117	9.83	0.26
8—24	6.11	0.30	6.05	0.297	13.5	0.67
24—48	0.44	N.M.*	0.41	—	0.91	—
				Total	39.34	1.43

*N.M. = ⩽ 0.05 μg/ml.

following a single 65-mg dose. The urine samples were collected immediately before and at 0—2, 2—4, 4—8, 8—24, and 24—48 h periods after the 65-mg dose. These samples were assayed for [I] and [III] using the ion-exchange and normal-phase HPLC methods, respectively. The results of the metabolic profile (Table II) showed approximately 40% of the dose excreted as [I] and approximately 1% of the dose as [III] over the excretion interval. Other subjects showed similar excretion profiles.

CONCLUSIONS

A sensitive and selective ion-exchange HPLC assay was developed for the determination of cibenzoline [I] in plasma and urine and was validated in the concentration range of 10 to 1000 ng/ml using UV detection at 214 nm. Removal of oxygen from the mobile phase through constant helium sparging and the use of special solvent lines is a requirement for the assay.

The assay was used to determine plasma and urine concentrations of [I] in human subjects following multiple oral dosing regimens of cibenzoline · succinate.

A normal-phase HPLC assay was also developed for the determination of the imidazole metabolite [III] and was applied to its quantitation in plasma and urine samples.

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DETERMINATION OF CEFTRIAXONE, A NOVEL CEPHALOSPORIN, IN PLASMA, URINE AND SALIVA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON AN NH_2 BONDED-PHASE COLUMN

V. ASCALONE* and L. DAL BÒ

Pharmacokinetics Laboratory, Department of Clinical Research, Prodotti Roche S.p.A., Piazza Durante 11, 20131 Milan (Italy)

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of a new cephalosporin antibiotic in plasma, urine and saliva (mixed saliva) using normal-phase technique and an NH_2 bonded-phase column. The eluent mixture was a combination of acetonitrile and an aqueous solution of ammonium carbonate. The rapid method involved precipitation of protein from fluids by means of acetonitrile followed by automatic injection of the supernatant. The detection limit was 0.4 μg/ml for plasma, 3 μg/ml for urine and 0.03 μg/ml for saliva using UV detection.

INTRODUCTION

The determination of cephalosporin antibiotics in biological fluids is often performed by microbiological assay procedures [1—4]. In recent years, high-performance liquid chromatography (HPLC) has proved to be a powerful analytical tool for measuring such drugs in biological fluids, because of its specificity, rapidity and sensitivity. HPLC methods for the determination of cephalosporins have been reported [5—7]; some of these deal with the use of ion-exchange columns [8], others with reversed-phase HPLC on C_{18} columns [9—12]. For the analysis of cephalosporins which are weak acids ($pK_a > 2$), reversed-phase chromatography with ion suppression is usually the method of choice [13]. For stronger acids ($pK_a < 2$), the ion-pairing technique is preferable [14—16].

According to various laboratory experiences [16, 17], ion-pair reversed-phase HPLC has some practical limitations: the salt content of the mobile phase can cause blocking in the pump and/or injector; R_4N^+ salts can be destructive to packing materials (even if buffered at pH 7), producing shrinkage

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Ceftriaxone (Rocephin®)

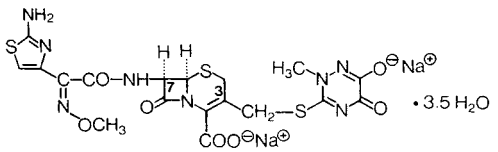

Fig. 1. Formula of ceftriaxone (Ro 13-9904).

of the reversed-phase material with deterioration of column performance. When low concentrations of ion-pairing salts are used, the time required for column—mobile phase equilibrium is often several hours at least; on the other hand, high ion-pair reagent concentrations are to be avoided because of possible column damage.

For pharmacokinetic studies with a new "third generation" parenteral cephalosporin, ceftriaxone (Ro 13-9904) (6*R*, 7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-{[(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-*as*-triazin-3-yl)-thio]methyl}-8-oxo-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2-carboxylic acid disodium salt (Fig. 1) with a potent in vitro activity against a wide range of bacteria [17—19] and long biological half-life [20, 21], we have developed a new HPLC method and compared it with the ion-pair HPLC method of Trautmann and Haefelfinger [16]. The method involves "normal-phase" HPLC performed on a polar alkylamino bonded-phase column (NH_2), connected to a UV spectrophotometric detector.

EXPERIMENTAL

Materials and reagents

Acetonitrile, HPLC grade was from E. Merck (Darmstadt, G.F.R.), ammonium carbonate, analytical reagent grade, from Carlo Erba (Milan, Italy); aqueous solutions of ammonium carbonate and the mobile phase for chromatography were prepared with HPLC-grade water produced by the Milli-Q system (Millipore, Bedford, MA, U.S.A.). Ceftriaxone (Ro 13-9904), pharmaceutical grade, was from Hoffmann-La Roche (Basel, Switzerland).

Stock solution of ceftriaxone

The solution contained 1 mg of ceftriaxone (the disodium salt, with 3.5 molecules of water of crystallization, Fig. 1) per ml of water and was stored at 0—5°C in the refrigerator. This solution was freshly prepared every week.

HPLC equipment and operating conditions

The analysis was carried out on a chromatographic system made up as follows: Model 410 constant-flow pump from Kontron (Zürich, Switzerland); Pye-Unicam variable-wavelength detector, Model 4020 (Cambridge, Great Britain) operating at a wavelength of 274 nm; ASI Model 45 sample processor (Kontron) with Rheodyne automatic injector (Berkely, CA, U.S.A.), a loop capacity of 20 or 50 μl; analytical column, 25 × 0.4 cm, Hibar® type filled with LiChrosorb (NH_2), 5 μm (Merck); precolumn (between the pump and

the injector) was 10 × 0.4 cm, filled with Corasil 37—50 μm from Waters Assoc. (Milford, MA, U.S.A.); guard column (between the injector and the analytical column), 3 × 0.4 cm from Merck, filled with Corasil 37—50 μm.

The eluent mixture containing acetonitrile—water—ammonium carbonate solution (10%, w/v) (70:26:4) was filtered through a 0.5-μm filter before use. The procedure was carried out at constant flow-rate of 1.5 ml/min, corresponding to a pressure of ca. 100 bar.

The UV detector was coupled to a chromatographic computer (Sigma 10, Perkin-Elmer, Norwalk, CN, U.S.A.) for the integration of peak areas and subsequent calculations, using the external standard method. The chromatograms were recorded on a PM 8252 recorder (Pye-Unicam). Under these conditions, the retention time of ceftriaxone was ca. 7.5 min.

During the night, the entire system was flushed with a mixture of acetonitrile—water (80:20, v/v) at a flow-rate of 0.1 ml/min; a mixture of methanol—water (90:10, v/v) was used to store the analytical column during periods of non-use, e.g., a weekend, and for long periods *n*-hexane is preferable.

Procedure for plasma samples

Test plasma (0.25 ml) was mixed with water (0.75 ml) either in a screw-capped test tube (PTFE-lined caps; Sovirel 13) for manual injection or in a cone-shaped vessel (3-ml vessels, cat. No. 3-39296, Supelco, PA, U.S.A.) for automated injection, and the mixture was homogenized by slow agitation. Acetonitrile (2 ml) was then added and the vial was shaken on a vortex mixer for about 30 sec. After centrifugation at 2200 *g* for 3 min, 20—50 μl of the clear supernatant were injected into the chromatographic system.

Specimens of control plasma (0.25 ml) and some (3—5) 0.25-ml specimens of the control which had been spiked with an aqueous solution of ceftriaxone giving plasma concentrations ranging from 5 to 250 μg/ml were processed along with unknown samples in the same way.

Procedure for urine samples

Urine samples were diluted with water (1—5 ml) and then processed as the plasma samples.

Procedure for saliva samples

Test saliva (0.5 ml) was mixed with acetonitrile (1 ml) in a cone-shaped vessel (3 ml) and shaken on a vortex mixer for about 30 sec. After centrifugation at 2200 *g* for 3 min, 100 μl of the clear supernatant were injected into the chromatographic system.

Specimen control saliva (0.5 ml) and some (2—3) 0.5-ml specimens of the control which had been spiked with an aqueous solution of ceftriaxone giving saliva concentrations ranging from 0.03 to 1 μg/ml were processed along with unknown samples in the same way.

The conditions for the saliva analysis had to be modified as follows: mobile phase, acetonitrile—water—ammonium carbonate (5% w/v) (70:26:4); UV detection wavelength 245 nm; loop capacity 100 μl. Under these conditions, the retention time of ceftriaxone was ca. 6.5 min.

Calculation

The Sigma 10 system computed peak areas; the corresponding concentrations of ceftriaxone were obtained by reference to an external standard calibration curve.

RESULTS AND DISCUSSION

Linearity

Using the external standard method, a linear correlation was obtained between the amount of ceftriaxone chromatographed and the relative peak area in the range 0.6—500 μg/ml of plasma (n = 8); r = 0.999972.

Sensitivity

The detection limit for plasma, urine and saliva samples was 0.4 μg/ml, 3 μg/ml and 0.03 μg/ml, respectively, with a signal-to-noise level of ca. 3:1.

Accuracy and precision

Accuracy experiments were performed on control human plasma and urine spiked with the drug. The accuracy for plasma, defined as [(the amount found)/(the amount added)] × 100, was found to be about 100% over a wide range of concentrations as shown in Table I. A similar extensive study has not yet been carried out for urine, although initial results were similar to those for plasma.

TABLE I

ACCURACY AND PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH CEFTRIAXONE

Quantity added (μg/ml)	n	Accuracy (%)	Intra-assay precision* (± %)	Instrument reproducibility** (± %)
225.0	6	96.4	0.7	0.4
112.5	2	97.3	—	—
45.0	6	95.5	0.6	0.45
22.5	2	94.7	—	—
12.0	2	99.0	—	—
6.0	6	100.2	1.2	1.0
2.4	2	102.4	—	—
0.6	6	100.0	4.0	2.5

*Refers to the standard deviation obtained after analysing six plasma samples having the same nominal concentration during one day.

**Refers to the standard deviation obtained by chromatographing each plasma extract six times over a one-day period.

The inter-assay precision, evaluated by analysing spiked plasma samples on different days over one week (n = 5), was found to be ± 0.7% and ± 5% for samples of nominal concentration 45 μg/ml and 0.6 μg/ml, respectively.

For saliva the overall accuracy was found to be 98% in the range 0.03—2 μg/ml (n = 6). The intra-assay precision was determined by analysing five

different spiked saliva samples on the same day containing 0.2 and 0.04 μg/ml; a relative standard deviation (C.V.) of ± 2% and ± 8%, respectively, was obtained.

The inter-assay precision was determined by analysing one specimen from each concentration (0.2 and 0.04 μg/ml) over five days within a period of two weeks; a relative standard deviation (C.V.) of ± 3.5% and ± 7.5%, respectively, was obtained.

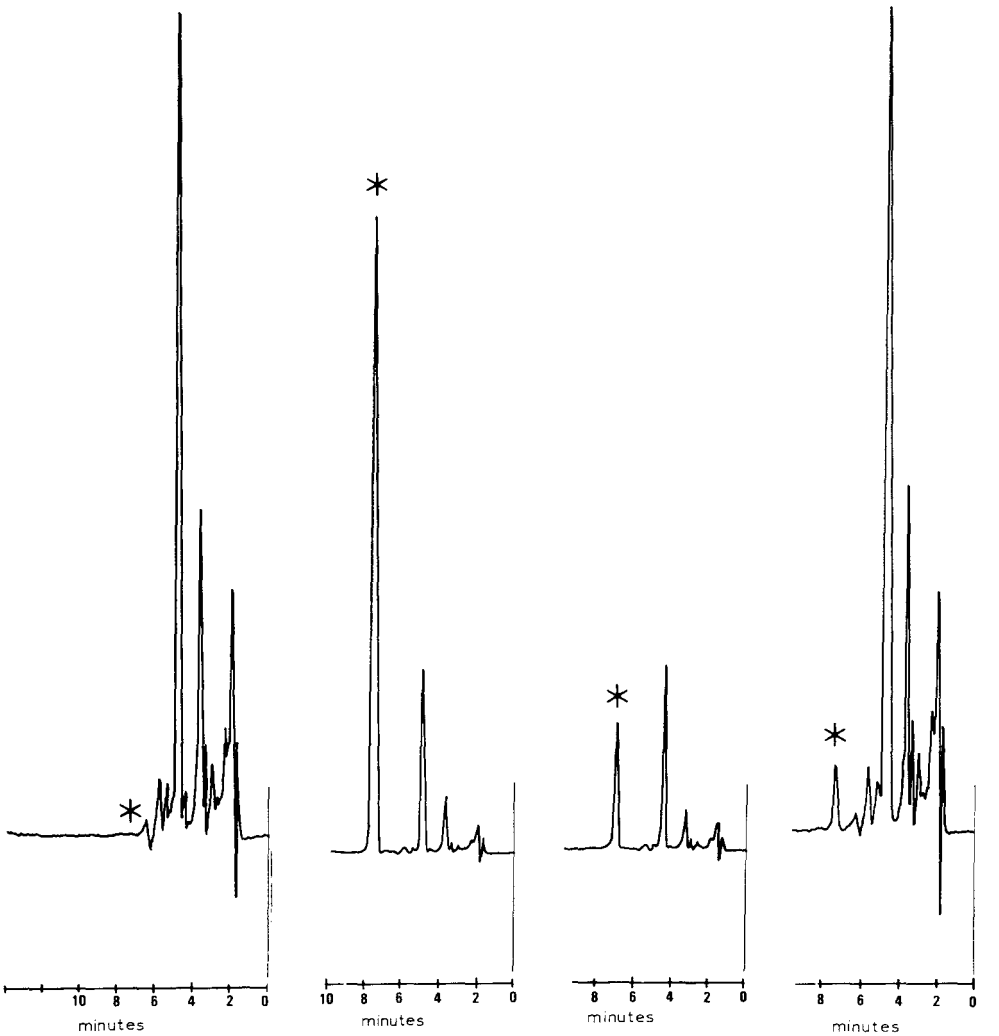

Fig. 2. Chromatogram of human drug-free plasma extract. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

Fig. 3. Chromatogram of authentic standard recovered from control plasma. Nominal concentration: 225 μg/ml. Loop = 20 μl, S = 0.08 a.u.f.s. (*), ceftriaxone.

Fig. 4. Chromatogram of authentic standard recovered from control plasma. Nominal concentration: 45 μg/ml. Loop = 20 μl, S = 0.08 a.u.f.s. (*), ceftriaxone.

Fig. 5. Chromatogram of authentic standard recovered from control plasma. Nominal concentration: 2.4 μg/ml. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

Stability

It has been reported that ceftriaxone is unstable in ethanol, and that sample solutions must be analyzed within two hours of preparation [16]. In the solution used in our method (acetonitrile—water) no stability problems were observed, either at 0—5°C after one week's storage or at room temperature after one day. In addition, these solutions were stable under normal laboratory lighting conditions.

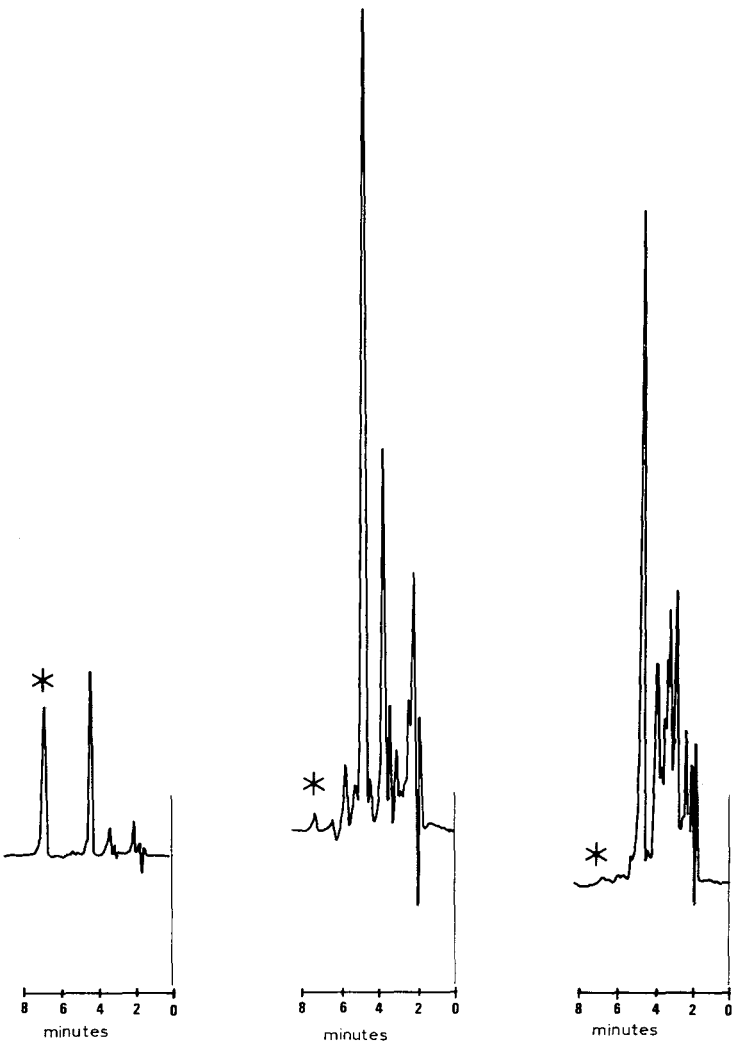

Fig. 6. Chromatogram of plasma extract from a patient receiving 1.0 g of ceftriaxone (intramuscularly) (first administration of a treatment), 8th-hour sample. Loop = 20 μl, S = 0.08 a.u.f.s. (*), ceftriaxone.

Fig. 7. Chromatogram from a spiked plasma sample (0.6 μg/ml) showing the detection limit of the method. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

Fig. 8. Chromatogram from a human drug-free urine extract. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

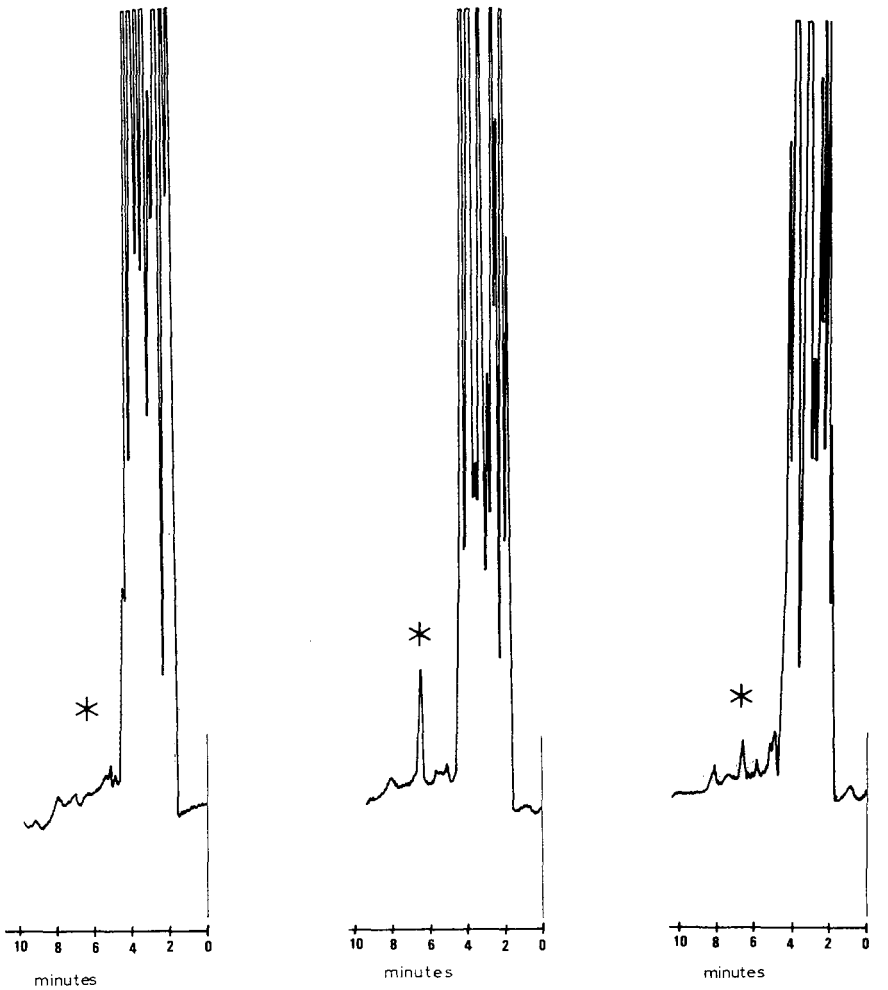

Fig. 9. Chromatogram of human drug-free saliva extract. Loop = 100 μl, S = 0.01 a.u.f.s. (*), ceftriaxone.

Fig. 10. Chromatogram of authentic standard recovered from control saliva. Nominal concentration: 0.2 μg/ml. Loop = 100 μl, S = 0.01 a.u.f.s. (*), ceftriaxone.

Fig. 11. Chromatogram of saliva extract from a volunteer receiving 1.5 g of ceftriaxone (intravenously); 8th hour after administration (concentration found: 0.06 μg/ml). (*), ceftriaxone.

Selectivity

A number of substances were investigated as possible internal standards. The following eluted with, or too close to, the solvent peak: sulfanilic acid, salicylic acid, 3,4,5-trimethoxybenzoic acid, nitrobenzoic acid, benzoic acid, phenoxyacetic acid, sulphanilamide, *p*-aminobenzoic acid. Salicylamide, barbituric acid, xanthanoic acid, 5-sulphosalicylic acid were retained on the column.

The selectivity of the method was tested with respect to other antibiotics.

Cefazolin, cefuroxim, desacetylcephalothin, 7-desacetylcephalothin, 7-aminocephalosporanic acid, cephalosporin C, and 7-desacetylaminocephalosporanic acid all eluted too close to, or with, the solvent peak. Ampicillin and 6-aminopenicillanic acid were retained.

Some examples of chromatograms are reported for plasma (see Figs. 2—7), urine (Fig. 8) and saliva (Figs. 9—11); these refer to the application of the method to control fluids, recovery studies and in vivo investigations.

Application of the method to biological specimens

The assay was applied to the quantitation of ceftriaxone in the plasma of a patient dosed (intramuscularly) with 1.0 g of ceftriaxone. In parallel, the determination of drug plasma levels was performed by ion-pair reversed-phase HPLC [16] (Table II). The results showed a good agreement between the two methods. No significant difference was found between the two methods

TABLE II

CEFTRIAXONE PLASMA LEVELS IN A PATIENT DOSED INTRAMUSCULARLY WITH 1.0 g OF CEFTRIAXONE

Time (h)	Concentration (μg/ml)	
	Normal-phase HPLC	Ion-pair HPLC
0.25	25.0	26.4
0.5	44.2	45.8
1.0	63.9	60.9
2.0	75.0	74.2
4.0	70.5	67.3
6.0	58.7	59.8
8.0	50.0	51.1
12.0	37.0	39.2
24.0	25.2	26.0

TABLE III

CEFTRIAXONE PLASMA AND SALIVA LEVELS IN A VOLUNTEER DOSED INTRAVENOUSLY WITH 1.5 g OF CEFTRIAXONE

Time (h)	Concentration (μg/ml)	
	Plasma	Saliva
0.25	236.8	0.35
0.5	211.8	0.31
1.0	181.2	0.27
2.0	143.3	0.20
4.0	112.5	0.16
6.0	84.4	0.11
8.0	71.0	0.12
12.0	45.7	0.08
24.0	25.9	0.05
36.0	8.0	0.03
48.0	2.0	Not detectable

at the 95% confidence level performing Student's t-test; the regression coefficient was $r = 0.99678$.

Another example is reported (Table III), dealing with plasma and saliva levels of a volunteer dosed (intravenously) with 1.5 g of ceftriaxone; in this case only our method was used since the ion-pair reversed-phase method [16] was not sensitive enough for saliva samples. Until now, very few antibiotics have been determined in mixed saliva [22] mainly because of the inadequate sensitivity, accuracy, and precision of the analytical methodology [23].

Tests performed on an NH_2 column and general comments

The NH_2 phase is frequently used for the separation of saccharides using an acetonitrile—water mixture [24]; in fact the NH_2 group displays a preferential interaction with the hydroxyl group of carbohydrates [25].

In so far as the retention of ceftriaxone is reduced with increasing water content in the mobile phase, the column is of the normal-phase type. In addition, increasing the proportion of ammonium carbonate in the eluent mixture reduced the retention time as reported (Table IV).

Many advantages resulted from the use of the proposed method in comparison to ion-pair HPLC methods; the column could be conditioned rapidly and it retained its performance over a three-week period, during which time 40 biological samples per day were analysed. No blocking of frits, phase shrinkage, or back-pressure problem was experienced.

TABLE IV

EFFECT OF AMMONIUM CARBONATE CONTENT IN THE MOBILE PHASE ON RETENTION TIME OF CEFTRIAXONE

Water (%)	Acetonitrile (%)	Ammonium carbonate (g per 100 ml of mixture)	Retention time (min)
25	75	—	Retained on column
25	75	0.1	Retained on column
25	75	0.2	22
25	75	0.3	14
25	75	0.4	12.5
25	75	0.5	11.5

After a month of non-use, the column was re-tested by analysing plasma and saliva specimens; the performance of the column had not deteriorated.

The method is rapid; no time-consuming sample preparation operations are required and the protein denaturation step is performed using an aqueous acetonitrile solution completely compatible with the mobile phase.

The retention of time of ceftriaxone did not change by more than 3% in one day. In our hands, much greater variation was observed using the ion-pair method.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF DIPOTASSIUM CLORAZEPATE AND ITS MAJOR METABOLITE NORDIAZEPAM IN PLASMA

PATRICK COLIN and GERARD SIROIS*

Faculté de Pharmacie, Université de Montréal, C.P. 6128, Succ. A, Montréal, Québec H3C 3J7 (Canada)

and

JACQUES LELORIER

Département de Pharmacologie, Faculté de Médecine, Université de Montréal, Québec H3C 3J7 (Canada)

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method is described for the quantitative analysis of dipotassium clorazepate (CZP) and its major metabolite nordiazepam (ND) in fresh human and dog plasma. The method consists of two separate selective ND extractions from a plasma sample without and with conversion of all the CZP to ND. For quantitation, diazepam (DZP) is used as the internal standard. The chromatographic phase utilized in a reversed-phase Hibar® EC-RT analytical column prepacked with LiChrosolv RP-18 with a solvent system consisting of acetonitrile—0.05 *M* sodium acetate buffer, pH 5.0 (45:55). The UV absorbance is monitored at 225 nm using a variable-wavelength detector. The mean assay coefficient of variation over a concentration range of 20—400 ng per ml of plasma is less than 3% for the within-day precision. Recoveries of ND, DZP and CZP (as ND) are essentially quantitative at all levels investigated. The calibration curves of ND are rectilinear ($r^2 = 0.99$) from the lower limit of sensitivity (2 ng/ml) to at least 2000 ng/ml in plasma. Applicability of the method to CZP and ND disposition studies in the anaesthetized mongrel dog is illustrated. When the two separate selective nordiazepam extractions from plasma cannot be performed immediately after blood sampling, an extrapolation kinetic method is suggested for the estimation of CZP concentration. In all previous in vivo studies, CZP has been determined only with gas—liquid chromatographic methods.

INTRODUCTION

Dipotassium clorazepate (CZP) is a 1,4-benzodiazepine antianxiety agent used in the management of many psychiatric disturbances [1]. The drug

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rapidly decarboxylates to nordiazepam (ND) in aqueous media, as can be found in the stomach [2]. Decarboxylation also occurs in the plasma but at a much lower rate. It is considered that CZP serves as a prodrug for active ND [3].

Different gas—liquid chromatographic (GLC) procedures, using electron-capture detection, have been suggested for the determination of CZP and/or ND in plasma with a reported sensitivity of 10 ng/ml after conversion of ND to a benzophenone [4] and 5 ng/ml without transformation of ND [5—7]. The plasma clorazepate levels reported from these GLC measurements were estimated from the difference between the total measured ND levels obtained by total acid conversion of CZP to ND, and the ND levels measured immediately after blood sampling and selective ND extraction [4—7].

Some papers have described reversed-phase liquid chromatography methods with UV detection at a fixed wavelength of 254 nm for the determination of ND in plasma with a sensitivity limit of 2, 20 and 50 ng/ml [8—10], respectively. The lowest sensitivity limit of 2 ng/ml was obtained for a 2-ml sample of plasma [8]. Brodie et al. [8] also report an interfering peak in control plasma having the same retention time of diazepam (DZP). In the method of Wallace et al. [10], a column temperature control system, which may not always be available, was used, and norchlordiazepoxide was reported to interfere with DZP under the column conditions described. With a variable-wavelength detector, a poor sensitivity limit of 30—80 ng/ml was reported at 230 nm [11] and 240 nm [12, 13].

In all previous in vivo studies [4, 5, 14], CZP has been determined only with GLC methods with a sensitivity limit of 5 ng/ml when expressed as ND [5—7]. In every case, the plasma sample had to be analyzed very shortly after blood sampling. On the other hand, it is to be noted that GLC methods are often associated with the problems of poor peak symmetry or tailing for ND. These latter difficulties are however eliminated with high-performance liquid chromatographic (HPLC) methods [10].

This paper describes a rapid, selective and more sensitive liquid chromatographic assay using a UV variable-wavelength detector set at 225 nm for analysis of ND in fresh human or dog plasma. It also shows its applicability for CZP determination in pharmacokinetic studies from measurement of total and actual ND plasma levels where the expression of total level is used for the combined levels of parent drug and metabolite measured as ND. In this procedure, the selective extraction of actual plasma ND concentration must be performed immediately after blood sampling. However, by considering the fact that this latter condition cannot always be met in clinical practice, an extrapolation kinetic method has been developed and is proposed here, in order to circumvent this difficulty. The technique is based on the equation for the first-order decarboxylation reaction kinetics of CZP [15].

EXPERIMENTAL

Reagents and chemicals

Diazepam, flurazepam, oxazepam and N-desalkylflurazepam were gifts of Dr. G. Caillé (Department of Pharmacology, University of Montreal). Nor-

diazepam, prazepam and pure dipotassium clorazepate were kindly donated by Hoffman-LaRoche (Vaudreuil, Canada), Parke Davis & Co. (Brockville, Canada) and J.J. McGilveray (Health Protection Branch, Ottawa, Canada), respectively. Tranxene® capsules were from Abbott Laboratories (Montréal, Canada). Pentobarbital and cimetidine were from C.D.M.V. (St-Hyacinthe, Canada) and Smith, Kline and French (Toronto, Canada). Acetonitrile, LiChrosolv grade, was obtained from BDH Chemicals (Toronto, Canada). Hexanes (mixture of isomers) and ethyl acetate, pesticide grade, were purchased from Fisher Scientific, Cat. No. H-300 and E-191, respectively (Montréal, Canada). Glacial acetic acid, analyzed reagent, aldehyde free, was purchased from J.T. Baker (Montréal, Canada). Glycine, reagent grade, was obtained from Fisher Scientific. All other reagents used were standard analytical grade.

The 0.05 *M* acetate buffer solution, pH 5.0, was prepared by adjusting the pH of an 800-ml aqueous acetic acid solution at 6.25×10^{-2} *M* by additon of a 1.0 *M* sodium hydroxide solution and of distilled water to complete the volume to 1 l. The 2.0 *M* glycine buffer solution, pH 9.0, was prepared by addition of a 1.0 *M* sodium hydroxide solution to an aqueous solution of glycine. The standard, DZP, was added to the extraction solvent mixture (60 ng/ml) to produce working standard plasma solutions with a concentration of 600 ng/ml.

Apparatus

The experiments were accomplished with a Varian liquid chromatograph Model 5010 (Varian, Palo Alto, CA, U.S.A.). The chromatograph was equipped with a Valco injector and a Varichrom variable-wavelength UV detector (Varian) set at 225 nm and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 56 recorder. The separations were performed on a reversed-phase Hibar® EC.RT column prepacked with LiChrosorb RP-18 (10 μm particle size, 250 × 4 mm I.D.; E. Merck, Darmstadt, G.F.R.).

Chromatographic conditions

The mobile phase, consisting of acetonitrile—0.05 *M* sodium acetate aqueous buffer, pH 5.0 (45:55, v/v) was delivered at a flow-rate of 1.5 ml/min. The resulting pressure was 50.6 bar. For the detector, the wavelength was set at 225 nm, the band width at 8 nm, the time constant at the slow position and the absorbance range at the 0.01—0.2 interval. The recorder was set at 1 mV full scale deflection and chart speed at 5 mm/min.

Glass tubes used in the extraction procedures were soaked for over 12 h in 70.4% nitric acid and rinsed in distilled water. All liquid volumes were delivered using Gilson automatic pipettes P20 to P1000.

Preparation of plasma standard solutions

A standard solution of ND was prepared in acetonitrile at a concentration of 5 mg/l. Aliquots of increasing volumes (10—300 μl) of this solution were evaporated to dryness at 38°C (Buchler rotary Evapo-mix; Buchler Instruments, Fort Lee, NJ, U.S.A.) in 15-ml screw-capped test tubes and 1 ml of fresh drug-free plasma (human or canine) was added in each to give samples for analysis in the range 50—1500 ng/ml.

Extraction procedure

To each of the above 1-ml plasma standard solutions (or samples to be assayed), 0.5 ml of 2 *M* glycine buffer, pH 9.0 was added with vortex mixing after each addition. A 10-ml aliquot of the solvent mixture of extraction (hexane—ethyl acetate, 70:30, v/v) containing 60 ng of DZP per ml were added and again each tube was mixed briefly. All tubes were then mixed in an Eberbach multi-tube reciprocal shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 10 min. All tubes were then centrifuged at 320 *g* for 5 min. A portion (approximately 8 ml) of the upper layer (organic phase) was transferred to a 15-ml centrifuge tube and the solvents evaporated to dryness at 38°C in the Buchler rotary Evapo-mix. To the dry residue, 500 μl of ethyl acetate were added, vortexed for 20 sec and evaporated to dryness. The latter step is done to make sure that all the ND and DZP possibly deposited on the upper inner walls of the tubes would be dissolved and drained in the bottom, allowing complete recaptation with mobile phase prior to injection in the liquid chromatograph. This residue was thus reconstituted in 100 μl of column eluent solution (acetonitrile—0.05 *M* sodium acetate buffer, pH 5.0, 45:55, v/v) and 50 μl were injected into the liquid chromatograph for quantitation of ND.

Determination of actual and total ND in dog plasma

Venous blood was withdrawn at different time intervals, in heparinised Vacutainer® tubes, from an anaesthetized mongrel dog after having received a single intravenous dose of 0.5 mg/kg of CZP. Blood samples were immediately centrifuged (within 1 min) at 560 *g* in a refrigerated centrifuge set at 5°C for 9 min. Here, a delay for blood centrifugation would increase the amount of in vitro CZP hydrolysis to ND. Four 1-ml plasma samples were immediately transferred into four different screw-capped test tubes where two of them contained 0.5 ml of cold 2 *M* glycine buffer, pH 9.

The two 1-ml plasma samples added to cold glycine buffer are promptly analyzed for actual ND. To each of these test tubes, the extraction solvent mixture (hexane—ethyl acetate) is added as described above for the quantitation of actual ND.

The other two 1-ml plasma samples are then analyzed for total ND (actual ND and ND from CZP hydrolysis). For this, 150 μl of 1 *N* hydrochloric acid were added to both tubes which were vortexed for 15 sec and then incubated at 37°C for 1 h to allow complete hydrolysis of unchanged CZP. After cooling the test tubes to room temperature, 150 μl of 1 *N* sodium hydroxide and 0.5 ml of 2 *M* glycine buffer, pH 9 were added with vortex mixing. The total ND is then extracted as described above and quantitated.

Determination of CZP in plasma

Plasma CZP concentrations were obtained from the difference between total and actual ND plasma concentrations. This difference must be multiplied by 1.51 to correct for difference in the molecular weight of CZP and ND.

Calculations for quantitation of ND

Standard curves were drawn using the measured peak height ratios of ND

to DZP against the known concentrations of ND in the standard plasma samples. The equations of the standard lines were obtained by least-squares regression analysis and were used to determine ND concentrations in unknown plasma samples.

Conversion of CZP to ND

A solution of pure CZP in bicarbonate buffer, pH 9.5 (7.5 mg per 100 ml) was prepared and diluted in fresh human plasma to give plasma concentrations of 75, 150, 300, 450 and 750 ng/ml of CZP that are equivalent to 50, 100, 200, 300 and 500 ng/ml of ND. These plasma samples were analyzed in duplicate for total ND after acid conversion of CZP to ND as described above.

Intravenous solution of CZP

The intravenous solution of dipotassium clorazepate was prepared, just before use, by dissolving the content of two 15-mg capsules of Tranxene® in 30 ml of a 0.005 *M* isotonic phosphate buffer, pH 7.0. The resulting solution was then immediately filtered on a HA type Millipore filter (Millipore, Mississauga, Canada) with pore sizes of 0.45 μm. This solution was found to be stable enough for in vivo work as no formation of ND could be measured within 10 min after its preparation.

Extrapolation kinetic method

A solution of pure CZP in bicarbonate buffer, pH 9.5 (20 mg per 200 ml) was prepared and 45—225 μl of this stable solution were immediately added to 30 ml of fresh drug-free human plasma to give plasma concentrations of 150, 300, 500 and 750 ng/ml of CZP. To three of these mixtures, a known amount of ND was added (100 ng/ml). The time t was noted and measured after CZP addition. The synthetic mixtures were kept at refrigerative temperature of 4°C and 1-ml plasma samples in duplicate were taken at times corresponding to 1, 3 and 5 h after their preparation. These specimens were then subjected to total and actual ND analysis as described above, in order to calculate the residual CZP concentration. The initial plasma concentration of CZP, C^0_{CZP}, was estimated from a linear regression analysis of the logarithm of the residual plasma concentration of CZP, C_{CZP}, versus time t, followed by an extrapolation of C_{CZP} to zero time:

$$\log C_{CZP} = \log C^0_{CZP} - k\, t/2.3$$

where k represents the first-order rate constant of transformation of CZP to ND.

RESULTS AND DISCUSSION

HPLC of some benzodiazepines has been performed on a reversed-phase column. A mobile phase was chosen by taking into consideraiton the following criteria: simplicity, retention times of the studied compounds, intracolumn pressure produced and solvent toxicity, polarity, viscosity and analysis sample solubility. A mixture of a 0.05 *M* sodium acetate buffer solution (pH 5)—acetonitrile (55:45, v/v) demonstrated the best resolution between

ND and DZP peaks with a total analysis time of less than 10 min (Fig. 1). The mobile phase was relatively insensitive to small variations of pH and in the acetate buffer concentration. The retention times of the drugs tested in this study are listed in Table I. Flurazepam, which has a retention time of 3.6 min here, was not eluted from the column or was found to have a retention time of 41 min under the conditions used by Brodie et al. [8] and MacKichan et al. [9], respectively. None of the drugs tested (Table I) represents a risk of interference with the analysis of ND or DZP. The absorbance of ND at 225 nm was found to be at a maximum under the conditions chosen here. At 254 nm, the wavelength commonly used in fixed-wavelength detectors, the absorbance represents 1/3 of the absorbance observed at 225 nm. For fifteen drug-free fresh human and canine blood samples processed, it was found that normal plasma components in the extract did not interfere with the peaks of ND and DZP. Although chromatographic background is negligible with fresh plasma, this was not always the case with frozen specimens. The buffers and solvent mixture of extraction chosen here were however found to lead to a minimum of interference in the analysis. On the other hand, benzene, when tested as a solvent of extraction, led to a significant interference in the assay

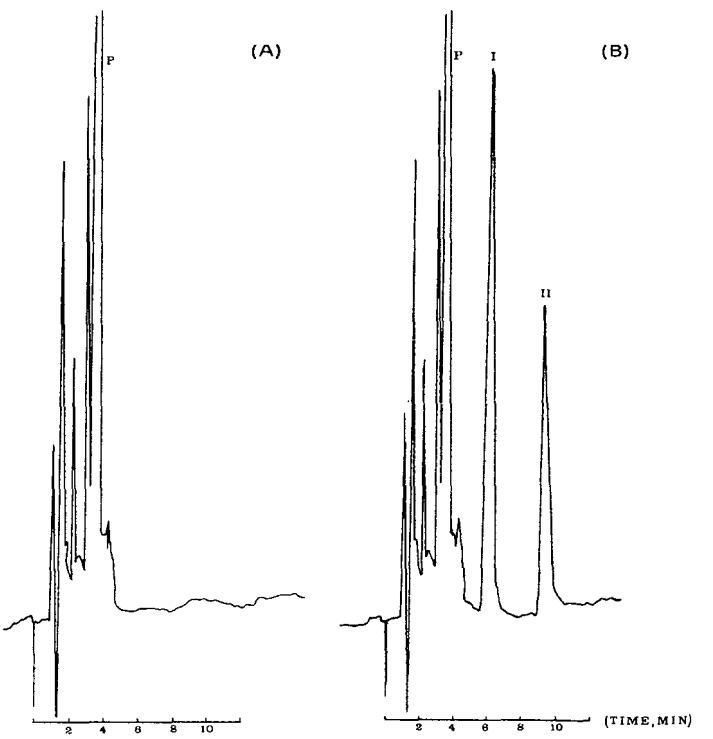

Fig. 1. Chromatograms of blank plasma extract and of a plasma extract of nordiazepam and diazepam. (A) Chromatogram of an extract of diazepam- and nordiazepam-free plasma; (B) chromatogram of an extract of the same plasma sample containing 600 ng/ml of nordiazepam and 600 ng/ml of diazepam. Peaks: P = pentobarbital; I = nordiazepam; II = diazepam.

TABLE I

RETENTION TIMES FOR SOME DRUGS

Drugs	Retention time (min)
Dipotassium clorazepate	2.1
Flurazepam	4.0
N-Desalkylflurazepam	3.8
Pentobarbital	3.6
Cimetidine	4.8
Oxazepam	5.0
Nordiazepam	6.2
2-Amino-5-chlorobenzophenone	7.5
Diazepam	9.2
Prazepam	14.5

on frozen plasma samples. Finally, it is to be noted that, in general, interference in frozen plasma samples was reduced by thawing them rapidly.

The precision of assay for ND was assessed by repeated analyses on fresh drug-free human plasma specimens spiked with known concentrations of ND. As shown in Table II, within-day and day-to-day precision of the method, as measured by the coefficient of variation, ranged from 1.65 to 2.84% and 2.94 to 9.82%, respectively.

TABLE II

REPRODUCIBILITY OF PLASMA NORDIAZEPAM ASSAY

	Concentration (ng/ml)	n^*	C.V. (%)
Within-day	20	10	2.84
	200	10	1.65
	400	10	2.42
Day-to-day	100	5	9.82
	300	5	2.94
	1200	5	4.26

*n = number of samples.

The minimum measurable concentration of ND for this procedure is 2 ng/ml of original plasma sample, allowing a signal-to-noise ratio of 5. The limits of detection of ND, setting the detector sensitivity at 0.005 full scale and allowing a signal-to-noise ratio of 2, is 1 ng/ml. The sensitivity of this HPLC procedure is thus found to be much higher than those obtained previously (30—80 ng/ml), when using a variable-wavelength detector [11—13]. With a fixed-wavelength detector, the lowest sensitivity reported (2 ng/ml), was obtained with a 2-ml plasma sample [8] instead of a 1-ml plasma sample.

The accuracy of the assay for ND was evaluated from three series of five

spiked fresh drug-free human plasma with concentrations of 85, 700 and 1250 ng/ml. The mean relative errors between the observed and the theoretical true concentration, were found to be 1.82, 2.34 and 5.34%, respectively.

For determination of analytical recovery of ND and DZP, the drugs were added to a solvent mixture of the mobile phase and to fresh drug-free human plasma to achieve concentrations of 50 and 500 ng/ml for ND and 500 ng/ml for DZP. The recovery for ND and DZP was then estimated by comparing peak heights of ND or DZP after the extraction step, where peak heights are corrected for the carefully measured aliquot taken for assay, with peak heights obtained after injection of the standard solutions containing known concentrations of ND or DZP. Recovery for ND and DZP ranged from 101.2 to 101.6%.

The calibration curves were found to be linear for a concentration of 2—2000 ng of ND per ml of fresh drug-free human or canine plasma. The cor-

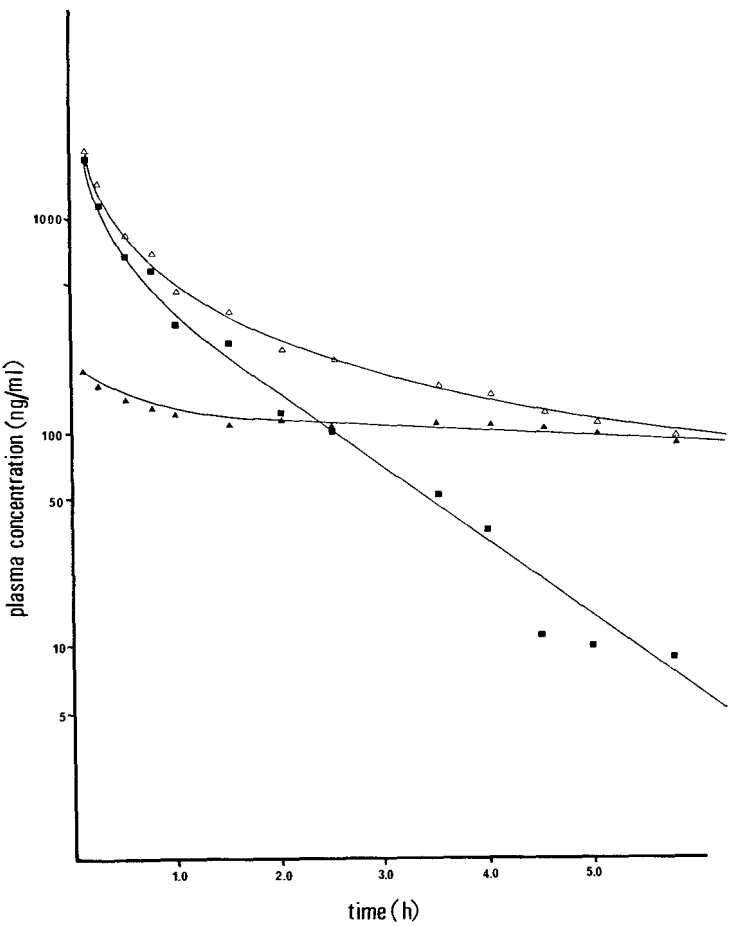

Fig. 2. Plasma concentration of dipotassium clorazepate, actual and total nordiazepam after a single intravenous dose of 0.5 mg/kg of dipotassium clorazepate in an anaesthetized mongrel dog. ■, CZP; ▲, actual ND; △, total ND.

relation coefficient obtained from the linear regression was always greater than 0.99. In a representative calibration curve, the linear equation, $Y = 0.0031 X + 0.0004$ (X = 50—1200 ng/ml, $n = 6$, $r = 0.9993$) was found. The 95% confidence intervals for the slope and the ordinate intercept were 0.0001 and 0.1091, respectively.

The lifetime of the column (which is 5 cm shorter and less expensive than those used in refs. 8—10) appears to be very good, as it is still in an excellent condition after a 1-year use in an applied research project.

The degree of acid conversion of CZP to ND was evaluated from fresh drug-free human plasma spiked with pure CZP to achieve concentrations of 75, 150, 300, 450 and 750 ng/ml. These samples were then analyzed in duplicate for ND according to the procedure of acid conversion of CZP to ND. Conversion to ND ranged from 100.5 to 102.4%. A detectable amount of the 2-amino-5-chlorobenzophenone, as a possible acid conversion product of ND, was not observed. Injection of 50 µl of a fresh solution of pure CZP (0.5 mg %) dissolved in a bicarbonate buffer pH 9.5 showed that the amount of ND was less than 1% when the solution was injected at 0, 5 and 10 min after solution preparation. These data indicate that the drug sample used was relatively pure and that CZP was relatively stable under these conditions of the HPLC method.

When applied to the collected plasma samples of an anaesthetized mongrel dog after receiving a single intravenous dose of CZP, it was found that the distribution and elimination of CZP could be described by an open two-compartment pharmacokinetic model (Fig. 2). The terminal elimination half-life of CZP, actual ND and total ND were estimated to be 0.84, 5.02 and 3.55 h, respectively.

The evaluation of the extrapolation kinetic method, for cases when the two separate selective ND extractions from plasma cannot be performed immediately, is illustrated in Fig. 3. Fig. 3 reveals that the kinetics of CZP

TABLE III

ACCURACY AND PRECISION OF THE EXTRAPOLATION KINETIC METHOD

Theoretical concentration* (ng/ml)			Percent of concentration found	
CZP	ND		Total ND**	CZP
750	0		108.45	95.46
500	100		101.76	94.43
300	100		107.10	93.82
150	100		111.66	86.42
		$\bar{X}$***	107.24	92.53
		S.D.	4.13	4.13
		C.V.	3.85	4.46

*CZP concentrations of 750, 500, 300 and 150 ng/ml are equivalent to 469.69, 331.13, 198.68 and 99.34 ng of ND per ml.

**Average of the values obtained at 1, 3 and 5 h.

***$\bar{X}$ = arithmetic mean; S.D. = standard deviation; C.V. = coefficient of variation (%).

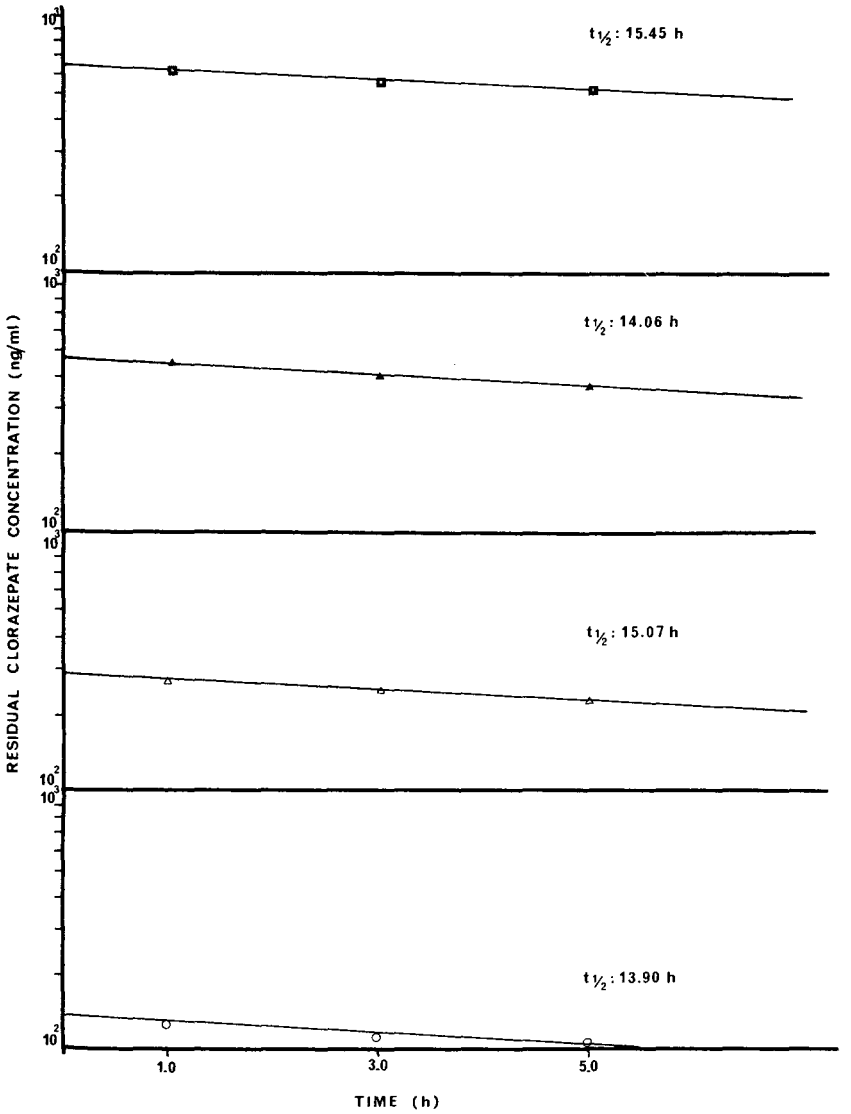

Fig. 3. First-order CZP decarboxylation plot for the estimation of CZP plasma concentration at time of blood sampling. Theoretical initial concentrations: ■, 750 ng CZP/ml; ▲, 500 ng CZP/ml and 100 ng ND/ml; △, 300 ng CZP/ml and 100 ng ND/ml; ○, 150 ng CZP/ml and 100 ng ND/ml.

decarboxylation is first-order at all CZP plasma concentrations tested here with or without addition of ND. The average half-life for the decarboxylation process in plasma at 4°C was found to be 14.62 h (S.D. = 0.76, C.V. = 5.18%). As shown in Table III, this study indicated that plasma concentrations of CZP and total ND can be determined with a relatively good accuracy and precision. The average deviations from theoretical plasma concentrations were found to be 7.47 and 7.24% for CZP and ND measurements, respectively.

In clinical practice, the application of this extrapolation kinetic method would imply that after blood sampling, the time would have to be recorded, the blood kept and centrifuged at 4°C, and the plasma separated and stored at 4°C until completion of the analysis on plasma specimens sampled at convenient and appropriate time intervals.

CONCLUSION

The HPLC procedure proposed here is a rapid, sensitive and reproducible technique for the determination of ND and CZP. The sensitivity of the method is particularly better than that reported previously with GLC for ND and CZP, and with HPLC for ND when using a variable-wavelength UV detector. The selectivity of the method, for benzodiazepines, may also represent an alternative to other HPLC procedures when an interaction with another compound is noted and found to be undesirable. The extrapolation kinetic technique appears to be a new valuable tool for estimating CZP when it is not possible to determine that compound immediately after blood sampling. Preliminary studies indicate that the method can be used in pharmacokinetic studies and can be adapted to analysis of other benzodiazepine compounds. The chromatograms are interference-free from normal components of fresh human and canine plasma as well as from some drug metabolites and commonly used drugs.

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SIMULTANEOUS DETERMINATION OF AMIODARONE AND ITS MAJOR METABOLITE DESETHYLAMIODARONE IN PLASMA, URINE AND TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

T.A. PLOMP*

Centre for Human Toxicology, University of Utrecht, Vondellaan 14, 3521 GE Utrecht (The Netherlands)

M. ENGELS and E.O. ROBLES DE MEDINA

Department of Cardiology, Academic Hospital, Catharijnesingel 101, Utrecht (The Netherlands)

and

R.A.A. MAES

Centre for Human Toxicology, University of Utrecht, Vondellaan 14, 3521 GE Utrecht (The Netherlands)

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method for the simultaneous assay of amiodarone and desethylamiodarone in plasma, urine and tissues has been developed. The method for plasma samples and tissue samples after homogenizing with 50% ethanol, involves deproteinization with acetonitrile containing the internal standard followed by centrifugation and direct injection of the supernatant into the liquid chromatograph. The method for urine specimens includes extraction with a diisopropyl ether—acetonitrile (95:5, v/v) mixture at pH 7.0 using disposable Clin-Elut 1003 columns, followed by evaporation of the eluate, reconstitution of the residue in methanol—acetonitrile (1:2, v/v) mixture and injection into the chromatograph. Separation was obtained using a Radial-Pak C_{18} column operating in combination with a radial compression separation unit and a methanol—25% ammonia (99.3:0.7, v/v) mobile phase. A wavelength of 242 nm was used to monitor amiodarone, desethylamiodarone and the internal standard. The influence of the ammonia concentration in the mobile phase on the capacity factors of amiodarone, desethylamiodarone and two other potential metabolites, monoiodoamiodarone (L6355) and desiodoamiodarone (L3937) were investigated. Endogenous substances or a variety of drugs concomitantly used in amiodarone therapy did not interfere with the assay.

The limit of sensitivity of the assay was 0.025 μg/ml with a precision of ± 17%. The

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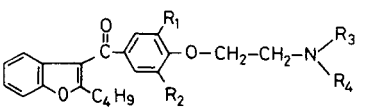
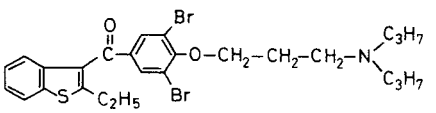
inter- and intra-day coefficient of variation for replicate analyses of spiked plasma samples was less than 6%. This method has been demonstrated to be suitable for pharmacokinetic and metabolism studies of amiodarone in man.

INTRODUCTION

Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)benzofuran, Fig. 1] has antianginal and antiarrhythmic properties [1]. It is used widely in Europe and South America for the treatment of ventricular and supraventricular arrhythmias, especially when resistant to conventional antiarrhythmic agents [2—7]. In addition, it is widely considered the drug of choice for treatment of tachycardias associated with the Wolff-Parkinson-White syndrome [4].



Compound	R_1	R_2	R_3	R_4
Amiodarone L3428	I	I	C_2H_5	C_2H_5
Desethylamiodarone L 32812	I	I	C_2H_5	H
L 6355	I	H	C_2H_5	C_2H_5
L 3937	H	H	C_2H_5	C_2H_5



Internal standard, L 8040

Fig. 1. Chemical structures of (top) amiodarone, desethylamiodarone, two potential metabolites, and (bottom) internal standard.

Amiodarone is well tolerated and does not usually cause serious untoward effects with maintenance doses of up to 600 mg per day. Adverse effects attributed to amiodarone include photosensitivity, a bluish skin discoloration, formation of corneal micro deposits and disturbances of thyroid function, which appear to be dose- and time-related and reversible upon cessation of treatment [1, 8—12]. From clinical practice it can be suspected that the drug is accumulated in the body and has a long "therapeutic" half-life since optimal antiarrhythmic effects may only be seen days to weeks after initiation of treatment and may persist for weeks after discontinuation of therapy [4]. However, little is known about plasma levels, elimination half-life and body distribution of the drug or the relationship between plasma concentrations and suppression of arrhythmias [13—19]. Amiodarone is metabolised by N-deethylation, and the desethyl metabolite (Fig. 1) is detectable in the plasma of patients on long-term treatment [13, 14]. Data on the pharmacokinetic properties of desethylamiodarone, however, are scarce [16, 18].

In order to assess the above-mentioned pharmacokinetic parameters of amiodarone and desethylamiodarone and to study the correlation between plasma levels and clinical effects, a fast, specific and sensitive method for the assay of amiodarone and desethylamiodarone in biological material is required. Up to now four high-performance liquid chromatographic (HPLC) methods for the determination of amiodarone in plasma or serum have been published [13—15, 19]. However, their applicability for the analysis of both amiodarone and desethylamiodarone in plasma, urine and tissues is questionable. The disadvantages of these methods include lack of use of internal standard [14], relatively complex and time-consuming sample preparation [15], applicability limited to amiodarone assay [13—15, 19] or plasma samples [13, 15] only. Furthermore, only one of the published methods has shown its feasibility for tissue analysis of amiodarone [19].

Therefore, a rapid, simple and sensitive HPLC procedure for the simultaneous determination of amiodarone and its major metabolite desethylamiodarone in plasma, urine and tissues has been developed.

EXPERIMENTAL

Chemicals and reagents

Amiodarone hydrochloride (L3428), desethylamiodarone oxalate (L3812), the internal standard [2-ethyl-3-(3,5-dibromo-4β-dipropylaminopropoxylbenzoyl)benzothiophene, L8040], and the additional potential metabolites L3937 and L6355 (Fig. 1), were obtained from Labaz (Maassluis, The Netherlands). Analytical reagent grade methanol and HPLC grade acetonitrile were purchased from Baker (Deventer, The Netherlands). Ethanol and diisopropyl ether were of analytical grade and were obtained from Merck (Darmstadt, G.F.R.).

Ammonia 25% and ethanol 50% were prepared by dilution with bidistilled water of ammonia 33% (Merck, Art. No. 5426) and ethanol (Merck), respectively. Phosphate buffer, pH 7.0, was prepared by dissolving 7.72 g of disodium hydrogen phosphate·$2H_2O$ (Merck) and 3.18 g of potassium biphosphate in 1 l of bidistilled water. Clin-Elut (CE 1003) extraction columns were obtained from Analytichem International (Lawndale, CA, U.S.A.).

Instruments

The HPLC system consisted of a Model 6000 A solvent delivery pump, a Model U6K injector, a guard column, a radial compression separation unit and a Model 450 variable-wavelength detector, all from Waters Assoc. (Milford, MA, U.S.A.). The radial compression separation unit consisted of a 10 cm × 8 mm I.D. Radial-PAK C_{18} (10 μm RP-18) cartridge and a Model RCM-100 module for compressing the cartridges. The separation unit was preceded by the guard column, packed with Bondapak C_{18}/Corasil (37—50 μm). The output of the detector was displayed on a Varian Model A-25 recorder. The output signal also was fed to a Hewlett-Packard 3353 data system for integration of peak areas.

An all-glass filter apparatus with appropriate 0.45-μm filters (Solvent Clarification Kit, Waters Assoc.) was used to filter the mobile phase before use. Other equipment included a Potter homogenizer (Type E 60, Heidolph Elektro

KG, Kelheim, G.F.R.), a vortex-type mixer, a Büchi Rotavapor and high-speed centrifuge.

Chromatographic conditions

All chromatography was done at ambient temperature. The mobile phase consisted of methanol—ammonia 25% (99.3:0.7, v/v). The flow-rate was 2.0 ml/min, maintained by a pressure of ca. 25 bars. The column effluent was monitored at 242 nm, the absorbance maximum of amiodarone, using a detector range of 0.01 absorbance unit full-scale (a.u.f.s.) and a chart speed of 20 cm/h.

Standards

A stock standard solution in methanol containing 100 μg/ml of amiodarone base and desethylamiodarone base and a stock internal standard solution of 250 μg/ml in acetonitrile were prepared. Standard solutions in methanol, plasma or urine containing amiodarone and desethylamiodarone at concentrations of 0.10, 0.25, 0.50, 1.00, 1.75, 2.50, 3.50 and 5.00 μg/ml were made by appropriate dilution of the stock solution with methanol, plasma or urine, respectively. The plasma and methanolic solutions were stable for one month if stored at 4°C in the absence of light in amber glass containers. An internal standard working solution was prepared by dilution of an aliquot of the stock solution with acetonitrile to contain 5.0 μg/ml.

Procedure for plasma, urine and tissues

Plasma. In a disposable polypropylene-capped glass centrifuge tube (16 × 100 mm) were introduced 1.00 ml of plasma and 2.00 ml of the solution of the internal standard in acetonitrile. The stoppered tube was vortexed for 30 sec, allowed to stand at room temperature for 15 min and centrifuged at 2000 *g* for 15 min. Subsequently the supernatant was transferred to a clean, disposable, glass tube and 50 μl of this solution were injected into the liquid chromatograph.

Urine. To a Clin-Elut 1003 disposable extraction column were successively added 2.00 ml phosphate buffer, pH 7, 40 μl of the stock internal standard solution and 1.00 ml of urine. After waiting for 3 min, 6 ml of a mixture of diisopropyl ether—acetonitrile (95:5) were added to the column. The eluate was collected in a 25-ml conical flask and after at least 3 min the extraction was repeated with one more 6-ml aliquot. After completion of the elution from the column, the collected combined eluates were evaporated to dryness under reduced pressure in a Büchi Rotavapor at room temperature. The sample residue then was reconstituted with 3.0 ml of a mixture of methanol—acetonitrile (1:2, v/v) and 50-μl aliquots were injected into the chromatograph.

Tissue. Tissue was finely minced, dried between Kleenex® tissues, and portions of about 100 mg of the dried minced tissue were homogenized for 10 min with 1.00 ml of 50% ethanol in a glass tube with a homogenizer. The tissue homogenate then was vortexed for 30 sec with 2.0 ml of the internal standard solution and thereafter completely transferred to a clean glass test tube and centrifuged at 2000 *g* for 15 min. From the supernatant 50 μl were injected into the liquid chromatograph. All samples were assayed in duplicate.

Quantitation. The concentration of amiodarone and desethylamiodarone in plasma, urine and tissues was determined from calibration curves of peak area ratios (amiodarone and desethylamiodarone to internal stadard) versus amiodarone and desethylamiodarone concentrations in plasma, urine and tissue standards carried through the described procedures.

RESULTS

Chromatograms from blank human plasma, urine, tissue and those spiked with known concentrations of amiodarone and desethylamiodarone are shown in Fig. 2. Chromatograms of a plasma sample, and a renal and heart tissue sample obtained, respectively, from a patient treated with 200 mg of amiodarone per day for more than a year and from a post-mortem case treated with the same dosage for more than a year are presented in Fig. 3. The peaks representing amiodarone, desethylamiodarone and the internal standard are symmetrical and well removed from the solvent front and interfering peaks from the biological material.

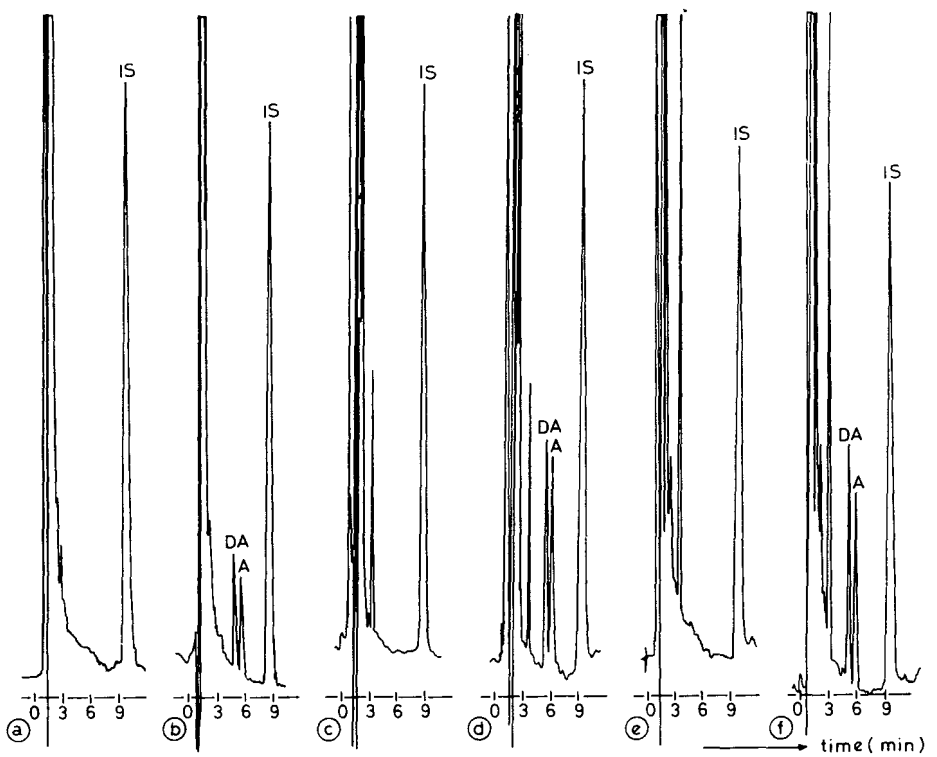


Fig. 2. Chromatograms of (a) blank human plasma, (b) plasma spiked with 1.0 μg/ml amiodarone (A) and desethylamiodarone (DA), (c) blank urine, (d) urine spiked with 2.5 μg/ml A and DA, (e) blank heart tissue, and (f) heart tissue spiked with 25 μg/g A and DA. Internal standard (IS) concentration 3.3 μg/ml. Injection volume: 50 μl. Detector sensitivity: 0.01 a.u.f.s. Recorder chart speed: 20 cm/h.

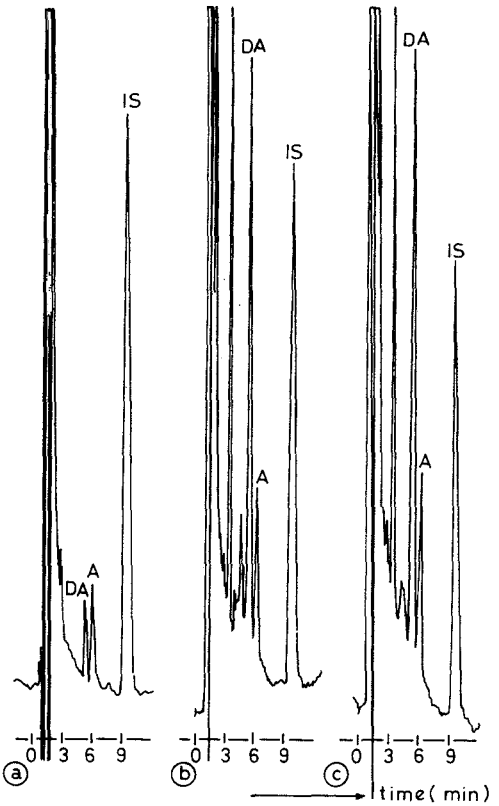


Fig. 3. Chromatograms of (a) plasma of a patient receiving 200 mg amiodarone for more than a year, containing 1.03 μg/ml A and 0.70 μg/ml DA, (b) kidney tissue containing 20.5 μg/g A and 57.4 μg/g DA, and (c) heart tissue containing 22.0 μg/g A and 56.1 μg/g DA from a post-mortem case who had received 200 mg of amiodarone for more than a year. Conditions as in Fig. 2.

The retention times of desethylamiodarone, amiodarone and internal standard were 5.3, 6.3 and 9.4 min, respectively. For the potential metabolites L3937 and L6355 retention times of 4.0 and 5.2 min were observed, respectively. The retention times of the compounds under study were quite stable as demonstrated by a coefficient of variation of less than 3% of the mean of the retention times of these compounds measured each day during a two-month period. A number of drugs were investigated for possible interference with the amiodarone assay. Verapamil, cinnarizine, quinidine and disopyramide were detectable if present at concentrations of about 5 μg/ml and showed in this system retention times of 2.5, 3.2, 4.7 and 4.8 min, respectively. However, they were all completely resolved from the compounds of interest in the chromatographic system. No interfering peaks were observed in the plasma of patients receiving amiodarone in combination with drugs such as acenocoumarol, carbamazepine, carbimazole, chlordiazepoxide, chlorthalidone, diazepam, diclophenac sodium, digoxin, dihydroergotoxine mesylate,

dipyridamole, furosemide, glaphenine, hydrallazine, isosorbide dinitrate, lorazepam, metoprolol, nicotinyl tartrate, nifedipine, phenprocoumon, prazosin, procaine, procainamide, propranolol, theophylline, tolbutamide, triamterene and valproate sodium.

Calibration curves of peak area ratio versus concentration were obtained by analyzing plasma and urine standards containing amiodarone and desethylamiodarone in concentrations ranging from 0.1 to 5.0 μg/ml. The calibration curves in plasma were established every week, and standard plasma samples were analysed during the week to validate the method. The equations of the curves were calculated by least-squares linear regression. For the curves in plasma and urine a good linear relationship was obtained in the concentration range studied for both drugs, with intercepts not significantly different from zero. The linear regression data for the mean calibration curves of amiodarone and desethylamiodarone in plasma and urine are summarized in Table I.

TABLE I

THE LINEAR REGRESSION PARAMETERS FOR THE MEAN CALIBRATION CURVES OF AMIODARONE AND DESETHYLAMIODARONE IN PLASMA AND URINE

Compound	Sample	n*	Linear regression parameters**		
			Slope	y-intercept	Correlation coefficient
Amiodarone	Plasma	8	0.1091	0.0032	0.9998
	Urine	4	0.1237	−0.0008	0.9979
Desethylamiodarone	Plasma	8	0.1100	0.0027	0.9999
	Urine	4	0.1161	0.0000	0.9992

*Number of calibration curves used.
**Peak area ratio of drug to internal standard plotted on the y-axis versus drug concentration in plasma or urine (in μg/ml) on the x-axis.

The plasma and urine curves were the average of eight and four standard curves, respectively, which were run during 2-month and one-month periods, respectively. The mean coefficient of variation of the peak area ratios of both drugs to internal standard for plasma and urine over the studied periods was 1.9% and 3.5% for the 5.0 μg/ml standards, 3.3% and 7.0% for the 2.5 μg/ml standards, 4.7% and 11.8% for the 1.0 μg/ml standards and 19.4% and 29.0% for the 0.10 μg/ml standards, respectively.

The precision of the assay was determined by replicate analyses of spiked plasma samples containing amiodarone and desethylamiodarone at concentrations of 0.75, 2 and 4 μg/ml. The intra-assay and inter-assay coefficients of variation are presented in Table II. The within-day and day-to-day variation at each concentration for both drugs was less than 6%. The sensitivity of the assay was 0.025 μg/ml for both drugs using an injection volume of 100 μl of plasma sample extract. The within-day coefficient of variation for spiked plasma samples containing 0.025 μg/ml was ± 17% (n = 5).

TABLE II

PRECISION DATA FOR THE DETERMINATION OF AMIODARONE AND DESETHYLAMIODARONE IN PLASMA

Concentration (μg/ml)	Coefficient of variation (%)			
	Within-day (n = 7)		Day-to-day* (n = 6)	
	Amiodarone	Desethylamiodarone	Amiodarone	Desethylamiodarone
0.75	3.1	4.6	5.6	5.6
2.00	1.5	3.4	3.5	3.7
4.00	1.2	1.4	2.2	2.8

*Analysis performed on six days during a three-week period.

The analytical recovery of amiodarone and desethylamiodarone from plasma, urine and various tissues was determined by comparison of the peak areas of amiodarone and desethylamiodarone obtained by analysis of 50-μl portions of methanolic standards mixed with internal standard solution (1:2, v/v) to those obtained from freshly prepared sample extracts. The results of the recovery studies are presented in Table III. The recovery of internal standard from plasma and urine was 107% (n = 70) and 76% (n = 40), respectively. For the two potential metabolites L3937 and L6355 (Fig. 1) recoveries in plasma of 103.3% (n = 4) and 103.8% (n = 4), respectively, were observed at a concentration of 1.0 μg/ml. In urine at a concentration of 1 μg/ml recoveries of 85.5% (n = 4) for L3937 and 66.3% (n = 4) for L6355 were found.

TABLE III

RECOVERY OF AMIODARONE (A) AND DESETHYLAMIODARONE (DA) FROM PLASMA, URINE AND TISSUES

Concentration (μg/ml or μg/g*)	Recovery (%)					
	Plasma (n** = 8)		Urine (n = 5)		Tissues*** (n = 12)	
	A	DA	A	DA	A	DA
0.10	99.7	104.0	84.2	76.2	—	—
0.25	102.3	105.6	85.3	86.4	—	—
0.50	100.7	105.5	84.4	74.8	—	—
1.00	104.8	106.7	90.2	85.2	86.1	89.3
1.75	102.2	104.3	90.9	77.7	—	—
2.50	104.2	100.8	81.0	72.4	97.0	94.7
3.50	103.5	101.5	89.2	81.6	—	—
5.00	98.9	102.0	90.5	75.6	99.6	98.2
Mean	102.0	103.8	87.0	78.7	94.2	94.1
S.D.	2.1	2.2	3.7	5.1	7.2	4.5

*Tissue concentrations in μg/g.
**Number of determinations at each concentration.
***Mean recovery data from aortic arch, esophagus, lung, heart, liver and renal tissue.

The described method was used to determine the plasma levels of amiodarone and desethylamiodarone after single oral and intravenous administration and after repeated oral administration of the drug. The plasma concentration—time curves of amiodarone obtained after single oral and intravenous administration of 400 mg to a healthy volunteer are shown in Fig. 4. The mean steady-state plasma levels of amiodarone and desethylamiodarone in seventeen patients receiving 200 mg a day for at least one month were 1.11 ± 0.33 μg/ml and 1.02 ± 0.31 μg/ml, respectively. After 400 mg a day, a mean level in seventeen patients of 1.70 ± 0.57 μg/ml and 1.40 ± 0.36 μg/ml for amiodarone and desethylamiodarone, respectively, was observed.

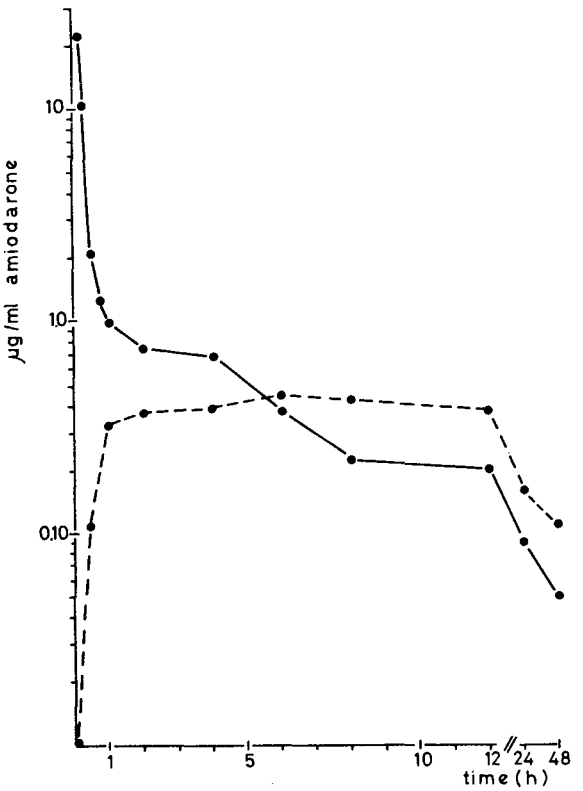


Fig. 4. Plasma concentration—time curves of amiodarone from a healthy volunteer after a single oral (•— — —•) and intravenous (•———•) dose of 400 mg of amiodarone.

Neither amiodarone nor desethylamiodarone were detected in urine samples of several patients on long-term amiodarone treatment. Very high levels were found in lung and liver tissue of a post-mortem case, with concentrations of 112 μg/g and 307 μg/g in the liver and 178 μg/g and 541 μg/g in the lung for amiodarone and desethylamiodarone, respectively. In other tissues investigated such as kidney, aortic arch, esophagus and heart, concentrations of amiodarone ranging from 8 to 16 μg/g and of desethylamiodarone ranging from 13 to 53 μg/g were observed.

DISCUSSION

Reversed-phase chromatography was used by several investigators for the separation of drugs like imipramine, maprotiline, chloroquine and lidocaine and their respective demethylated or deethylated metabolites [20—23]. In this type of liquid chromatography the dealkylated metabolites will usually be eluted before the parent drugs, which is advantageous with respect to the detection limit of these metabolites.

As desethylamiodarone, the major metabolite of amiodarone, is more polar and always present in considerable concentrations in plasma and tissues of patients on long-term amiodarone treatment, it is obvious that for the simultaneous assay of these compounds reversed-phase liquid chromatography should be chosen as the separation method. After investigation of several reversed-phase systems, a Radial-PAK C_{18} column in combination with a radial compression separation system (RCSS, Waters Assoc.) and a mobile phase consisting of methanol—0.7% ammonia 25% (v/v) was found most suitable for the separation of amiodarone, desethylamiodarone and the internal standard. The Radial-PAK C_{18} column was preferred to the conventional Bondapak C_{18} steel column because of its superior economy and convenience, enhanced reproducibility and reliability, high efficiency at higher flow-rates for faster analysis, lower back-pressure, and complete elimination of column voiding and channelling. For the normal routine analysis of steady-state levels of amiodarone and desethylamiodarone the 10-μm cartridge was preferred to the 5-μm one, because it has sufficient efficiency for complete separation of both compounds, it operates at a much lower back-pressure (25 versus 70 bars), it is far less prone to contamination with biological material from the sample and it has a substantially lower price. The 5-μm C_{18} cartridge was used in cases where higher efficiency (about 9000 versus 5000 plates for the 10-μm C_{18} column) and detection of low levels ($<$ 100 ng/ml of plasma) are required, for instance with metabolic and single-dose pharmacokinetic studies.

In order to determine the optimum chromatographic conditions, the influence of the ammonia concentration in the mobile phase on the retention behaviour and the capacity factors of amiodarone, its metabolites, desethylamiodarone, L6355, L3937, and the internal standard was investigated. The effect of the increase of the ammonia content in the mobile phase from 0.05% to 1% on the capacity factors (k') of amiodarone and its metabolites is shown in Fig. 5. It can be seen that the k' values decrease sharply from 0.05% (v/v) to about 0.35% (v/v) of ammonia 25% and then decrease gradually with further increase in the amount of ammonia. At an ammonia content of 0.05% all compounds were separated; however, under these conditions, retention times were long (L3937, 8.1 min; L6355, 9.9 min; amiodarone 11.4 min; desethylamiodarone 12.9 min; and internal standard, 20 min) and the peaks were unacceptably broad. Increase of the ammonia content to 0.1% resulted in a reduction of the retention times of about 26% for all compounds; however, the peaks were still rather broad and no separation was observed between amiodarone and desethylamiodarone with retention times of 8.6 min and 9.2 min, respectively. A further rise in the amount of ammonia to 0.35% (v/v) resulted again in a substantial decline of the retention times by another

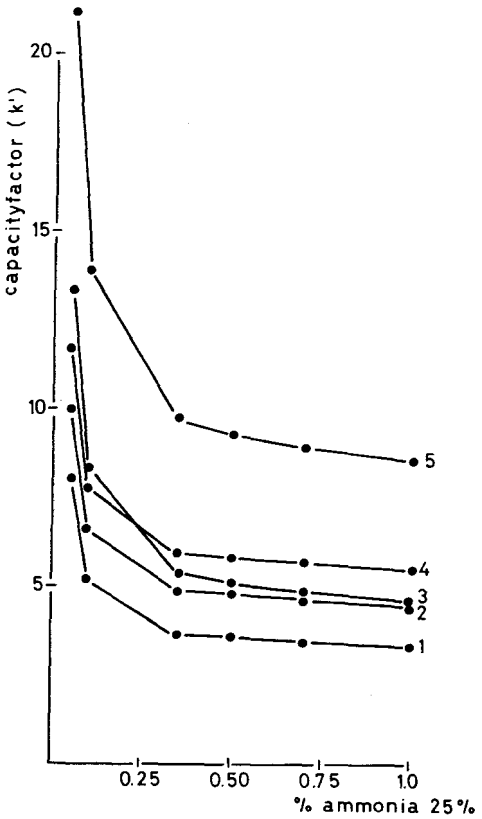


Fig. 5. Plot of capacity factors (k') of amiodarone (4), desethylamiodarone (3), two potential metabolites, L6355 (2) and L3937 (1), and the internal standard (5) against ammonia 25% content of the mobile phase. Unretained compound: methanol. Flow-rate: 2.0 ml/min. Chromatographic conditions as described in the text.

29% for all substances. At this concentration, however, the elution order of amiodarone and the desethyl metabolite was reversed with retention times of 6.5 min and 6.0 min, respectively, and the compounds showed a partial separation with L6355 (t_R = 5.5 min) and a complete separation with L3937 (t_R = 4.3 min) and the internal standard (t_R = 10.0 min). Using a mobile phase with an ammonia content of 0.70% (v/v) a further decrease in retention times of all compounds by ca. 12% was observed and sharp symmetrical peaks were obtained. At this ammonia concentration desethylamiodarone and amiodarone were completely separated (k'_{DA} = 4.88, k'_{A} = 5.71, R = 1.02 and α = 1.17); however, no separation could be obtained between desethylamiodarone and L6355 (k' = 4.65). A further increase of the ammonia content to 1.0% (v/v) resulted only in a small decrease of ca. 2% of the retention times of the compounds investigated and did not lead to a markedly better separation between amiodarone and its desethyl metabolite (R = 1.11) or between the latter compound and L6355 (Fig. 5). The effect of the ammonia increase in the mobile phase on the chromatographic behaviour of the drugs involved may be explained by a suppression of the dissociation of these compounds by the in-

creasing ammonia concentration, resulting in a separation on the reversed-phase column based upon the respective lipophilic character of the drugs using almost pure methanol as eluant. In addition, the increase of the ammonia content to 1% also led to a pH of the mobile phase of 11.0, which may cause gradual deterioration of the column efficiency and reduction of the column life. Consequently, methanol with 0.70% ammonia 25% (v/v) was chosen as the final mobile phase for the simultaneous assay of amiodarone and desethylamiodarone. Using this condition, however, it should be noted that the potential metabolite L6355 may interfere with the assay of desethylamiodarone. This possible risk of interference, however, is insignificant in clinical practice, since in plasma and tissue samples only amiodarone and desethylamiodarone have been detected so far and, furthermore, in urine specimens neither amiodarone nor any of its potential metabolites have been found [13, 14]. If the presence of L6355 is suspected, samples can be analysed using a mobile phase with 0.35% ammonia.

For the detection of amiodarone three different wavelengths, 242, 254 and 280 nm, have been described [13—15, 19]. Using these different wavelengths it was shown that the highest assay sensitivity was at 242 nm, the λ_{max} of amiodarone in the mobile phase, and therefore this wavelength was selected for the detection of amiodarone and its potential metabolites.

Several procedures have been reported for the extraction of amiodarone from plasma or serum [13—15, 19]. The procedure of Flanagan et al. [13] involved acidification of the sample to pH 6.0 with phosphate buffer, followed by a single extraction with diisopropyl ether and direct injection of the supernatant on to the HPLC column. Cervelli et al. [15] and Lesko et al. [19] used a double solvent extraction with diethyl ether and hexane, respectively, after acidification of the plasma to pH 3.8 with an acetate buffer. After evaporation of the organic layer and reconstitution in mobile phase, the solutions were injected into the liquid chromatograph. Instead of extraction of amiodarone from plasma with an organic solvent, Andreasen et al. [14] used a simple deproteinization of plasma with ethanol, then centrifugation, followed by direct injection of the supernatant on to the HPLC column. Recoveries reported for these various procedures varied from about 80% for the extractions at pH 3.8 with diethyl ether and hexane to about 99% for the extraction at pH 6 with diisopropyl ether and for the deproteinization with ethanol. Using the same procedures for the extraction of desethylamiodarone, recoveries of about 8% at pH 3.8 and of 82% at pH 6.0 were observed, suggesting that for quantitative recovery of both drugs a pH of at least 6 or even higher should be used. This observation was supported by preliminary experiments of Flanagan et al. [13], indicating pH 7.4 as optimal for the extraction of desethylamiodarone. For the above-mentioned reason and to prevent losses due to extraction, a simple deproteinization with acetonitrile at the pH of plasma (7.4) was chosen as the method for the simultaneous assay of amiodarone and its desethyl metabolite in plasma. Acetonitrile was selected as protein precipitant because it is the most effective of the organic solvents used for deproteinization and it provides virtually complete removal of plasma proteins [24]. Using this protein precipitating method a quantitative recovery was observed for both amiodarone and desethylamiodarone, as shown in

Table III, and also for the potential metabolites L6355 and L3937. In addition an adequate precision (Table II), sufficient sensitivity and identical mean calibration curves in plasma (r = 0.9999) were obtained for both drugs. Besides, preliminary experiments have shown that the described method may easily be applied to 200 μl of plasma sample, using 400 μl of acetonitrile and 50 μl of injection volume, with the same sensitivity, accuracy and precision as obtained by the use of 1.0-ml plasma samples. Endogenous substances or a variety of drugs concomitantly used in amiodarone therapy have not been found to interfere with the analysis of amiodarone and its metabolites, indicating the versatility of the method.

Using the deproteinization method for urine specimens, even after injection of 10 μl of the supernatant a very broad solvent peak in the chromatogram was observed, caused by endogenous UV-absorbing substances from urine and limiting the detection of amiodarone and desethylamiodarone in urine to about 2 μg/ml.

To obtain cleaner urine extracts and a better sensitivity, an extraction at pH 7 with a mixture of diisopropyl ether—acetonitrile (95:5) using disposable Clin-Elut columns appears to be the most suitable. Using this procedure an acceptable solvent peak was shown in the chromatogram (Fig. 2), recoveries for amiodarone and its metabolites were adequate (Table III) and urine levels of 0.1 μg/ml for both drugs could easily be detected. In addition, by reconstitution of the residue with 750 μl of solvent instead of 3 ml a sensitivity of about 0.03 μg/ml could be obtained.

In contrast to urine, the direct plasma deproteinization method was applicable with appropriate sensitivity and selectivity to the analysis of amiodarone and its metabolites in various tissues (Figs. 2 and 3). The mean recoveries of both compounds of ca. 94% in the various tissues investigated (Table III) and the lack of interference from endogenous substances (Fig. 2) showed that the homogenization with 50% ethanol and the subsequent deproteinization with acetonitrile is an efficient and effective procedure, which has proved its feasibility for post-mortem tissue analysis. The data on amiodarone plasma concentrations after single and repeated oral administration are in good agreement with the levels observed by Andreasen et al. [14] and Flanagan et al. [13]. No comparable data are available on the concentration of desethylamiodarone in plasma after repeated oral administration. Our data indicate that in the steady-state, plasma levels of both compounds were in the same range, whereas in tissues concentrations of desethylamiodarone were approximately 2.5 times higher than the amiodarone concentration. So far, in urine, no measurable concentration of amiodarone and desethylamiodarone was found after single or repeated oral administration, which agrees with data reported previously [13, 14].

Furthermore, no detectable amounts were present ($\leqslant$ 0.05 μg/ml or μg/g) of the two potential metabolites L3937 and L6355 (Fig. 1) in urine and tissues of patients on amiodarone treatment.

Finally, the simultaneous assay of amiodarone and desethylamiodarone has been used for over a year and has shown to be simple, reliable and accurate. The Radial-PAK C_{18} column may be continuously used for about

three months with 40 injections daily, without any apparent loss in column efficiency.

The method could be a valuable tool for the further elucidation of amiodarone disposition and pharmacokinetics. In addition, it may be suitable for routine monitoring of plasma levels of amiodarone as well as its major metabolite desethylamiodarone, which is of great importance for establishing the relation between these levels and clinical effects or possible side-effects during chronic amiodarone therapy.

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Note

A new internal standard suitable for the gas chromatographic determination of 3-methoxy-4-hydroxyphenylethyleneglycol

PIER ANTONIO BIONDI*, GU NIU-FAN*, TATJANA SIMONIC and CAMILLO SECCHI

Istituto di Fisiologia Veterinaria e Biochimica, Via Celoria 10, 20133 Milan (Italy)

and

ADA MANZOCCHI

Istituto di Chimica, Facoltà di Medicina e Chirurgia, Via Saldini 50, 20133 Milan (Italy)

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In order to improve the precision of the gas chromatographic (GC) analysis of (3-methoxy-4-hydroxyphenyl)ethane-1,2-diol (3-methoxy-4-hydroxyphenylethyleneglycol, MHPG) a proper internal standard should have a structure as similar as possible to MHPG itself. In this connection in recent procedures for MHPG determination by GC with electron-capture detection (GC—ECD), 3-methoxy-4-hydroxyphenylethanol [1] and the MHPG isomer 3-hydroxy-4-methoxyphenylethyleneglycol [2] have been used as internal standard, but both the former compound to a major extent [3] and the latter compound [4] are shown to be present in urine as endogenous metabolites and do not allow the final MHPG evaluation to be sufficiently accurate without additional adjustments.

Synthetic internal standards that are not so similar to MHPG as the compounds mentioned above, *o*-hydroxyethylresorcinol [5] and tryptophol [6], have been successfully used in other methods for MHPG estimation, again with the GC—ECD technique. In our experience, anyway, also the less sophisticated flame ionization detection (FID) has been shown to be suitable for GC analysis of urinary MHPG, provided that derivatization selectivity is increased by the use of boronic acids, which are specific reagents for

*Present address: Shanghai Psychiatric Hospital, Wan Ping Nan Lu 600, Shanghai, China.

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diol groups [7]. In our method, phenanthrene was used as reference compound added only before injection, but the assay precision was shown to be rather low.

In this connection a synthetic non-endogenous internal standard containing both MHPG functional groups, phenolic and glycolic ones, was indispensable to improve the reproducibility of the GC—FID method without any loss in specificity. The lack of such a compound has been stated to be cumbersome also in a recent MHPG determination by high-performance liquid chromatography (HPLC) carried out with fluorescence detection [8].

A substance with the above-mentioned features not being available, our aim was to obtain it from inexpensive reagents by means of an easy synthesis. For this purpose the MHPG homologue 3-(3-methoxy-4-hydroxyphenyl)propane-1,2-diol (3-methoxy-4-hydroxybenzylethyleneglycol, MHBG) was obtained in the simple pathway shown in Fig. 1 from 3-(3-methoxy-4-hydroxyphenyl)-propene (eugenol, Eu).

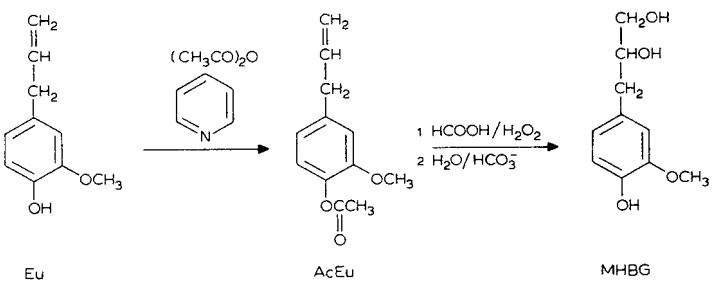

Fig. 1. Scheme of the synthesis of MHBG from Eu.

MATERIALS AND METHODS

The solvents and inorganic and organic reagents used were purchased from Carlo Erba (Milan, Italy); Eu and piperazine were from Fluka (Buchs, Switzerland).

MHBG synthesis

In the first step the Eu phenolic group was protected as acetate: Eu (10 ml) was kept overnight at room temperature in acetic anhydride and pyridine (20 ml each). The reaction mixture was poured into crushed ice and extracted with diethyl ether. The organic phase, washed with 1 *N* HCl and 5% $NaHCO_3$, was dried and evaporated to give acetyl-Eu (AcEu).

In the second step the glycol moiety was introduced by stirring at room temperature AcEu (1 g) with 98% formic acid (5 ml) and 35% hydrogen peroxide (0.8 ml). The reaction was complete within 2 h, as shown by the disappearance of the AcEu spot in thin-layer chromatographic analysis carried out on silica gel 60 F_{254} (0.2 mm) (Merck, Darmstadt, G.F.R.) (mobile phase, toluene—ethyl acetate (8:2); detection, UV lamp at 254 nm). The glycol monoformates so obtained were directly hydrolyzed by diluting the reaction mixture

with water (20 ml), adding $NaHCO_3$ to pH 8 and heating for 3 h at 80°C. During this treatment both formyl and acetyl groups were released from MHBG. The solution was then extracted with ethyl acetate which was finally evaporated.

The oily residue containing MHBG was dissolved in acetone—hexane (2:1) mixture and a saturated solution of piperazine in the same mixture was added to give as precipitate the bis-MHBG-piperazine salt, finally recrystallized in the same solvent mixture (m.p. 106°C). Elemental analysis calculated for $C_{24}H_{38}O_8N_2$: C, 59.74; H, 7.94; N, 5.81. Found: C, 60.25; H, 8.02; N, 5.76. Overall yields ranged from 25% to 30%.

In the NMR spectrum of free MHBG, obtained by extracting the acidic solution of the piperazine salt, the expected signals appeared: Ph-CH_2 (2.67, 2H, d); CH_2OH (3.45, 2H, m); CHOH (3.65, 1H, m); OCH_3 (3.77, 3H, s); aromatic hydrogens (6.78, 3H, m).

The same derivative used in our method for the MHPG determination, acetyl-MHBG-*n*-butaneboronate (Ac-MHBG-BuB), was obtained according to the previously described procedure and analyzed by gas chromatography—mass spectrometry (GC—MS) using a Finnigan Model 3100 instrument with the same GC conditions described below and with an electron energy of 70 eV. As well as for MHPG derivative [9] the mass spectrum showed a low molecular peak (*m/e* 306) and a high peak (95% of *m/e* 138 base peak) corresponding to the fragment from CH_2=C=O loss (*m/e* 264).

Urine analysis

For the treatment of urine samples, the only difference in comparison with the described method was the addition of MHBG (10 μg) to the urine specimen (15 ml) from the 24-h collections.

GC analysis was carried out on a Dani (Monza, Italy) Model 3600 B apparatus equipped with a glass column (2 m × 2.5 mm I.D.) packed with 3% SP-2100 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The injector and detector temperatures were 260°C and 310°C, respectively. The column temperature was programmed from 220°C to 300°C at 12°C/min. The carrier gas (nitrogen), hydrogen and air flow-rates were 42, 35 and 400 ml/min, respectively.

Calibration curves were obtained by adding different amounts (5—40 μg) of MHPG and constant amounts of MHBG (10 μg) to urine samples and carrying out the whole procedure described elsewhere [7]. The MHPG peak was corrected for its endogenous content.

RESULTS

The methanolic standard solutions of MHBG (0.1 mg/ml as free MHBG) were kept in the refrigerator and showed no content variations up to two weeks.

In the GC conditions used the MHPG retention time relative to MHBG was 0.91 ± 0.06 (n = 20) and the zone of MHBG peak was free from interfering peaks when MHBG was not added to the urine samples, as shown in Fig. 2.

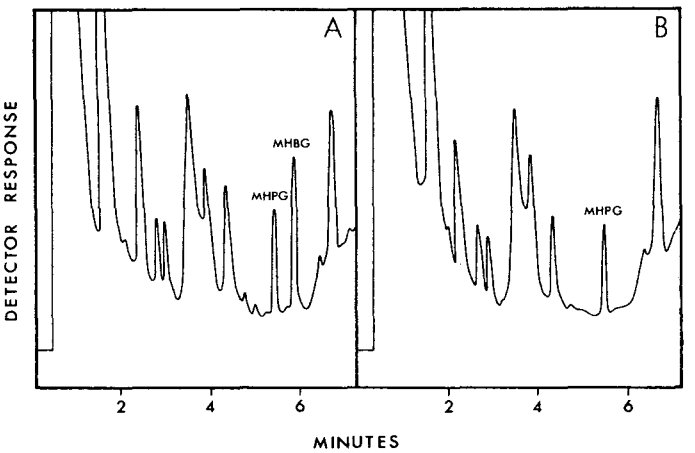

Fig. 2. Typical GC profiles obtained from a urine sample with (A) and without (B) MHBG.

Routine regression lines were obtained for each preparation of MHBG with r values ranging from 0.997 to 0.999. The slope of the standard curves (peak height ratio between MHPG peak and MHBG peak/MHPG amount) was 0.109 ± 0.008 (n = 5).

Within-run precision was estimated by analyzing seven samples of a 24-h urine collection. The MHPG content and the standard deviation was 535 ± 12 μg/l. Day-to-day precision was measured on three urine samples analyzed ten times in a month. The MHPG mean contents and the standard deviations were 415 ± 10 μg/l, 917 ± 19 μg/l and 1228 ± 24 μg/l.

CONCLUSIONS

The data on statistical validation of the use of MHBG clearly showed higher precision in comparison to that obtained with phenanthrene as reference compound.

In conclusion, the use of a "true" internal standard made the MHPG analysis by GC more simple, rapid and reliable. In spite of our experience being restricted to the GC—FID technique, MHBG could be an ideal analytical tool suitable for the evaluation of MHPG in biological fluids by other techniques such as GC—ECD and HPLC with electrochemical or fluorescence detection.

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Note

Determination of glycerol in biological samples

ROSA MARINA MATARESE*

Institute of Biological Chemistry, University of Rome and Centro di Biologia Molecolare del C.N.R., Rome (Italy)

and

CARLO ZAMPONI

Institute of Neurosurgery, University of Rome, Rome (Italy)

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The use of glycerol in neurosurgery was first reported in 1961 [1] and since then it has been widely recommended as a safe and effective means of acutely reducing intracranial pressure [2—4]. The great therapeutical success of glycerol induced us to begin a study of its mechanism of action as a cerebral dehydrating agent. For this purpose we worked up a method for qualitative and quantitative analysis of glycerol in biological tissues and fluids.

In this note we describe the gas chromatographic determination of glycerol in biological samples.

MATERIALS AND METHODS

Biological samples, serum, cerebrospinal fluid and cerebral tissue were from patients hospitalized in the Neurosurgical Clinic of the University of Rome. Cerebral tissue was from surgical removal of cerebral tumors with a surrounding part of sound tissue, in patients either treated with glycerol (1 g/kg oral glycerol every 5 h) or not so treated.

For extraction and gas chromatographic analysis of glycerol we modified the method reported by Slansky and Moshy [5].

*Correspondence address: Istituto di Chimica Biologica, Unversità degli Studi, Piazzale Aldo Moro, 00100 Rome, Italy.

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We added to the sample (serum or cerebrospinal fluid) methanol in a 1:10 ratio (v/v). For cerebral tissue, samples were homogenized in a Potter homogenizer with methanol and then further diluted 1:10 (w/v). Methanol has a twofold function: to deproteinize the sample and to extract glycerol. Samples were well shaken and left standing at room temperature overnight. Then they were centrifuged and an aliquot of supernatant, with a suitable quantity of erythritol diluted in methanol added as internal standard, was evaporated to dryness by a nitrogen stream at room temperature.

One milliliter of Tri-Sil/N,O-bis(trimethylsilyl)-acetamide (BSA), Formula D, was added to each sample; the test tube was tightly closed with a screw cap fitted with a Teflon liner, and well shaken. After 1 h at room temperature, 2 μl of reaction mixture were injected into the gas chromatograph.

Analyses were carried out on a Perkin-Elmer 990 gas chromatograph fitted with a flame ionization detector. The glass column (1.8 m × 2 mm I.D.) was packed with 3.5% SE-30 on Gas-Chrom Q AW DMCS, 80—100 mesh. Column temperature was 80°C for 3 min, then programmed to reach 150°C at a rate of 3°C/min. Injector and detector temperature was 220°C. Flow-rates were 30 ml/min for nitrogen carrier gas, 20 ml/min for hydrogen and 300 ml/min for air.

Glycerol anhydrous extra pure and erythritol were obtained from Merck (Darmstadt, G.F.R.); Tri-Sil/BSA, Formula D, in vials, was obtained from Pierce Chemical Co., Rockford, IL, U.S.A.

RESULTS AND DISCUSSION

Gas chromatograms of glycerol, with erythritol as internal standard, in standard solution and biological material are reported in Figs. 1 and 2, respectively. Data for glycerol concentration in the various biological samples are reported in Table I. The recovery of glycerol was tested by analysing a sample of cerebrospinal fluid containing 0.041 mg/ml glycerol after the addition 0.050 mg of glycerol: recovery was total. Also intra-assay variability was tested: it was lower than 7%. Evaporation of the solvent by a stream of

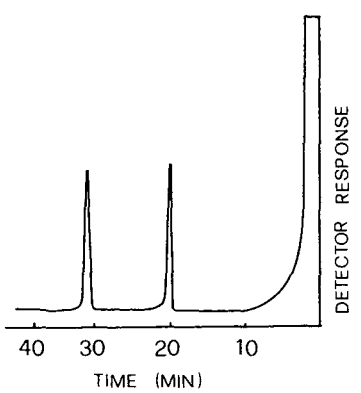

Fig. 1. Gas chromatogram of glycerol with erythritol as internal standard in standard solution: retention times are 20 and 31 min, respectively. For conditions of analysis see text.

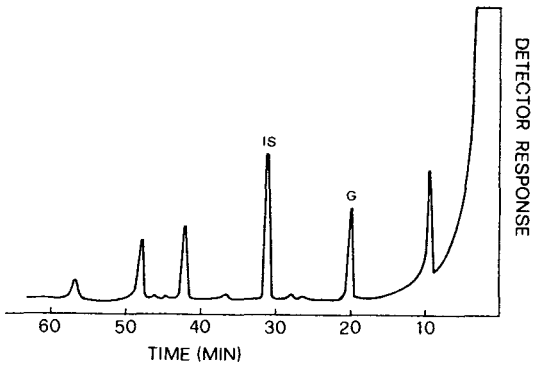

Fig. 2. Gas chromatogram of a sample of a tumor (paraventricular glioma) of a patient treated with glycerol (operation was performed about 12 h after last dose of glycerol). For conditions of analysis see text. A 2-μl volume of reaction mixture was injected, corresponding to 0.2 mg of tissue. G = glycerol, IS = internal standard; other peaks were not investigated.

TABLE I

MEAN VALUES OF GLYCEROL CONCENTRATIONS IN SOME BIOLOGICAL SAMPLES FROM PATIENTS NOT TREATED OR TREATED WITH GLYCEROL

Biological sample	Untreated patients	Treated patients
Serum (mg/ml)	0.05*	0.26 (0.05—0.65)**
Cerebrospinal fluid (mg/ml)	0.02*	0.13 (0.04—0.42)
Tumoral cerebral tissue (mg/g)	0.20 (0.05—0.44)	0.44 (0.11—0.89)
Sound peritumoral cerebral tissue (mg/g)	0.21 (0.12—0.35)	0.23 (0.11—0.44)

*Single value.
**Range in parentheses.

nitrogen and without heating or vacuum is a guarantee against any loss of sample. The use of erythritol as internal standard assures accuracy of analysis. The calibration graphs were linear in the experimented range (0.01—10 mg/ml of reaction mixture). The sensitivity of the method was 5 ng in standard solutions. In biological samples the lower value we obtained was 0.02 mg/ml of cerebrospinal fluid; but the method allows a lower limit of detection because the glycerol and erythritol internal standard peaks are isolated from the other unknown peaks of biological material.

Results with this method are very reproducible. The technique is simple and suitable for our research, which requires screening of a large number of samples.

Our first results have not allowed us to draw any conclusions about a possible mechanism of action of glycerol as a means of acutely reducing intracranial pressure, but they are very encouraging for the continuation of this research.

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Note

Rapid extraction of arachidonic acid metabolites utilizing octadecyl reversed-phase columns

J.R. LUDERER*, D.L. RILEY and L.M. DEMERS

Departments of Medicine, Pharmacology and Pathology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U.S.A.)

(Received September 15th, 1982)

Prostaglandins (PGs), thromboxanes (TXs) and other arachidonic acid metabolites are usually extracted from acidified plasma, urine or tissue samples with organic solvents such as diethyl ether, ethyl acetate or chloroform [1]. The solvents are then evaporated, the residues resuspended and oftentimes purified further with silicic acid column chromatography prior to high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), or gas chromatography—mass spectrometry (GC—MS). If it is necessary to remove neutral lipids, the aqueous samples must first be extracted with petroleum ether prior to acidification and further extraction. Such procedures employing multiple solvent extraction steps and column chromatography are time consuming and frequently require the evaporation of relatively large volumes of organic solvent.

We have developed a sample preparation procedure which eliminates solvent extraction by utilizing the Baker 10 Extraction System (J.T. Baker, Phillipsburg, NJ, U.S.A.). This system was originally developed for the extraction of drugs from plasma samples and consists of a specially designed vacuum manifold capable of processing up to ten columns simultaneously (see Fig. 1). Commercially available, disposable columns are prepacked with octadecyl (C_{18}) silane bonded to silica gel. Acidified samples (plasma, urine, cell suspensions) containing prostaglandins and related compounds can be applied directly to the column without prior solvent extraction and selectively eluted. This technique is more rapid than the conventional silicic acid column chromatography utilized in separating out prostaglandins. The samples pass through the columns with the assistance of a vacuum and compounds can be eluted with small volumes (< 500 μl) of solvent. In addition to preparing samples for RIA and

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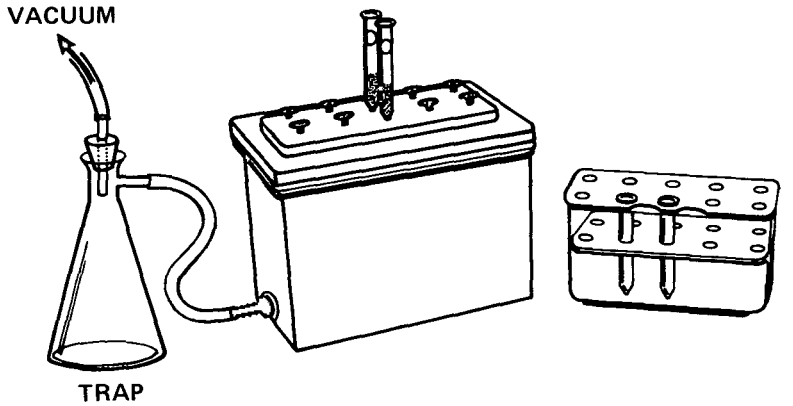

Fig. 1. Baker 10 Extraction System. Up to ten disposable extraction columns can be processed simultaneously. Solvents and samples pass through the columns by application of a vacuum. Sample eluents are collected in tubes positioned in a removable rack placed inside the manifold.

HPLC, we have also utilized this technique in lieu of organic solvent extraction to remove substances from aqueous HPLC mobile phases prior to derivatization for GC–MS. Finally, an HPLC method utilizing radial compression columns is also described for the separation of human granulocyte lipoxygenase products of arachidonic acid metabolism.

MATERIALS AND METHODS

[^{14}C]Arachidonic acid and tritiated standards of arachidonic acid, PGB_2, PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, TXB_2, 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, Leukotriene C_4 (LTC_4), Aquasol and radioimmunoassay kits for TXB_2 were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]6-Keto $PGF_{1\alpha}$ was purchased from Amersham (Arlington Heights, IL, U.S.A.). [^{3}H]LTB_4 was biosynthesized from human granulocytes and purified by HPLC as described. Unlabelled arachidonic acid (purity > 99%), and nordihydroguaiaretic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Diazomethane was prepared from N-methyl-N-nitroso-N-nitroguanidine (Sigma). Ionophore A23187 was purchased from Calbiochem (La Jolla, CA, U.S.A.). Ficoll–Paque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). HPLC solvents were purchased from Fisher (Pittsburgh, PA, U.S.A.) and J.T. Baker and passed through a Millipore filter (0.5 μm) prior to use. Indomethacin was a gift from Merck Sharp and Dohme (West Point, PA, U.S.A.). LTB_4 standard was synthesized in the laboratory of E.J. Corey and was a generous gift from Dr. Susan Levinson (Ortho Pharmaceuticals, Rahway, NJ, U.S.A.). 5-HETE was also a gift from Dr. Levinson.

Preparation of columns and extraction procedure

Octadecyl (C_{18}) extraction columns (3 ml) were placed in the luer fittings of the Baker-10 Extraction System manifold cover (see Fig. 1). A vacuum (380–500 mm Hg) was applied and the column washed with methanol (2–3 ml)

followed by 2—3 ml of phosphate buffered saline (pH 3), and the vacuum turned off so as not to allow the sorbent to dry. The methanol wash is necessary to condition the packing material to allow aqueous samples to pass through it. The conditioning flush with buffer is necessary to prepare the packing material surface for adsorption. A pH 3 buffer is used for the conditioning step since aqueous samples were applied to the column at this pH. The volumes delivered to the column in these conditioning steps are not critical and were added from plastic squeeze bottles. Plasma, buffer, or urine samples were acidified (pH 3) with either 1 *N* hydrochloric acid or 2 *N* formic acid. The plasma (1 ml) and buffer (1 ml) samples were applied directly to the column. Small urine volumes (10 ml) could be applied directly, but when larger volumes (> 50 ml) were processed, the acidified urine was centrifuged (1000 *g*) before application to the column since urine sediment has a tendency to clog the column. Large volumes of urine (400 ml) were processed using a 6-ml octadecyl (C_{18}) column fitted with a reservoir which attaches to the top of the column to accommodate the larger sample size.

High-performance liquid chromatography

Chromatography was performed with a Waters Assoc. HPLC system (U6K injector, two 6000A pumps, data module, system controller, Z-module radial compression system). Radial-Pak μBondapak C_{18} cartridges (10 cm × 8 mm, 10 μm) or fatty acid analysis stainless-steel columns (30 cm × 3.9 mm) from Waters were used. Linear solvent gradients from 45% acetonitrile (with 0.1% glacial acetic acid) to 65% acetonitrile (with 0.1% glacial acetic acid) were delivered at a flow-rate of 3 ml/min for the separation of lipoxygenase products. Absorbance was monitored with a Waters 440 detector (280 nm) and a Schoeffel (SF 770) variable-wavelength detector (235 nm) connected in series. Fractions were collected with a Gilson (B-200) automatic fraction collector. A guard column packed with μBondapak C_{18} was also employed.

Preparation of platelets and granulocytes

Blood was obtained from normal volunteers who had not ingested aspirin or nonsteroidal anti-inflammatory agents for at least two weeks. Washed platelet suspensions were prepared as previously described [2, 3]. To prepare granulocytes, whole blood was drawn into a plastic syringe containing heparin (500 U in 0.5 ml) in 6% clinical dextran (3.13 ml per 15 ml blood). After gentle mixing the cells were allowed to sediment for 90 min at room temperature. The plasma was removed from the sedimented red cells and centrifuged (180 *g*) for 8 min. The cell pellet was resuspended and washed twice in Tris-buffered saline (pH 7.4; 0.15 *M* sodium chloride) containing heparin (5 U/ml). The washed cells were then layered onto a Ficoll—Paque gradient (sp.gr. = 1.078) and centrifuged at 20°C according to the method of Boyum [4]. The lower granulocyte layer was then resuspended in 5 ml Tris-buffered isotonic ammonium chloride (pH 7.2) and incubated for 4 min (37°C) to lyse contaminating red cells [5]. After centrifugation (180 *g*) for 4 min the cells were washed again in ammonium chloride, and finally resuspended in phosphate-buffered saline (PBS). The PBS was prepared as previously described [6]. The cell suspension contained > 98% granulocytes which were > 95% viable as assessed by trypton-blue dye exclusion.

Platelet and granulocyte incubations

Washed platelet suspensions (0.4 ml; 10^9 cells/ml) were stirred in a platelet aggregometer (1100 rpm) at 37°C. Following the addition of arachidonic acid (1 μg) the platelet incubation was continued for 15 min. Granulocyte suspensions (0.5 ml; 10^6 cells/ml) were stirred in a constant temperature circulating water bath at 37°C. Ionophore A23187 was added (final concentration, 20 μM) and 5 min later arachidonic acid (final concentration, 75 μM) was added and the incubation continued for 15 min. Preliminary studies in our laboratory have indicated that these reaction conditions result in maximal production of LTB_4. The incubation was terminated by the addition of methanol (1 ml) and 2 *N* formic acid (to adjust the pH to 3—3.5). After centrifugation the supernatant was applied to the octadecyl column as described and eluted sequentially with water (2 ml), 25% methanol (2 ml) and 100% methanol (2 ml). The methanol fraction was taken to dryness (in vacuo) and resuspended in methanol (100 μl) prior to HPLC.

RESULTS AND DISCUSSION

Table I lists the recoveries (mean ± 1 S.D.) of radiolabelled standards extracted from buffer. The recoveries range from 73 to 98% with an average recovery of 84%. These values represent the percent of radioactivity recovered in the methanol fraction after the prepared columns were eluted sequentially with water (2 ml), 25% methanol (2 ml) and 100% methanol (2 ml). The usual procedure of solvent extraction followed by silicic acid column chromatography commonly results in recoveries of less than 70% [1]. The majority of the unrecovered radioactivity was found in the eluate obtained when the

TABLE I

RECOVERIES OF STANDARDS EXTRACTED FROM BUFFER USING OCTADECYL (C_{18}) 3-ml COLUMNS

Columns were prepared as described in Methods and eluted sequentially with 100% water (2 ml) 25% methanol—water (2 ml), and 100% methanol (2 ml).

Compound	Recovery (%)* (Mean ± S.D., n = 4)
Arachidonic acid	79 ± 6
PGB_2	87 ± 2
PGD_2	88 ± 2
PGE_2	82 ± 2
$PGF_{2\alpha}$	88 ± 4
6-Keto $PGF_{1\alpha}$	73 ± 4
TXB_2	79 ± 2
LTB_4	98 ± 2
5-HETE	77 ± 10
12-HETE	88 ± 9

*Recovery in the 100% methanol fraction.

samples were applied to the column. Lesser amounts of radioactivity were recovered in the fractions resulting from elution with water and 25% methanol. Washing the column with water and 25% methanol was found to be necessary when preparing incubations from granulocytes or platelets for HPLC. These clean-up steps removed interfering UV-absorbing peaks and proteinaceous material. Fig. 2 shows a representative chromatogram obtained from the methanol fraction of a granulocyte incubation carried out with [^{14}C]-arachidonic acid. [^{3}H]PGB_2 (400 ng, 50,000 cpm) was added as an internal standard following the incubation to calculate recoveries.

The identification of the peak designated as LTB_4 in Fig. 2 was based upon the following observations: (1) As depicted in Fig. 2, this peak incorporated a ^{14}C-label when incubations were performed with [^{14}C]arachidonic acid. (2) This peak was inhibited when granulocyte incubations were carried out in the presence of the lipoxygenase inhibitor nordihydroguaiaretic acid (30 μM) [7] but not with indomethacin (10^{-4} M). (3) When this peak was collected, separated from the aqueous mobile phase [using 3-ml octadecyl (C_{18}) extraction column as before] and derivatized with ethereal diazomethane, the resultant product after HPLC purification, showed a typical triene UV absorption pattern with peaks at 259, 270, and 281 nm. This is in agreement with spectral data for the methyl ester of LTB_4 as reported by Samuelsson et al. [8]. (4) Finally, the retention time of this peak corresponded with that of authentic, chemically synthesized, LTB_4. Moreover, when this peak was collected and mixed with authentic LTB_4 and rechromatographed, the mixture

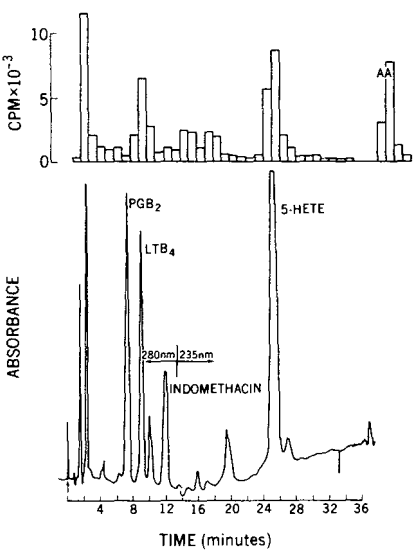

Fig. 2. HPLC separation of human granulocyte lipoxygenase products. Granulocyte incubations were carried out in the presence of indomethacin (10^{-4} M) and [^{14}C]arachidonic acid. The incubation medium was processed through an octadecyl (C_{18}) silane column as described in Methods and the methanol fractions subjected to HPLC. PGB_2 was added as an internal standard. A Waters radial compression μBondapak C_{18} cartridge (10 cm × 8 mm, 10 μm) was used. A linear solvent gradient from 45% to 65% acetonitrile in water (with 0.1% glacial acetic acid) was delivered at a flow-rate of 3 ml/min.

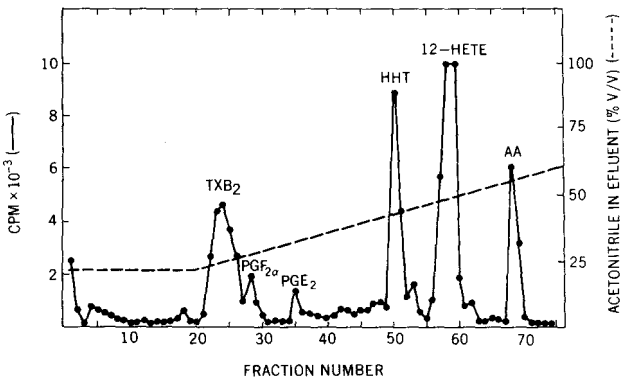

Fig. 3. HPLC separation of reaction products from human platelets incubated with [^{14}C]-arachidonic acid. The incubation medium was processed through an octadecyl (C_{18}) silane column as described in Methods and the methanol fraction subjected to HPLC. A Fatty Acid Analysis stainless-steel column (30 cm × 3.9 mm) was used. The initial eluent was 23% acetonitrile (with 0.1% glacial acetic acid), and after 20 min the acetonitrile was increased over a linear gradient as shown. The flow-rate was 2 ml/min. Fractions were collected and radioactivity determined by liquid scintillation counting.

eluted as a single component. The identity of 5-HETE was similarly established by means of incorporation of [^{14}C]arachidonic acid, inhibition with nordihydroguaiaretic acid, and co-elution with chemically synthesized 5-HETE and purchased [^{3}H]5-HETE.

Fig. 3 shows a representative chromatogram obtained when washed human platelets were incubated with [^{14}C]arachidonic acid and illustrates the utility of this extraction technique in experiments utilizing platelets. The incubation medium was processed exactly as outlined for the granulocytes. The identification of the peaks was as previously described [2].

Table II lists the recoveries obtained from plasma samples spiked with radiolabelled standards. This technique was developed for processing plasma samples

TABLE II

RECOVERIES OF STANDARDS FROM HUMAN PLASMA USING OCTADECYL (C_{18}) 3-ml COLUMNS

Columns were prepared as described in Methods and eluted sequentially with water (2 ml), benzene (2 ml), and ethyl acetate (2 ml).

Compound	Recovery (%)* (Mean ± S.D., n = 4)		
	Water	Benzene	Ethyl acetate
Arachidonic acid	5.0 ± 2.6	35.1 ± 9.9	6.8 ± 1.8
PGE_1	0.9 ± 0.1	0.3 ± 0.1	79.9 ± 4.2
PGE_2	2.8 ± 0.2	1.3 ± 0.2	74.4 ± 2.9
$PGF_{2\alpha}$	1.5 ± 0.1	0.9 ± 0.1	74.8 ± 2.1
TXB_2	2.2 ± 0.2	1.1 ± 0.1	79.1 ± 5.0

*Counts used in calculating recoveries were corrected for quenching.

for RIA. As shown, when acidified plasma (pH 3) was applied to the column and eluted with water, the majority of the radioactivity remained on column. This aqueous wash step, however, removed water-soluble components in unextracted plasma that could interfere with the subsequent RIA. The columns were then eluted with benzene, and as demonstrated using arachidonic acid as a marker, this step removes fatty acids while allowing the prostaglandins and thromboxanes to remain on column thus avoiding the double solvent extraction described in previous methods [1]. Finally, Table II demonstrates that the prostaglandins of interest can then be eluted in high yield with ethyl acetate. The ethyl acetate can then be volatilized easily under a stream of nitrogen. With the specificity of antibodies that are now available, the fact that the various prostaglandins were present in a mixture has not presented a problem. If desired, it would probably be possible to devise a more selective solvent system to separate the various prostaglandins. As can be seen in Table II, only about 42% of the arachidonic acid can be accounted for in the benzene and ethyl acetate fractions combined. The majority of the remaining arachidonic acid was eluted when the plasma sample was applied to the column. This probably results from the fact that a significant portion of the arachidonic acid is bound to protein that is not retained by the column, since this loss was not observed when arachidonic acid was applied in a protein-free buffer (Table I).

When urine samples (10 ml) were spiked with [^{3}H]PGE_2 and processed exactly as the plasma samples, 91 ± 1% (mean ± S.D., n = 4) of the added radioactivity was recovered in the ethyl acetate fraction. However, when large volumes (400 ml) of urine were processed through a single column (6 ml, C_{18}), the recovery of PGE_2 was poor (16—31%); moreover, the columns tended to clog from the accumulation of amorphous sediment resulting in a significant prolongation of the time required to carry out an extraction. This problem of column obstruction could be lessened but not totally eliminated by centrifuging the urine prior to application to the column. Thus, our experience would indicate that this method is not very efficient for recovering trace amounts of prostaglandin from large urine volumes. On the other hand, when large volumes of protein-free buffer (400 ml) were processed through a single column (6 ml, C_{18}), the recovery of added PGE_2 was usually greater than 80%. These results would suggest that this method might be of utility in extracting trace amounts of prostaglandin from large volumes of buffer such as might be obtained from an organ perfusion.

This improved sample preparation procedure using Baker disposable columns is simpler and more rapid than our previous procedure which involved solvent extraction and chromatography on silicic acid [9, 10]. We have investigated this procedure most extensively in regard to TXB_2 and PGE_2. When whole blood was allowed to clot and the serum (which contained high levels of TXB_2) was serially diluted and processed through the Baker columns and analyzed by RIA, parallelism could be demonstrated. Processing a buffer blank through the Baker columns results in insignificant assayable levels of immunoreactive material (< 5 pg/ml of TXB_2 or PGE_2). This is in contrast with previous attempts employing Amberlite XAD-2 to avoid solvent extraction [1]. With Amberlite columns, significant amounts of polymer leach from the resin resulting in inhibition of antigen—antibody binding thereby precluding its use

in processing RIA samples even though this extraction method is efficient, reproducible and rapid. However, XAD-2 Amberlite can be used in preparing prostaglandin samples for GC—MS.

Powell [11] and Müller et al. [12], utilizing Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.), have described sample extraction procedures similar to that reported here. Müller et al. [12] combined the use of normal- and reversed-phase columns to extract PGE_2, $PGF_{2\alpha}$ and PGE-M from plasma and urine prior to GC—MS. Powell [11] used reversed-phase C_{18} columns to extract several arachidonic acid metabolites from plasma, urine and tissue homogenates. Neither of these previous studies reported recovery data for 5-HETE, 12-HETE or LTB_4. Such procedures employing the Waters Sep-Pak and the Baker Extraction System illustrate the potential and growing use of this approach as an alternative to solvent extraction in processing biological samples containing arachidonic acid metabolites.

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Note

High-performance liquid chromatography of the provitamin A β-carotene in plasma

YEI-MEI PENG* and JON BEAUDRY

Section of Hematology/Oncology, Department of Medicine and the Cancer Center, College of Medicine, University of Arizona, Tucson, AZ 85724 (U.S.A.)

DAVID S. ALBERTS

Section of Hematology/Oncology, Department of Medicine and the Cancer Center, and Department of Pharmacology, University of Arizona, Tucson, AZ 85724 (U.S.A.)

and

THOMAS P. DAVIS

Department of Pharmacology, University of Arizona, Tucson, AZ 85724 (U.S.A.)

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Vitamin A, its naturally occurring provitamins (i.e., α- and β-carotene, Fig. 1), and synthetic analogues have become important drugs in ongoing chemoprevention and treatment trials of human cancers. These compounds have been shown to prevent or cure several chemically induced benign and neoplastic animal tumors [1—5]. In humans, the retinoids have caused regressions of premalignant lesions such as actinic keratosis [6, 7] and leukoplakia [8]. Recent epidemeologic studies have shown that the risk of developing epithelial cancers is inversely related to consumption of retinol and the provitamin, β-carotene [9, 10]. On these bases, large clinical trials of β-carotene are now being organized to evaluate its chemopreventive activity against a variety of epithelial cancers. Unfortunately, little data exist concerning the plasma pharmacokinetics of β-carotene after long-term dosing in humans.

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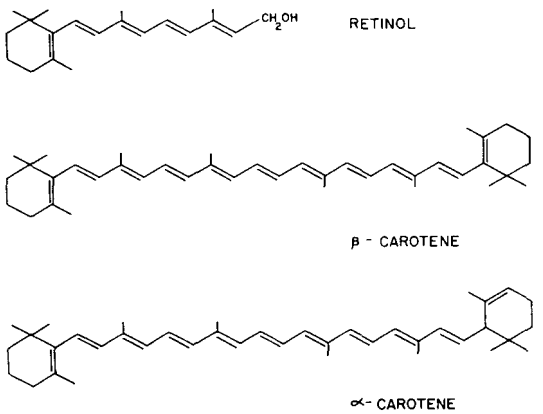

Fig. 1. Chemical structures of α- and β-carotene.

Published analytical methods for provitamin A have included colorimetry [11], open column chromatography [12] and thin-layer chromatography [13]. These have proven tedious, imprecise and fail to resolve β-carotene from its geometrical isomers (i.e. α-carotene and other carotenoids). In addition, gas chromatography has proven impractical because of the thermal instability of carotenoids [14]. Recently, Zakaria et al. [15] described a high-performance liquid chromatographic (HPLC) method for the measurement of carotene in tomatoes. The extraction method is tedious, time consuming, and not suitable for the assay of β-carotene in plasma and other biological fluids.

We have developed a rapid, simple, selective, and sensitive sample cleanup procedure and HPLC assay for the measurement of β-carotene in human plasma. Using this method we have studied the in vitro plasma stability of β-carotene and have shown that low concentrations can be assayed following oral dosing in humans.

EXPERIMENTAL

Materials

α- and β-carotene, obtained from Sigma (St. Louis, MO, U.S.A.), were dissolved in tetrahydrofuran (THF) and stored at −80°C in brown glass vials. All organic solvents were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and filtered through an 0.45-μm fluoropore filter (Millipore, Bedford, MA, U.S.A.) prior to use. Aqueous solvents for HPLC were filtered through an 0.45-μm cellulose acetate filter prior to use. Ammonium acetate (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.), and perchloric acid was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Sample cleanup procedure

To an 0.5-ml aliquot of plasma in a microcentrifuge tube were added 100 μl of 5% perchloric acid and mixed rapidly. An 0.5-ml aliquot of THF—ethyl acetate (1:1, v/v) mixture was added and the samples again mixed for 60 sec and

centrifuged with a microcentrifuge (Fisher Scientific) at 13,000 *g* for 1 min. A 50-μl aliquot of the resulting organic layer was analyzed by HPLC.

HPLC analysis

HPLC analysis was performed using two Model M45 solvent delivery systems, a Model 710B WISP autoinjector, a Model 441 UV detector, a Model 730 data module and a Model 720 system controller (Waters Assoc., Milford, MA, U.S.A.). A Beckman (Berkeley, CA, U.S.A.) Ultrasphere-ODS 5-μm (25 cm × 4.6 mm I.D.) reversed-phase column preceded by a guard column (Waters Assoc.) packed with CO:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used for all analyses. The mobile phase consisted of 88% acetonitrile—THF (3:1, v/v) and 12% of methanol—ammonium acetate (1%) (3:2, v/v) at a flow-rate of 3.0 ml/min. Carotenes were detected at 436 nm. Quantitation was done by the external standard method.

Peak identification

Initial peak identification was based on retention times and comparison with the standards as well as co-chromatography with the standards. Identifications were additionally confirmed by mass spectrometry using a Finnigan Model 3300 mass spectrometer coupled to an Incos Model 2061 data system (Finnigan Instruments, Sunnyvale, CA, U.S.A.). Complete spectra were obtained for β-carotene isolated from the HPLC columns and compared to reference β-carotene spectra.

Recovery studies

Various amounts of standard β-carotene were added to an 0.5-ml aliquot of human plasma at room temperature. The plasma samples were prepared immediately for HPLC analysis as described above. Recovery was calculated by comparing the peak areas of the spiked samples to that of the standards. All experiments were carried out in triplicate on different days.

Stability studies

Stability studies of β-carotene were carried out in THF, THF—ethyl acetate (1:1, v/v) and human plasma. Samples stored at 25°C, 37°C, and -80°C were assayed for β-carotene concentration at time intervals.

RESULTS AND DISCUSSIONS

Fig. 2 shows representative HPLC chromatograms of α- and β-carotene extracted from human plasma: (a) endogenous α- and β-carotene, and (b) 1000 ng/ml standard β-carotene added to plasma. The precision and recovery data for the assay are shown in Table I. The average recovery of β-carotene from plasma was 106 ± 2.65% with a coefficient of variation (C.V.) of less than 5% (Table I). Recovery was linear from 50 to 2000 ng/ml. The assay had a detection limit of 10 ng/ml, determined on the basis of a signal equal to twice the noise level.

β-Carotene was stable in human plasma for more than 24 h at 25°C and 37°C in laboratory light. Reference standard stored in THF at —80°C was stable for

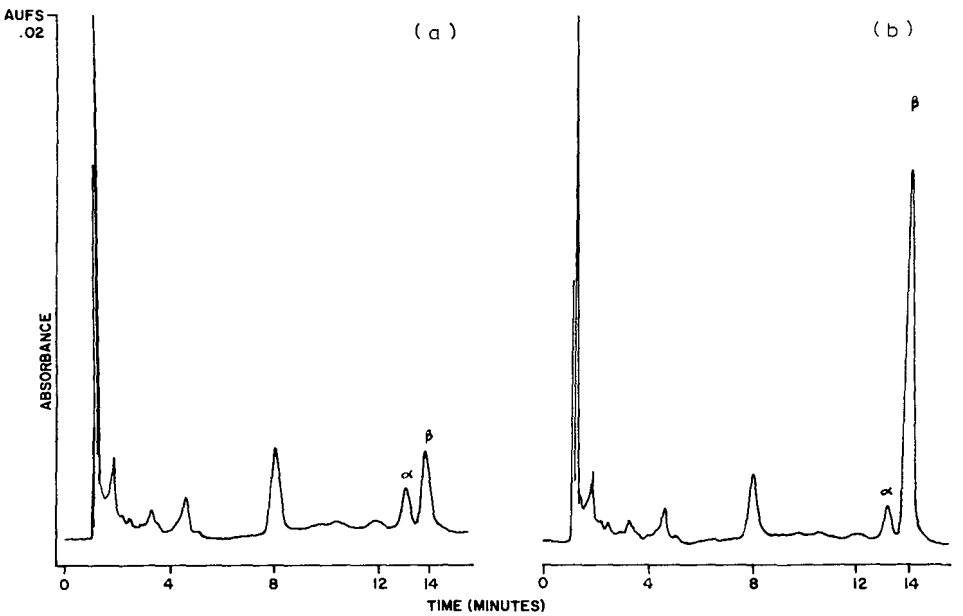

Fig. 2. HPLC chromatograms of α- and β-carotene extracted from human plasma. (a) Endogenous α- and β-carotene; (b) sample spiked with 1000 ng/ml standard β-carotene.

TABLE I

RECOVERY OF β-CAROTENE FROM PLASMA

Recovery of β-carotene at 25°C. All experiments were carried out in triplicate on different days.

β-Carotene plasma concentration (ng/ml)	Recovery (%) ($\bar{x}$ ± S.D.)	C.V. (%)*
1000	105 ± 3.15	3.0
500	109 ± 4.58	4.2
100	104 ± 4.35	4.2
Average	106 ± 2.65	2.5

*C.V. = S.D./$\bar{x}$, where S.D. is the standard deviation and $\bar{x}$ is the mean of three analyses on different days.

at least three months; however, β-carotene was stable for only 1 h in the extraction solvent (THF—ethyl acetate, 1:1, v/v) at 25°C. Thus, HPLC analysis should be performed within 1 h after extraction of β-carotene from plasma samples.

Our assay differs significantly from that of Zakaria et al. [15]. Saponification of the sample and evaporation of the extraction solvent are not required. These two procedures, used by Zakaria et al., are tedious, time consuming and tend to decrease drug recovery and assay precision. Additionally, we have im-

proved chromatographic separation by using 88% acetonitrile—THF (3:1, v/v) and 12% methanol—ammonium acetate (1%) (3:2, v/v). We believe that the present assay will prove useful for monitoring β-carotene concentrations in biological samples, and will help us study its pharmacokinetics and metabolism in cancer patients and normal subjects entered into chemoprevention trials.

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Note

Improved high-performance liquid chromatographic method for isolation of platelet-activating factor from other phospholipids

M.L. BLANK and FRED SNYDER*

Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, TN 37830 (U.S.A.)

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High-performance liquid chromatography (HPLC) with silicic acid columns has been utilized for the separation of various classes of intact phospholipids [1—13]. HPLC methods employing ion-exchange columns have also been used for the separation of phospholipid classes [14—16]. Detection of lipids in these systems was based on radioactivity [8, 12], flame ionization [1, 2, 7, 14], collection of fractions followed by phosphorus analysis [6], and UV in the 200—210 nm range [3—5, 9—11, 13, 15, 16]. These methods described a wide range of solvent mixtures; however, the solvents used with the UV detectors were necessarily limited to those that have low absorbance between 200 and 210 nm.

We were primarily interested in selecting a HPLC system that would separate the recently discovered, biologically active 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkylacetyl-GPC) (platelet-activating factor) from other normally encountered phospholipids; alkylacetyl-GPC can cause platelet aggregation, hypotension, allergic responses, and anaphylaxis [17—19]. HPLC methods are superior to thin-layer chromatography (TLC) in the separation of alkylacetyl-GPC from other phospholipids by the better resolution from closely related analogues (e.g., lysophosphatidylcholine, LPC) and by having the potential of providing a shorter time for a single analysis of a complex phospholipid mixture.

Earlier, HPLC (using a silica column and a chloroform—methanol—water system) had been used as a final step in the purification of alkylacetyl-GPC [20], however, the alkylacetyl-GPC fraction contained some sphingomyelin (SPG) and LPC as impurities [21]. Alkylacetyl-GPC has also been resolved from total phospholipids by using two separate HPLC runs with different

solvent systems [22]. Recently, tritium-labeled alkylacetyl-GPC was separated from several other radioactive lipids on a silica column with two different solvent mixtures in a single chromatographic run; the time required was about 100 min [12].

Our method uses a gradient elution system, since previous work [4, 5, 9] had suggested that gradients could provide good HPLC separations of phospholipids in the shortest time. Gradient elution systems can be used with UV detectors but since the saturated alkylacetyl-GPC shows little or no absorption at 206 nm, it was also important to find lipid markers that possess UV absorbing properties to serve as reference points for detecting the elution of alkylacetyl-GPC. The advantages of our method over the other HPLC techniques reported earlier for the isolation of PAF are the shorter time (40 min or less), good solvent absorption characteristics, the lack of necessity for any preliminary isolation procedures, and the development of lipid standards as markers with excellent UV-absorbing qualities.

EXPERIMENTAL

Materials

Phosphatidylcholine (PC) from soybean, phosphatidylethanolamine (PE) from egg, phosphatidylglycerol (PG) derived from egg PC, phosphatidic acid (PA) prepared from egg PC, phosphatidylinositol (PI) from soybean, phosphatidylserine (PS) from beef brain, and SPG from egg were all purchased from Sigma (St. Louis, MO, U.S.A.). On the basis of a purity check by TLC on Silica Gel HR plates developed with chloroform—methanol—glacial acetic acid—water (50:25:8:2), only PS had to be further purified (by preparative TLC) before use. LPC was prepared from soybean PC by hydrolysis with phospholipase A_2 [23]. A portion of TLC-purified LPC was acetylated to yield acylacetyl-GPC, which was also purified by TLC [24]. All of these phospholipids contained sufficient unsaturation to be easily detected at levels of 30—50 μg per peak with the UV detector set at 206 nm. 1-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (hexadecyl-[^{3}H]acetyl-GPC) was prepared as previously described [25].

Methods

HPLC was performed using a dual-pump Beckman Model 324 M system fitted with a 250 × 4.6 mm Ultrasphere-Si (5 μm) column, which was connected to a Model 155-40 variable UV—visible detector (206 nm) and a Model C-R1A recorder—integrator (all purchased from Beckman Instruments, Norcross, GA, U.S.A.). All samples were dissolved in isopropanol—hexane (1:1) and injected on the column using an Altex Model 210 injector (Beckman Instruments) fitted with a 20-μl sample loop. HPLC grade solvents were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). One of the solvent systems used (I) was similar to that of Geurts van Kessel et al. [4] starting with 96% A [isopropanol—hexane (1:1)] and 4% B (water) with a linear gradient to 8% B over a 15-min period after injection. A second solvent system (II) consisted of 96% A [isopropanol—hexane (1:1) containing 0.005% glacial acetic acid] and 4% B (water containing 0.005% glacial acetic acid); the percent B was increased

linearly to 8.5% over a 20-min period. Solvent system III was the same as system II except 0.005% concentrated ammonium hydroxide was substituted for the acetic acid. The flow-rate was 2 ml/min with all solvent systems. At the end of a chromatographic separation, the column was equilibrated with 20 ml of the starting solvent mixture before injecting the next sample. When switching from one solvent system to another, the column was always equilibrated with about 100 column volumes of the new solvent system before use.

For the separation of hexadecyl-[^{3}H]acetyl-GPC, a fraction collector (Model 1220, Instrumentation Specialties Company, Lincoln, NE, U.S.A.) was attached to the UV flow cell and fractions collected at 0.2-min intervals. The amount of tritium in each fraction was determined with a liquid scintillation spectrometer and the radioactivity per fraction was plotted manually at the appropriate position on the UV recorder tracing.

RESULTS AND DISCUSSION

Hexadecyl-[^{3}H]acetyl-GPC was completely separated from other commonly encountered phospholipids with all three solvent systems (Figs. 1—3). It was eluted between SPG and LPC with a retention time of about 30 min in solvent systems I and III and at 36 min in system II. The resolution of hexadecyl-[^{3}H]acetyl-GPC, acylacetyl-GPC, and LPC was better using either the neutral system (I) or the basic system (III) than using the acetic system (II). Regardless of whether small amounts of acetic acid or ammonia were included in the solvents, PE was eluted at the same time (ca. 10 min). However, elution times of PG, PI, and PA were increased when acetic acid was included in the solvents (Fig. 2), i.e., relative to their retention times in the neutral system (Fig. 1).

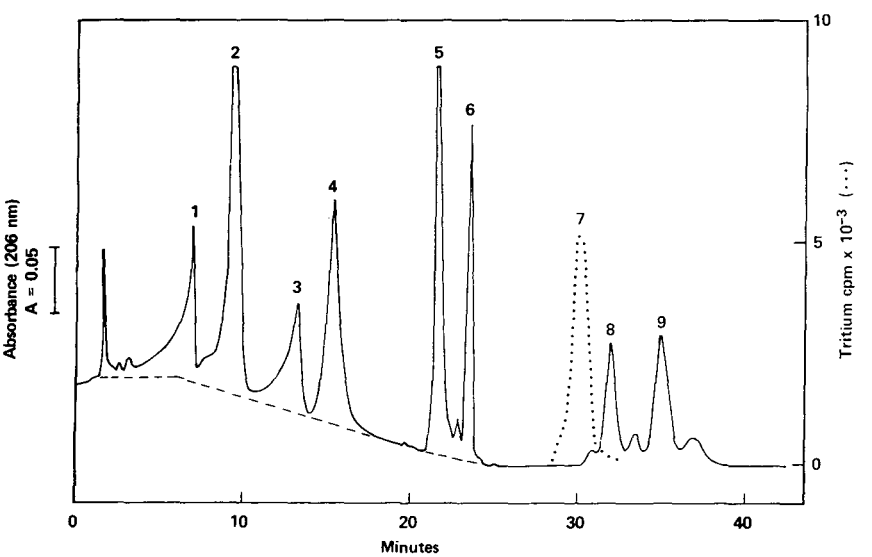

Fig. 1. HPLC separation of (1) PG, (2) PE + PI, (3) PA, (4) PS, (5) PC (and some SPG), (6) SPG, (7) hexadecyl-[^{3}H]acetyl-GPC, (8) acylacetyl-GPC, and (9) LPC using solvent system I described in Methods.

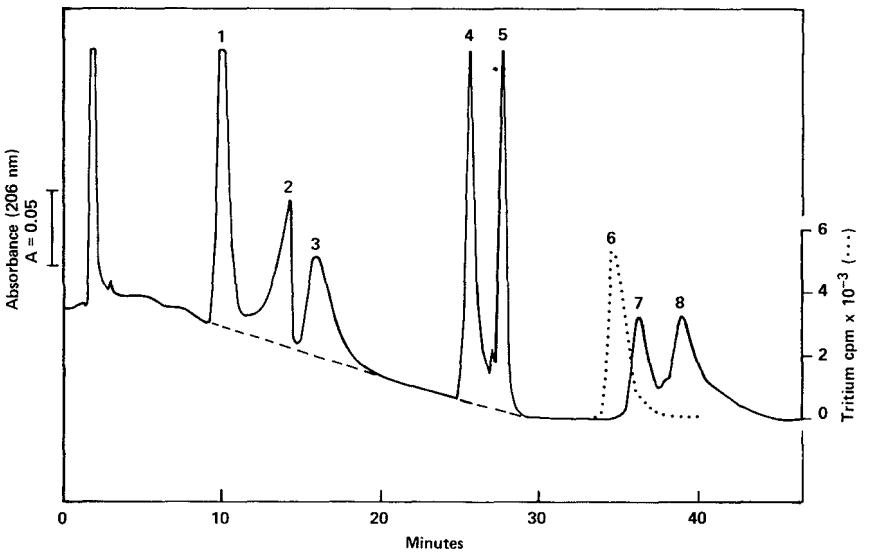

Fig. 2. HPLC separation of (1) PE + PG, (2) PI, (3) PS (also PA if present), (4) PC (and some SPG), (5) SPG, (6) hexadecyl-[^{3}H]acetyl-GPC, (7) acylacetyl-GPC, and (8) LPC using solvent system II described in Methods.

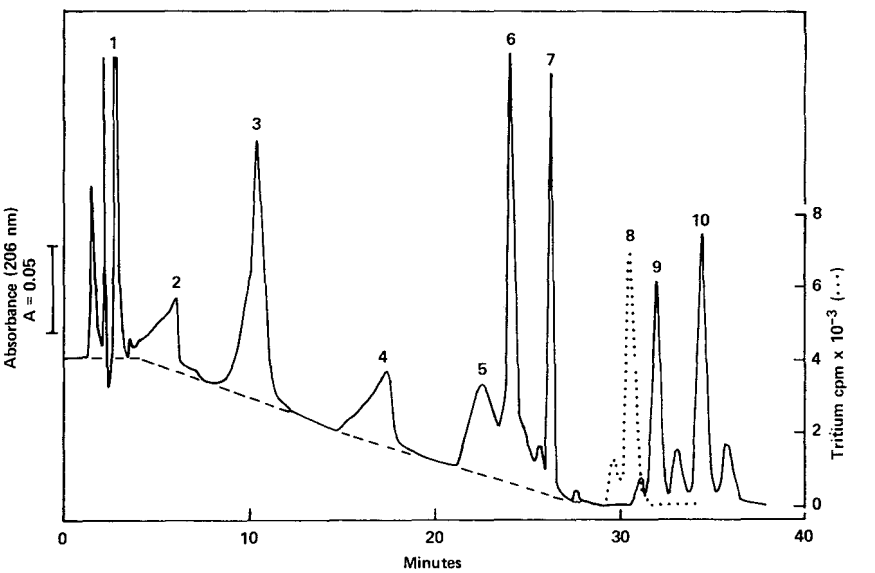

Fig. 3. HPLC separation of (1) fatty acids, (2) PG, (3) PE + PI, (4) PA, (5) PS, (6) PC (and some SPG), (7) SPG, (8) hexadecyl-[^{3}H]acetyl-GPC, (9) acylacetyl-GPC, and (10) LPC using solvent system III described in Methods.

When ammonium hydroxide was in the solvents, the retention of PS was greatly increased (Fig. 3) compared to its retention volume in either of the other solvent systems. However, the acetic system (II) produced better separations of PE, PI, and PS than systems I and III. SPG from egg and the acylacetyl-GPC or LPC prepared from soybean PC serve as good, UV-absorbing

reference peaks for the isolation of alkylacetyl-GPC by HPLC in the solvent systems described. Although the UV recorder tracing shows good separation of PC and SPG in all solvent systems, there was a significant amount (ca. 16%) of SPG eluted with PC as shown by TLC of the collected HPLC peak; this may be related to the extent of unsaturation and acyl chain lengths in the SPG species.

The use of the Ultrasphere-Si column and solvent systems I—III provide a rapid and apparently complete separation of alkylacetyl-GPC (platelet-activating factor) from other commonly encountered phospholipids. The time required to separate alkylacetyl-GPC from other phospholipids, using solvent systems I—III, is shorter than other published HPLC systems [12, 20—22]. SPG from egg and acylacetyl-GPC or LPC prepared from soybean PC are sufficiently unsaturated to provide useful reference compounds that can be used with UV detection to "bracket" the elution time of the non-UV-absorbing alkylacetyl-GPC; this could not be done with some of the systems previously described for the separation of alkylacetyl-GPC because the solvents were not transparent at 200—210 nm [12, 20, 21].

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Note

Estimation of polyethyleneglycols in human urine for studies of intestinal absorption

R.W.R. BAKER* and JEAN FERRETT

Department of Chemical Pathology, Guy's Hospital Medical School, London SE1 9RT (Great Britain)

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In studies of human intestinal permeability, it has been customary to administer orally some probe molecule and to measure the amount of probe which reaches the bloodstream. In many cases, the probe molecules are rapidly excreted in the urine and an assessment of urinary excretion provides the advantages of non-invasive investigation. Substances used have included mannitol and cellobiose [1], lactulose and L-rhamnose [2] and polyethyleneglycols [3—5], and in all cases the molecular weights involved have not exceeded 600. Since it was desired to extend the study to include larger molecules (e.g. MW 4000), with polyethyleneglycol as the probe material of choice, it was necessary to devise a method for estimation in this wider range of molecular weights.

With polyethyleneglycols (PEG) of molecular weights of 400—500 or less, the use of gas chromatography has proved entirely acceptable [4, 5] but this method cannot readily be used with higher polymers. We have devised a method of detection and measurement of various molecular-sized PEG fractions in urine which combines separation by gel chromatography [6, 7] with differential measurement of refractive index. PEG 600 and PEG 4000 were combined in a single oral dose and the urinary excretion over 24 h was measured. It was found that the degree of absorption of PEG 4000 in healthy subjects did not exceed 2.5% of the amount ingested.

EXPERIMENTAL

Materials and equipment

Bio-Gels P-4, P-6 and P-10, all 200—400 mesh, were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.), Polyethyleneglycols (PEG) with mean

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molecular weights (MW) 200, 300, 400 and 1540 were from Koch-Light Labs. (Colnbrook, Great Britain). Ethyleneglycol and PEG of mean MW 600, 1000, 4000 and 6000 were from BDH Chemicals (Poole, Great Britain) as were sodium azide and the non-ionic detergent Brij 35. Chromatography columns from Wright Scientific (Stonehouse, Great Britain) were used, with valves (SRV4) from Pharmacia (Uppsala, Sweden). The differential refractometer (Model 1109) and Milton-Roy pump were from Laboratory Data Control (Stone, Great Britain). An LKB (Bromma, Sweden) strip-chart recorder (Model 2210) was used. Urine for analysis was collected directly into polythene bottles containing 10 ml 25% (v/v) hydrochloric acid as preservative, and after measurement of the volume a filtered aliquot was stored at 2° C if not handled at once.

Analytical method

Two jacketed columns were used, the first (178 × 16 mm) of Bio-Gel P-4, and the second (250 × 16 mm) of Bio-Gel P-10. They were connected by two 4-port valves so that they could operate in series or with elution only through the second column while the first was back-washed under gravity from the magnetically stirred reservoir of eluent, which was water containing Brij 35 (60 mg/l) and sodium azide (20 mg/l), in a thermostat at 45°C. Water from the thermostat was circulated through the column jackets. The load-loop, also constructed from two valves as above, was of volume approximately 1.50 ml. Urine, warmed and shaken at 45° C to reduce dissolved air, was loaded with the columns in series and elution proceeded at approximately 0.7 ml/min until all PEG had passed out of the first column (25 ml). The elution was then continued in the second column while urea, etc. were back-washed off the first (30 ml), after which series-flow was resumed for a following load. Eluate from the system passed through the refractive index monitor to a 5-ml syphon delivering to waste. Each operation of the syphon was shown by an event-mark on the strip-chart of the recorder registering the output of the refractometer. Connected at the output of the pump by a stainless-steel capillary T-joint was a vertical (50 cm × 10 mm I.D.) tube of stainless steel filled with air, having at its upper end a pressure gauge; pulses were thus damped and pressures were indicated. Working pressures did not exceed 1 kg/cm^2.

Quantitation

Peaks on elution charts were reduced to equivalent triangles either by simply drawing in tangents after insertion of the baseline, or by constructing chords (a mm) at $c = 0.75\ h$ and (b mm) at $0.5\ c$ for peaks of height h mm, giving the area $A = c\ (b - \frac{a}{2})^2/(b - a)$. Areas found ($A$ mm^2) were corrected to standard chart-speed (1 mm/min). Flow-rates (F ml/min) were evaluated from the syphon volume and event-marks on the chart, and the product AF was taken as estimator of the concentration of PEG in the load applied. Values of AF corresponding to 1 mg/ml PEG in the original loads were determined by passing through the process a standard solution, usually 1.5 mg/ml PEG 600 with 0.75 mg/ml PEG 4000 dissolved in elution liquid or urine.

RESULTS

Analytical method

Typical elution patterns given by direct chromatography of urine are shown in Fig. 1. Results of recovery trials are shown in Table I. There was a linear relationship between the function AF (above) and the mass of PEG applied to the analytical system in a constant volume, and this extended well beyond the range 0—1500 μg/ml of either polymer [r = 0.999, 7DF (degrees of freedom)], whereas in analysis of urine samples the loads should not exceed 1000 μg of PEG 600 or 250 μg of PEG 4000.

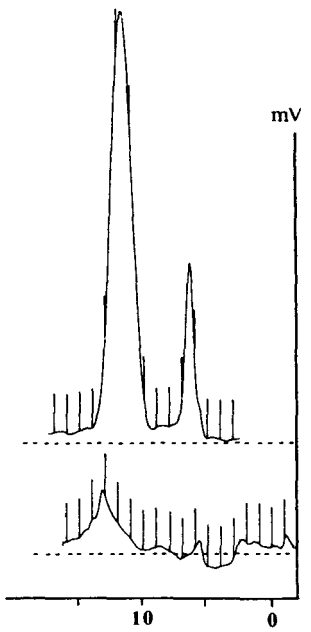

Fig. 1. Elution pattern for blank urine and urine with added PEG 600 (1500 μg/ml, tallest peak) together with PEG 4000 (300 μg/ml). Refractive index monitor attenuated × 50; 10 mV full scale. Recorder zero shown as dotted lines. Fractions were each 5.06 ml.

TABLE I

RECOVERIES FROM POSTPRANDIAL URINE OF ADDED POLYETHYLENEGLYCOLS (PEG, μg/ml)

Added		Found	
PEG 600	PEG 4000	PEG 600	PEG 4000
150	30.0	190	30.0
250	50.0	208	39.3
500	100	444	87.1
1000	200	1024	197
1500	300	1505	301
0	503	0	524
0	1005	0	1030
0	1508	0	1484

The 24-h urinary excretion of healthy adult subjects was estimated in 27 cases after an oral load containing 2.5 g PEG 600, and was found as the 95% confidence interval to be 18.7—66.3% of the load. Because of the difficulty in detecting very low levels of PEG 4000, 5 g of the higher polymer were incorporated in the solution ingested and 24-h excretions of 0.39—2.67% were obtained [8] as determining the 95% confidence interval.

DISCUSSION

In routine analysis, speed of operation and time taken over manipulations are important factors, and thus enforce a compromise between rapidity and the excellence which itself indicates the use of long columns and slow flow-rates. In the present work, the fastest flow-rates consistent with stable and low back-pressures were chosen and the columns were of minimum heights. In the preliminary treatment there is a positive exclusion of urinary solutes of lower molecular weights, but since PEG 4000 elutes near the void volume of Bio-Gel P-10, a proportion of larger molecules, e.g. proteins, may appear and in some cases a small peak, imperfectly resolved from that given by PEG 4000 on the final chromatogram, may be seen. However, this appeared not to introduce appreciable error and can be corrected for by processing a blank sample of urine voided before ingestion of the probe. For this reason, more recently introduced filtration media [9], including those capable of operating under high pressures, are being investigated. The method presented here has been used to study intestinal absorption in abnormal subjects; the results will be described elsewhere.

In preliminary work, a buffer solution (0.05 *M* phosphate, pH 7.3 with 0.1 *M* sodium chloride) was used as column eluent, but appeared to offer no advantage over the solution finally adopted. The use of a 24-h collection period was specified because excretion of PEG was always observed after the first 12 h had passed but did not continue significantly beyond 24 h after ingestion of the load. The use of increased temperature for chromatography reflects an attempt to secure maximal flow-rate without exceeding an arbitrarily set pressure in the system.

Recoveries of PEG added to normal urine to give concentrations similar to those anticipated to be found in disease were as shown in Table I. From the regression equations between prepared (x) and estimated (y) concentrations,

$y = 1.005x - 5.874$ ($r = 0.998$) for PEG 600,
$y = 0.999x - 0.224$ ($r = 0.999$) for PEG 4000,

it is seen that mean recoveries were essentially quantitative. Since the standard errors of the regression coefficients are small, variability of replicates is ascribed almost entirely to variance about the regression. This variance is such that 95% of replicates would be expected to exhibit random errors for PEG 6000 of less than 80 μg/ml, or $\pm < 10\%$ for a typical specimen. Similarly, 95% of results for PEG 4000 should be in error by $\pm < 12$ μg/ml, or $\pm < 30\%$ for normal excretions, but most estimates will of course exhibit appreciably smaller errors for both polymers.

More desirably, an internal standard such as PEG 1540 might be employed, but would require improved resolution in chromatography with subsequent increase in the time required for each estimation. If an extraction of PEG with organic solvent could be incorporated without undue prolongation of analysis, PEG 400 could be used as internal standard, as found in preliminary trials using chloroform—methanol (2:1, v/v) as extractant. It should be feasible to convert the present method into an automatic system controlled by signals from the syphon, provided piston valves were incorporated in place of those described.

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Note

Rapid micromethod for the determination of Evan's blue in human plasma by high-performance liquid chromatography

THOMAS P. GREEN* and RANDAL P. MARCHESSAULT

Division of Clinical Pharmacology, Departments of Pediatrics and Pharmacology, Box 363, Mayo Hospital, University of Minnesota, Minneapolis, MN 55455 (U.S.A.)

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Evan's blue (T-1824) is an azo dye of unusual biomedical importance. Because of the high affinity of this compound for plasma albumin [1, 2], Evan's blue is used in the clinical determination of plasma volume [3—9] and in studies of albumin distribution [10] and vascular permeability [7, 8, 10]. Currently, spectrophotometric techniques for the determination of the concentrations of Evan's blue lack sensitivity and specificity. We report a method employing high-performance liquid chromatography (HPLC) for the rapid assay of Evan's blue in plasma samples. The method has distinct advantages over conventional techniques and will permit studies employing Evan's blue in a wide variety of clinical problems.

METHODS

Extraction of Evan's blue from plasma

A 150-μl sample of plasma collected in the presence of EDTA disodium salt, or a smaller volume of sample diluted to 150 μl with 154 m*M* sodium chloride solution, was placed in a microcentrifuge tube. The pH was increased to 11—12 by the addition of 120 μl of 0.1 *N* sodium hydroxide, after which 120 μl of magnesium oxide suspension (2 mg/ml) and 25 μl of Trypan blue solution (40 mg/l) were added. The mixture was agitated briefly, centrifuged in a high-speed table top centrifuge for 2 min, and the supernatant discarded. The pellet was washed twice with water by resuspending the pellet in 50 μl water, agitating, centrifuging, and discarding the supernatant. The pellet was then dissolved in 75 μl of 0.2 *N* hydrochloric acid, and the mixture neutralized with 75 μl of 0.2 *M* Tris. An aliquot of this solution was then injected in the HPLC system.

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High-performance liquid chromatography

The principal features of this system included an Altex 110A pump, a Rheodyne 7125 loop injector, an Alltech 300 × 5 mm, C_{18} reversed-phase column (10 μm particle size), Schoeffel SF770 variable-wavelength detector with tungsten lamp and a Beckman 10-mV strip chart recorder. The eluent was 66% (v/v) methanol and 34% aqueous phase (120 m*M* Tris, pH 8.0, 40 m*M* magnesium chloride) and was pumped at 1 ml/min. The effluent was monitored at 590 nm.

Quantitation of Evan's blue in plasma samples

Chromatographic standards of Evan's blue were made by appropriate dilutions of Evan's blue in 120 m*M* Tris, pH 8.0, 40 m*M* magnesium chloride, and standard curves were constructed by plotting peak height against concentration.

RESULTS

Evan's blue had a retention time of 5 min and produced a sharp symmetrical peak (Fig. 1). Trypan blue, added to the samples for visualization of the precipitate during the extraction procedure, was not retained on the column.

The relationship between chromatographic peak height and dye concentration was slightly non-linear for concentrations between 0.5 and 20 mg/ml; in a typical experiment, the ratio of peak height to concentration increased from 2.43 at 0.5 mg/l to 3.64 at 20 mg/l (Fig. 2). A linear plot of concentration vs. peak height visually appeared linear (r = 0.990) but was perceptably convex (Fig. 2A). The data were best represented by an exponential plot:

$$C = 10.0\ (P/P_{10})^{0.899}$$

where C is concentration (mg/l), P is the peak height for the sample, and P_{10} is the peak height of the standard containing 10 mg/l (Fig. 2B; r = 0.9996, n = 27). This formula was found to accurately describe peak height—concentration relationships on subsequent standard curve determinations. Four sets of concentration standards, each set containing 11—16 samples, had absolute sample mean errors (error/concentration) that ranged from 3.0 to 5.5%. Error was independent of concentration within the concentration range of 0.5—20 mg/l. The threshold of accurate detection (signal-to-noise ratio greater than 2) was 0.5 mg/l with a 0.050-ml injection volume (25 ng).

Recovery from extracted samples was 81.3% with a coefficient of variation of 4.8% (n = 16). Recovery was independent of concentration and recovery of samples from serum and 154 m*M* sodium chloride solution gave similar values.

No peaks with a retention time similar to Evan's blue have been observed in serum samples from subjects who had not received this compound. In addition, the following commonly used drugs were tested in the system and did not interfere with the determination of Evan's blue concentration: ampicillin, cephalothin, gentamicin, furosemide, acetazolamide, digoxin, caffeine, phenytoin, riboflavin, and indocyanine green. Twenty to thirty samples have been processed on a typical day.

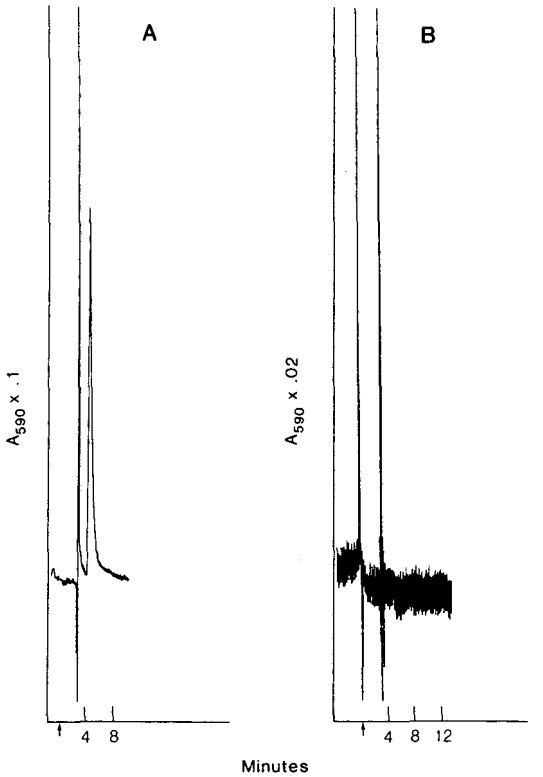

Fig. 1. HPLC of plasma samples. (A) Plasma containing 10 μg/ml of Evan's blue; attenuation 0.1. (B) Plasma containing no Evan's blue; attenuation 0.02. Plasma extraction procedures and chromatography conditions as described in text. In both cases injection volume was 0.050 ml. Injection performed as indicated at arrow; retention time of Evan's blue 5 min.

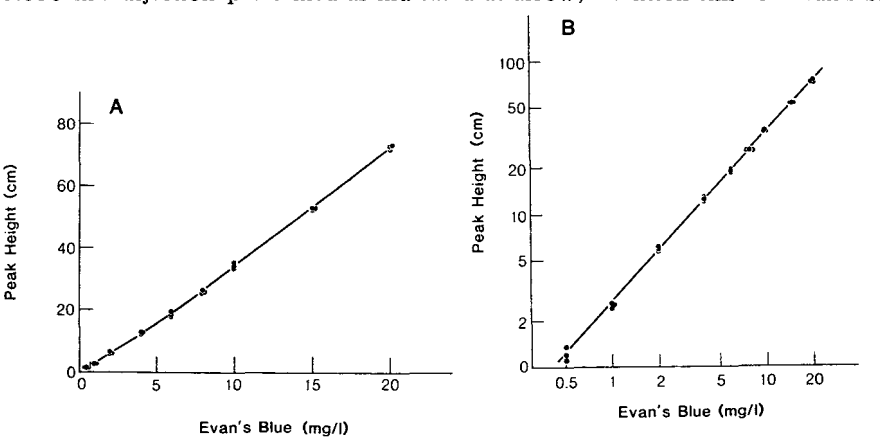

Fig. 2. Relationship between Evan's blue chromatographic peak height and concentration of dye injected (three points at each concentration, injection volume 0.050 ml; attenuation 0.04). (A) Linear plot: although linear plot fit of data is good (r = 0.990), there is a distinct convexity to the curve. Correspondingly, peak height to concentration ratios increase with concentration. (B) Logarithmic plot: fit is independent of concentration (r = 0.9996). Concentration accurately predicted by regression equation $C = 10\ (P/P_{10})^{0.899}$, where C is the concentration of the unknown, P is the peak height of the unknown and P_{10} is the peak height of a standard containing 10 μg/ml (see text).

DISCUSSION

Two analytical problems were encountered during the development of this assay technique. The first was the separation of Evan's blue from albumin in plasma, necessary because the Evan's blue—albumin complex had markedly different chromatographic properties from the free dye and since virtually all the dye in plasma (>99%) exists in the protein-bound form [2]. Conventional protein denaturation procedures did not quantitatively release free dye. This problem was solved by alkalinization of the plasma in the presence of excess metallic cation (Mg^{2+}) producing a dye lake that was insoluble in plasma. The principle of this procedure is similar to that used in methods in which other azo dyes are used for the quantitative determination of Mg^{2+} concentrations [11]. Once the lake was washed free of plasma proteins, the precipitate was redissolved in acid, the solution neutralized, and an aliquot chromatographed.

The second difficulty encountered was the non-linearity of the concentration—peak height relationship. Although the deviation from linearity was small, clinically unacceptable errors (greater than 5—10%) would be introduced at the lowest concentrations using linear assumptions. Alternatively, laborious, complete standard curves that do not assume any format, linear or exponential, could be developed with each day's determinations. We found, however, that the exponential formula experimentally developed and presented above gave excellent accuracy and precision (coefficient of variation of less than 5%) at concentrations achieved with clinically used doses of dye at 0.1—0.5 mg/kg. The likely explanation for the non-linearity is that increasing amounts of cation relative to dye decrease the affinity of the lake formation because of cation—cation interactions [12]. This hypothesis was supported by experiments in which we found a decreased recovery of dye from serum when the amount of magnesium oxide in the initial step of the extraction procedure was increased.

The clinical measurement of plasma volume is currently performed by determining the dilution volume of intravenously administered radiolabelled albumin, radiolabelled red cells, or Evan's blue. These techniques have been validated and protocols with normal values have been published [3—9]. The radioisotopic methods necessitate radiation exposure, which can be minimized by using larger blood sample volumes and avoiding repeated determinations in the same patient. The use of Evan's blue eliminates the radiation hazard and repeated determinations carry no hazard.

This technique presents significant advantages over other assay methods for Evan s blue. The high specificity of the method accrues from the extraction and chromatographic separation of the dye from other plasma constituents. Spectrophotometric methods are subject to interference in hemolyzed, turbid or lipemic sera [13]. Other attempts to eliminate the interference have involved the quantitation of the interfering substances [9] or more laborious chromatographic procedures [13]. The high sensitivity and accuracy of this assay procedure are due both to the lack of even minute amounts of interfering substances and to the high resolution characteristics of HPLC. This improvement in sensitivity over previous techniques will allow the reduction in sample size thereby permitting its application to even the smallest patient.

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Note

Thin-layer chromatographic determination of L-asparaginase in the presence of human serum

SHAM L. PAHUJA and TED W. REID*

Yale University School of Medicine, Department of Ophthalmology and Visual Science, New Haven, CT 06510 (U.S.A.)

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L-Asparaginase is an aminohydrolase which causes the hydrolysis of asparagine to aspartic acid and ammonia. There is a great deal of interest in studying L-asparaginase from *E. coli* because of the fact that the enzyme has been used extensively for the management of acute lymphocytic leukemia over the last decade [1—5]. The antineoplastic activity is probably due to depletion of circulating pools of asparagine by asparaginase. Despite the therapeutic significance of this enzyme, there is no easy method for its determination.

Assay methods generally measure the enzyme activity either by the determination of ammonia [6] or measurement of NADH formation after coupling aspartate with other enzymes [7—9]. Determination of ammonia by the Nessler reagent [15] is less sensitive and is time consuming. The enzyme coupling methods require the use of coupling enzymes in adequate purity. The radioisotope method based on the separation of aspartic acid from asparagine by ion-exchange chromatography [10] is specific and sensitive but suffers from disadvantages such as being time consuming, lengthy and subject to interferences such as changes in pH.

This paper describes a simple, specific, sensitive and rapid assay for L-asparaginase which is also applicable in the presence of human serum. The enzyme activity is measured by incubating L-asparaginase with the assay mixture and separating the product by thin-layer chromatography (TLC). Under the conditions employed, aspartate moves near the origin, while asparagine moves with the solvent front resulting in a complete separation of both the components.

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MATERIALS AND METHODS

Materials

Plastic sheets precoated with Ionex SB-Ac were obtained from Brinkman (Westbury, NY, U.S.A.). Prior to use, 1.2 × 9.0 cm strips were cut and equilibrated with 0.05% acetic acid for 30 min as recommended by Devenyi [11]. Unlabelled asparagine, aspartate, and *E. coli* L-asparaginase (Grade 1, 30 U/mg of protein) were obtained from Sigma (St. Louis, MO, U.S.A.). Aqueous counting scintillant was obtained from Amersham (Arlington Heights, IL, U.S.A.). L-[^{14}C(U)]Asparagine (150 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Due to impurities present in the commercial preparation of radiolabelled asparagine, it was further purified as follows: A 20-μl aliquot was applied 1 cm from the bottom of the strip (2.5 × 9.0 cm) and ascending chromatography was carried out as described below. Asparagine was eluted from the strip with 1% formic acid. The resin was removed by filtration through a Pasteur pipet plugged with glass wool and the filtrate lyophilised. The residue was dissolved in 0.5 ml of distilled water and lyophilised again. This asparagine was used as a substrate in the assay mixture.

Procedure for incubation

Assays were performed at 37°C in a final volume of 0.05 ml in the polypropylene microcentrifuge tubes. The incubation mixture contained 50 m*M* Tris, pH 8.6, 5 m*M* [^{14}C]asparagine (800—2000 cpm/nmol). The reaction was initiated by the addition of enzyme to the incubation mixture and terminated by rapidly cooling in ice. In the experiments where human serum was added to the enzyme assays, the final concentration of serum was 20%. The blank usually contained the assay mixture with the denatured enzyme (100°C, 10 min) incubated at 37°C. One international unit of enzyme activity (IU) is equivalent to the amount of enzyme that causes the hydrolysis of 1 μmol of asparagine per min at 37°C. Specific acitivity is defined as U/mg of protein.

Thin-layer chromatography

Radiolabelled aspartate formed by the enzymatic reaction was separated from the unreacted substrate by ascending TLC. Immediately after cooling, 5 μl of the incubation mixture were spotted and dried at an origin 1 cm from the bottom of the strip. The strip was next placed in a developing tank containing ethyl acetate—acetic acid—water (8:0.05:91.95). After the solvent had migrated about 7 cm from the origin, the strips were removed and dried. Reference strips were also run with unlabelled asparagine and aspartate under identical conditions. Spots corresponding to asparagine and aspartate were cut and placed in the scintillation vials. In cases where reference strips were not run, the strips were cut into 1-cm pieces and placed in separate vials. Radioactivity was determined after eluting amino acids from the resin by adding 3.0 ml of scintillation fluid followed by 0.5 ml of 20% formic acid.

Protein was determined by the dye-binding method using bovine serum albumin as a standard [12].

RESULTS

Separation of aspartate and asparagine

The separation of asparagine and aspartate with ethyl acetate—acetic acid—water (8:0.05:91.95) in 30 min is seen in Fig. 1. Aspartate moved near the origin and asparagine moved near the solvent front. R_F values of asparagine and aspartate in three separate runs were 0.97 ± 0.02 and 0.04 ± 0.01, respectively. This separation of asparagine from aspartate was used to develop an assay for L-asparaginase.

Application to the determination of L-asparaginase

Fig. 2 shows the separation of aspartate produced by L-asparaginase after incubation with the radiolabelled substrate. Serum alone when assayed under identical conditions did not reveal any enzyme activity. Addition of serum to the assay mixture did not affect the mobility of asparagine or aspartate as a similar separation was obtained when L-asparaginase without any serum was

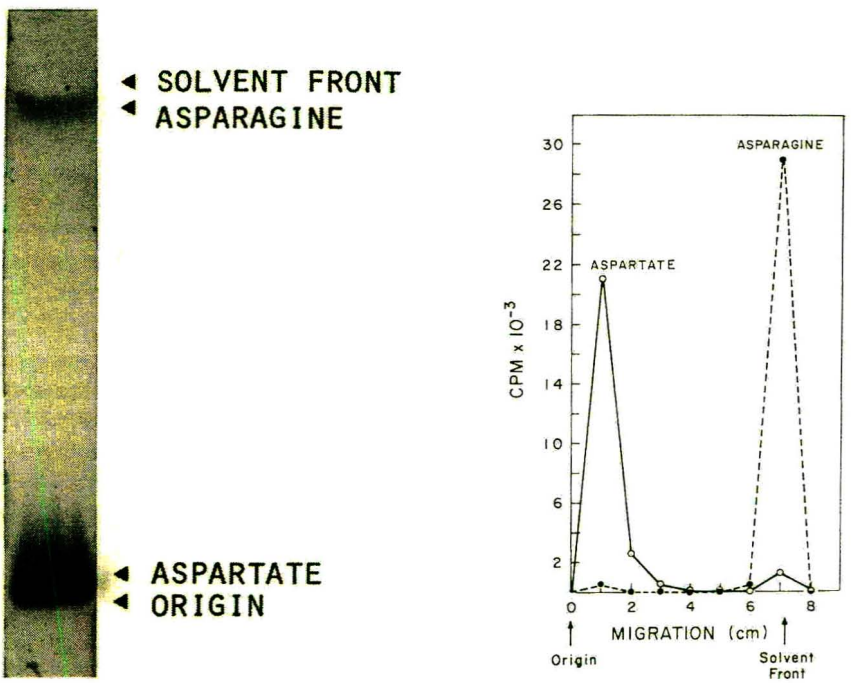

Fig. 1. Separation of asparagine from aspartate. A 2-μl aliquot containing 5 μg of asparagine and aspartate in Tris buffer, pH 8.6, was applied. After development, the strips were dried, sprayed with 0.2% ninhydrin in butanol—acetic acid (95:5) and spots visualised after brief exposure at 70°C.

Fig. 2. Separation of radiolabelled aspartate (○——○) formed from [^{14}C]asparagine by 0.8 μg of *E. coli* L-asparaginase added exogenously to a 10 μl of human serum. The assay was performed at 37°C for 10 min and the sample was chromatographed as described in Materials and methods except the strip was cut into 1-cm pieces and radioactivity counted. Mobility of [^{14}C]asparagine (•— — —•) on the strip after the assay mixture was incubated with heat-inactivated enzyme at 37°C for 10 min.

used in the assay mixture. The assay also showed a linear relationship for aspartate formation with incubation times ranging up to 30 min, and had a correlation coefficient of 0.998.

Recovery and reproducibility

To determine the assay precision, 100 ng and 400 ng of L-asparaginase were added to 10 μl of human serum. The enzyme activity was determined after incubation with the assay mixture at 37°C for 10 min. The results are shown in Table I. The amount of enzyme added was directly proportional to the amount of enzyme measured.

TABLE I

ACCURACY OF DETERMINATION OF *E. COLI* L-ASPARAGINASE IN THE PRESENCE OF HUMAN SERUM

Asparaginase added (ng)	Asparaginase found (ng)	Coefficient of variation (%)	Recovery (%)
100	103.1 101.5 98.4		101.0
Mean	101.0 ± 2.43	2.40	
400	448.4 382.0 405.6 410.4 418.4 398.0		102.6
Mean	410.5 ± 22.3	5.43	

DISCUSSION

TLC is a simple, rapid and inexpensive analytical technique for resolving metabolites. Recently, Dunlop et al. [13] used paper chromatography to separate asparagine from aspartate. Aspartate migrated 5—7 cm from the origin, asparagine migrated 10—14 cm from the origin and separation was achieved in 18 h. Pataki [14] lists different solvent systems as to their ability to separate asparagine from aspartate by TLC. However, the differences in R_F values are not large enough to adopt one of these separations as a regular, reliable assay for L-asparaginase. With the present method, aspartate remains near the origin, while asparagine moves with the solvent front and separation is obtained in 30 min.

The present method measures aspartate formation directly and is more sensitive than Nessler's method for ammonia determination or enzyme coupling methods. As little as $4 \cdot 10^{-5}$ IU of L-asparaginase can be detected easily with this method as compared to $1 \cdot 10^{-2}$ IU of enzyme required for Nessler's method or $5 \cdot 10^{-4}$ IU of enzyme required for the enzyme coupling method

[9]. The sensitivity of the present assay was determined using [^{14}C]asparagine (2000 cpm/nmol) with 500 cpm as a reliable detectable change in radioactivity. The method could be made more sensitive by increasing the specific activity of the substrate. In comparison to the ion-exchange column method, the present method is simple, rapid, and separation of asparagine from aspartate is not affected by changes in pH of the assay mixture, ionic strength or by constituents present in serum.

In conclusion, we feel that this technique provides a rapid, sensitive and reliable assay for L-asparaginase. The assay should be useful in enzyme clearance studies in patients receiving L-asparaginase therapeutically.

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Note

Sensitive and quantitative determination of plasma doxepin and desmethyldoxepin in chronic pain patients by gas chromatography and mass spectrometry

T.P. DAVIS*, S.K. VEGGEBERG, S.R. HAMEROFF and K.L. WATTS

Departments of Pharmacology and Anesthesiology, University of Arizona, College of Medicine, Tucson, AZ 85724 (U.S.A.)

(First received June 25th, 1982; revised manuscript received October 20th, 1982)

Doxepin (N,N-dimethyl-3-dibenz[*b*,*e*]oxepin-11-(6H)-ylidene-1-propanamine) is a tricyclic antidepressant with anti-anxiety and antihistamine (sleep, sedation) properties. Although documentation is limited, it has also been found effective in chronic pain states which are often accompanied by depression [1]. The purpose of this study was to develop a precise and selective gas chromatography—mass spectrometry (GC—MS) method to analyze doxepin and its major metabolite desmethyldoxepin and to correlate the peripheral plasma concentrations with therapeutic analgesic, antidepressant, and sleep effects of doxepin. These clinical effects were observed in a double blind comparison between doxepin and placebo in 30 depressed, chronic pain patients [2].

Many analytical methods have been utilized to measure tricyclics in general and doxepin in particular. Gas—liquid chromatography (GLC) with flame ionization detection [3], GLC and capillary GLC with nitrogen—phosphorus detection [4—6], high-performance liquid chromatography (HPLC) (7, 8], radioimmunoassay [9], and GC—MS with mass fragmentography detection [10, 11]. These methods have proven to be adequate for high concentrations of doxepin but GC—MS offers the necessary precision, accuracy, selectivity, sensitivity and low sample volume requirements for clinical analyses. The method presented in this paper provides the necessary precision, selectivity, low sample volume (i.e., 1.0 ml) and a sensitivity of less than 1 ng/ml for the presented clinical studies of pain patients.

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MATERIALS AND METHODS

Standards and reagents

Doxepin hydrochloride (76.0% *trans*, 12.8% *cis* isomer and desmethyldoxepin *trans* isomer) were supplied by Pfizer (Central Research, Groton, CT, U.S.A.). Amitriptyline and nortriptyline were used as the internal standards and supplied by Wyeth Laboratories (New York, NY, U.S.A.). Hexane, methanol and isoamyl alcohol were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Trifluoroacetic anhydride (Regis Chemical, Morton Grove, IL, U.S.A.), sodium hydroxide and hydrochloric acid were all ACS reagent grade. All glassware was rinsed previously with 10% isoamyl alcohol in hexane. This rinse prevents adsorption of the secondary and tertiary amines to the glassware.

Sample extraction and derivatization procedure

Blood samples were collected into Vacutainer tubes, kept on ice and centrifuged within 1 h. Plasma was transferred to plastic tubes and stored at −80°C for up to six months, prior to analysis, without loss of drug. After thawing, 1.0 ml of plasma was transferred to a 12-ml screw top test tube with a PTFE-lined cap and 50 μl of the internal standard (I.S.) (amitriptyline—nortriptyline, 60 ng:50 μl) in water was added. The mixture was made alkaline by the addition of 50 μl of 6 *N* sodium hydroxide followed by the addition of 5 ml of hexane—isoamyl alcohol (95:5, v/v). The tubes were shaken on an automatic shaker for 20 min and the phases were separated by centrifugation at 1700 *g*. The organic phase was then transferred to a graduated conical centrifuge tube. To selectively extract the tricyclics, 500 μl of 0.1 *N* hydrochloric acid was added to the organic phase and the samples were placed on the shaker for 10 min. The hydrochloric acid was then transferred to a 1.0-ml reacti-vial (Regis Chemical) and reduced to dryness at 45°C under a stream of ultra pure nitrogen. Derivatization of the desmethyldoxepin was achieved by the addition of 300 μl of a 5% trifluoroacetic anhydride (TFAA) solution and reacted at room temperature for 30 min. Higher concentrations of TFAA or higher reaction temperatures tend to degrade tricyclics or give multiple reaction products [11]. The reaction converted virtually 100% of desmethyldoxepin to its N-trifluoroacetyl derivative without degradation losses of doxepin. The reaction mixture was then taken to dryness under ultra-pure nitrogen at room temperature, redissolved in 25 μl ethyl acetate and 10 μl were injected directly into the GC—MS system for analysis.

Gas chromatography—mass spectrometry analyses

A Finnigan 3300 quadrupole mass spectrometer coupled to a Finnigan 9500 gas chromatograph and a 6000 data system were used to perform the analyses (Finnigan Instruments, Sunnyvale, CA, U.S.A.). The following chromatographic conditions were used: glass column, 1.1 m × 2 mm I.D., packed with 3% OV-17 on 100—120 Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). The column temperature was maintained at 220°C; injector, 260°C; separator, 300°C. The carrier gas (helium) was maintained at a flow-rate of 20 ml/min. The column was prepared for analyses by priming

with three injections of 10 μg each of doxepin and desmethyldoxepin trifluoroacetyl derivative. As previously reported [12], this is an essential step to avoid variable adsorption of the secondary and tertiary amines and ensure optimal chromatographic reproducibility. The mass spectrometer was operated at an electron energy of 70 eV, filament emission of 800 μA, and an ion source temperature of 75°C. The data system was programmed to scan the ions *m/e* 58.1 (doxepin), 165.1 (doxepin and desmethyldoxepin), 232.2 (nortriptyline) and 234.2 (desmethyldoxepin) at a rate of 1 sec per scan.

In parallel with each set of patient samples, a set of calibration samples were processed. Plasma (1 ml) was spiked with 10, 20, 50 and 100 ng of doxepin and desmethyldoxepin. These samples were analyzed first in duplicate and from the resulting area integration values the ratios vs. the internal standard were generated, plotted and used to quantitate subsequent clinical samples. It is essential to include adequate calibration points in the normal therapeutic range of 20—100 ng/ml doxepin, and 10—60 ng/ml desmethyldoxepin, to ensure validity of reported values. We used an internal standard ratio calibration curve generated daily to quantitate our daily set of samples. This curve varied by only ± 7% over a week's period of analyses.

Clinical study

A double blind comparison between doxepin and placebo in 30 depressed patients with chronic lumbar or cervical pain was undertaken. Dependent variables included Hamilton depression scale, Profile of mood states, Global assessment, Visual analog scale ratings of average pain severity, Percent time pain felt, Muscle tension, Effects of pain on sleep, mood, and activity, as well as plasma β-endorphin and enkephalin-like activity. Plasma levels of doxepin and desmethyldoxepin as described in this study were also correlated with these variables [2].

RESULTS AND DISCUSSION

A typical mass fragment chromatogram of a patient's sample is shown in Fig. 1. The ions chosen were based upon our analysis of the mass spectra of the reference material shown in Fig. 2. We used *m/e* 58.1 for doxepin and amitriptyline and *m/e* 232.2 and 234.2 for desmethyldoxepin and nortriptyline, respectively. The utility and validity of the developed methodology for providing clinically significant data was verified in a double blind study of patients receiving varying amounts of doxepin for pain. Significant improvements in doxepin-treated patients compared to placebo patients were observed in several variables including Hamilton depression scale, global assessment, percent time pain felt, muscle tension, sleep and mood. Maximal therapeutic effects occurred when combined plasma doxepin and desmethyldoxepin levels were near 70 ng/ml as determined by the assay technique described. Presented in Figs. 3 and 4 are linear correlations of the daily oral doxepin dosage taken by patients versus the concentration of doxepin (Fig. 3) and desmethyldoxepin (Fig. 4) found in their plasma utilizing our newly developed GC—MS procedure. After analyses were complete, it was determined that Hamilton depression scores which are used to determine the depth of depres-

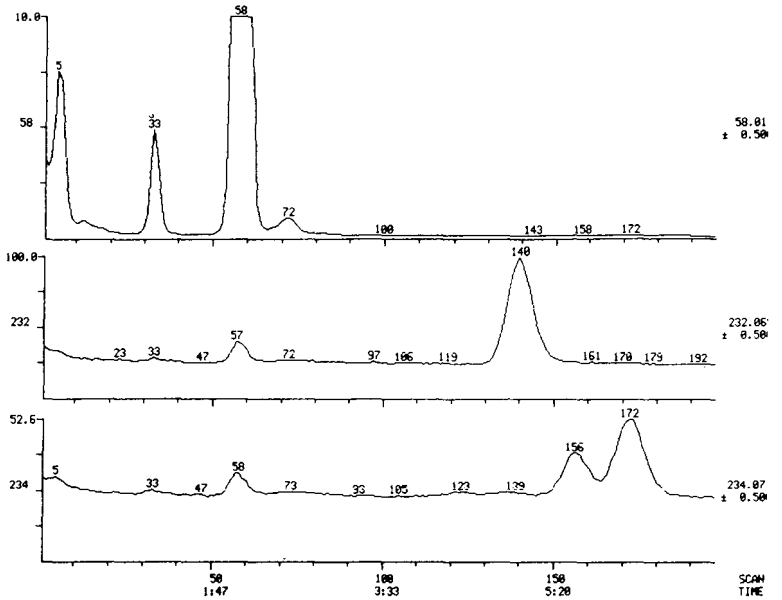

Fig. 1. Selected ion monitoring chromatogram of amitriptyline (I.S.), scan 58; doxepin, scan 72; nortriptyline (I.S.), scan 140; *cis*-desmethyldoxepin, scan 156; and *trans*-desmethyldoxepin, scan 172. This injection represents 0.4 ml (equivalent) of patient's plasma onto the column.

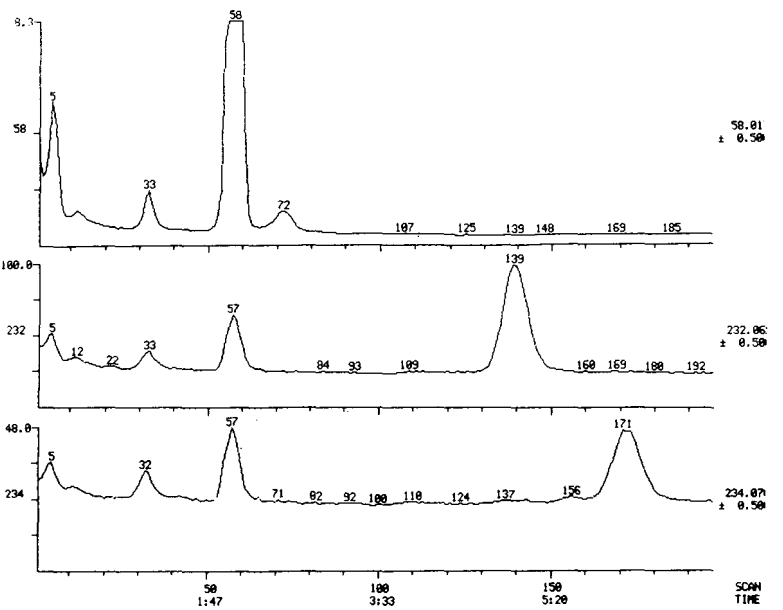

Fig. 2. Selected ion monitoring chromatogram of amitriptyline (I.S.), scan 58; doxepin, scan 72; nortriptyline (I.S.), scan 139; and *trans*-desmethyldoxepin, scan 171. This is a blank plasma spiked with drug.

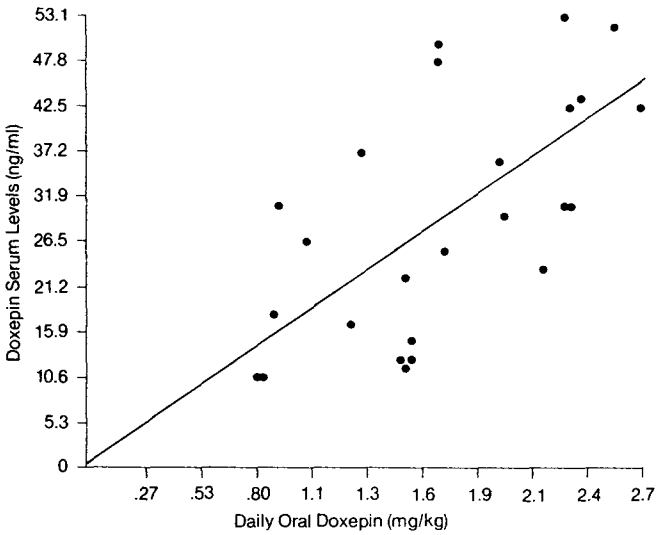

Fig. 3. Doxepin plasma levels (ng/ml) in depressed, chronic pain patients at varying daily oral doxepin doses. $y = 0.5 + 17.1\ x$ (x = oral dose), $r = 0.88, p < 0.001, n = 39$. Linearity suggests consistent absorption and metabolism.

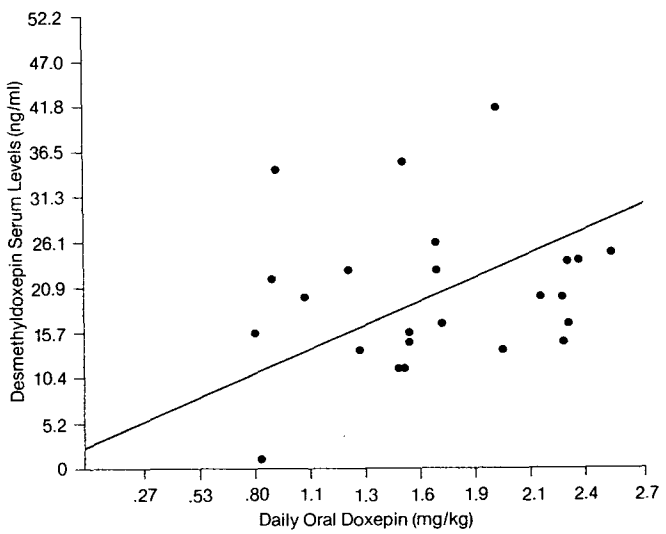

Fig. 4. Desmethyldoxepin plasma levels (ng/ml) in depressed, chronic pain patients at varying daily oral doxepin levels. $y = 2.25 + 10.3\ x$ (x = oral dose), $r = 0.72, p < 0.001$, $n = 39$. Values include both *cis* and *trans* isomers.

sion were significantly ($p < 0.01$) decreased by doxepin. Doxepin and the desmethyl metabolite were also significantly ($p < 0.01$) associated with a decrease in the percent of time pain was felt.

Therefore, utilizing an improved GC—MS procedure which uses only 1 ml plasma and has a sensitivity of less than 1 ng/ml, we found an 88% correlation of patient plasma doxepin concentration to daily oral doxepin and a 72% correlation with the plasma desmethyl metabolite formation. Therefore, this

newly developed GC—MS procedure can be used to study various clinical effects of doxepin as well as patient compliance to the drug due to its inherent sensitivity and low sample volume necessary for analysis.

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Note

Comparison of three methods for the extraction of aflatoxins from human serum in combination with a high-performance liquid chromatographic assay

S.M. LAMPLUGH

Department of Tropical Paediatrics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA (Great Britain)

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Four major aflatoxins B_1 (AFB_1), B_2, G_1 and G_2, produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*, occur as frequent contaminants of many food commodities such as peanuts, corn and rice with AFB_1 usually found in the greatest concentration [1]. The AFB_1 metabolites aflatoxins M_1, M_2 and aflatoxicol have also been identified, aflatoxins M_1 and M_2 are excreted in the milk of animals fed rations containing aflatoxins B_1 and B_2 [1].

The ability of aflatoxin B_1 and its related compounds to act as potent carcinogens in many species has been well documented [2, 3] and there is considerable evidence that these compounds may be primary causes of human liver cancer in certain areas [4—8]. AFB_1 has also been cited in the aetiology of Reye—Johnson syndrome [9—11].

In order to facilitate investigation of the role of ingested aflatoxin in human disease a sensitive method for the detection of aflatoxin in small volumes of human sera is required. Radioimmunoassay [12] and ELISA techniques [13, 14] fulfill the requirements but both methods involve the use of specific antibodies. These antibodies are not commercially available and their production requires special resources including facilities for animal work, which are not available in many laboratories, especially in countries where aflatoxin contamination of food is most prevalent.

In order to identify the most consistently reliable method for the detection of AFB_1 in small serum samples comparisons were made between the methods of Nelson et al. [11], Romer [15], and Van Egmond et al. [16] for extracting AFB_1 from 1 ml or less of human sera spiked with known amounts of AFB_1 using high-performance liquid chromatography (HPLC) as the detection

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system. This paper reports the results of these comparisons and the applicability of the chosen method for the extraction of other aflatoxins B_2, G_1, G_2, M_1, M_2 and aflatoxicol from serum.

MATERIALS AND METHODS

Aflatoxin B_1 (20 μg/ml in chloroform) was obtained from Dr. Van Egmond, Rijksinstituut voor de Volksgezondheid (Bilthoven, The Netherlands). Aflatoxicol (0.1 mg/ml) in methanol and aflatoxins B_2, G_1, G_2, M_1 and M_2 in solid form were obtained from Sigma (Poole, Great Britain). Standard stock solutions were prepared by dissolving solid standards in a mixture of acetonitrile—benzene (1:9) to give concentrations of 0.1 μg/ml or by diluting the aflatoxin B_1 and aflatoxicol solutions to give concentrations of 0.1 μg/ml. All other chemicals were obtained from BDH (Poole, Great Britain).

Blood specimens were obtained, by antecubital venipuncture, from normal healthy adult volunteers. After allowing the blood to clot at room temperature for 2 h serum was separated by centrifugation. Spiked sera samples were prepared by transferring aliquots of the aflatoxin standard solutions containing known amounts of aflatoxin to tubes and evaporating to dryness under a stream of nitrogen. Serum (10 ml) was added to the tubes and incubated at 30°C for 1 h with gentle swirling. The resultant serum containing known amounts of aflatoxin standard was either used immediately or stored at 4°C until use.

HPLC

The sample components were separated on an ODS 5-μm column, 25 cm × 5 mm (HPLC Technology, Macclesfield, Great Britain) and detected by fluorescence detector (Kratos, Schoeffel Instruments) fitted with a 365-nm excitation filter and a 418-nm emission filter. The mobile phase consisted of water—methanol (50:50) at a flow-rate of 2 ml/min and pressure of 241 bar.

Extraction techniques

(1) Hexane—chloroform extraction. This is the method according to Nelson et al. [11]. Initially, 1 ml hexane was added to a 1-ml aliquot of aflatoxin spiked serum and gently mixed for 2 min, the mixture was centrifuged for 5 min at 2000 *g* and the upper hexane layer containing the serum lipids, removed. This procedure was repeated twice with further 1-ml aliquots of hexane, and the hexane layer removed after each centrifugation. The serum was then extracted four times with 1-ml aliquots of chloroform, by the addition of 1 ml of chloroform to the serum, vigorous shaking for 4 min, centrifugation for 10 min at 2000 *g* and removal of the lower chloroform layer. The chloroform extracts were pooled, evaporated to dryness under a stream of nitrogen, redissolved in 50 μl methanol—water—acetonitrile (25:25:50) and 20 μl of the resultant solution analysed by HPLC.

(2) Chloroform extraction with pentane clean-up. This method is that used by Van Egmond et al. [16] for determination of aflatoxins in liver samples.

Serum (1 ml) was mixed on a wrist-action shaker with 20 ml chloroform for 30 min. Phosphoric acid (4 mol/l), 0.4 ml, was added and the mixture shaken

for a further 1 min, then filtered and the filtrate blown to dryness. The extract was cleaned by sequential washings with (a) 7 ml *n*-pentane, 2.5 ml methanol and 0.25 ml 1.2 *M* sodium chloride; (b) 3.5 ml 1.2 *M* sodium chloride and 2.5 ml pentane; (c) 1 ml pentane, 1.5 ml diethyl ether, and 0.05 ml 4 *M* hydrochloric acid. After each addition the extract was shaken for 1 min and the pentane layer discarded. Finally 0.45 ml hydrochloric acid was added and the aflatoxin extracted with 1-ml aliquots of chloroform four times. The chloroform extracts were pooled, and then analysed by HPLC as described in (1) above.

(3) Acetone—ferric gel—chloroform extraction. This method is based on the Romer method [15] as used by the Tropical Products Institute (London, Great Britain).

Serum (1 ml) was added to 5 ml acetone and shaken on a wrist-action shaker for 30 min. The mixture was filtered and 0.3 g basic cupric carbonate added, mixed with a glass rod and then allowed to stand for 2.5 min. Ferric chloride (0.41 *M*, 3 ml) was added to 17 ml 0.2 *M* sodium hydroxide to form a gel which was immediately added to the sample, mixed well and allowed to stand for 2 min with occasional swirling. The mixture was filtered. The filtrate was placed in a separating funnel with 22 ml 0.0054 *M* sulphuric acid and 1 ml chloroform and shaken vigorously for 1 min. The layers were allowed to separate and the chloroform layer run off through a filter funnel containing anhydrous sodium sulphate. The upper aqueous layer was further extracted with three 1-ml chloroform aliquots. The extracts were pooled, blown to dryness and then analysed by HPLC as described above.

Procedure

Unspiked serum samples were extracted by all three methods and analysed by HPLC to detect fluorescing compounds which might interfere with aflatoxin B_1 analysis. None were detected, as shown in Fig. 1.

The lower limits of detection were measured by the lowest concentration at which the aflatoxin would produce a peak 2% of full scale deflection on the recorder. For each extraction method the lower limit of detection of AFB_1 was checked by extracting and analysing serum samples spiked with AFB_1 in concentrations ranging from 200 to 800 pg/ml. The lower limits of detection for methods (1), (2) and (3) were 250, 300 and 400 pg/ml, respectively.

Following the above procedures six serum samples spiked with AFB_1 in concentrations ranging from 571 to 8000 pg/ml were analysed by the three methods. One additional serum sample containing AFB_1, 296 pg/ml was analysed by method (1) only. The reproducibility of each method was checked by replicate analysis at each concentration. The standard deviation associated with the mean value for six estimations was expressed as the coefficient of variation.

RESULTS

Results are presented in Tables I, II and III. Recovery of AFB_1 by method (1) ranged from 80—95%, by method (2) from 59—82% and by method (3) from 33—67%. The mean coefficient of variation was similar for the three

TABLE I

REPRODUCIBILITY OF AFLATOXIN MEASUREMENTS USING EXTRACTION METHOD (1)

In all cases $n = 6$.

Aflatoxin concentration (ng/ml)		Recovery (%)	Coefficient of variation (%)
Actual	Recovered (mean ± S.D.)		
8.0	6.43 ± 0.21	80.4	3.3
8.0	7.61 ± 0.302	95.1	4.0
4.0	3.51 ± 0.07	87.8	2.0
4.44	4.09 ± 0.27	92.1	6.6
2.96	2.65 ± 0.31	89.7	11.6
0.571	0.471 ± 0.054	82.5	11.5
0.296	0.28 ± 0.022	94.8	7.8
	Mean	88.9	Mean 6.7

TABLE II

REPRODUCIBILITY OF AFLATOXIN MEASUREMENTS USING EXTRACTION METHOD (2)

In all cases $n = 6$.

Aflatoxin concentration (ng/ml)		Recovery (%)	Coefficient of variation (%)
Actual	Recovered (mean ± S.D.)		
8.0	4.77 ± 0.25	59.6	5.2
8.0	4.86 ± 0.31	60.7	6.3
4.0	2.57 ± 0.08	64.2	3.1
4.44	3.46 ± 0.15	77.9	4.3
1.0	0.87 ± 0.12	87.0	13.8
0.571	0.47 ± 0.04	81.8	8.3
	Mean	71.9	Mean 6.8

methods. The coefficient of variation was slightly greater for the lowest concentrations of AFB_1, by all methods. Percentage recovery of AFB_1 by method (2) seemed to be inversely proportional to the serum concentration of AFB_1 rising from ca. 60% in the highest concentration to ca. 80% in the lowest concentrations.

HPLC analysis of the hexane discarded in method (1) and *n*-pentane discarded in method (2) showed no AFB_1.

Storage of the spiked sera and the extracts at 4° C for periods up to one week did not affect the recovery or measurement of AFB_1.

TABLE III

REPRODUCIBILITY OF AFLATOXIN MEASUREMENTS USING EXTRACTION METHOD (3)

In all cases $n = 6$.

Aflatoxin concentration (ng/ml)		Recovery (%)	Coefficient of variation (%)
Actual	Recovered (mean ± S.D.)		
8.0	3.78 ± 0.21	47.3	5.6
8.0	4.76 ± 0.41	59.5	8.6
4.0	2.67 ± 0.19	66.8	7.1
4.44	1.46 ± 0.15	32.8	10.3
2.96	1.33 ± 0.07	44.9	5.3
0.571	0.36 ± 0.04	62.7	11.1
	Mean	52.3	Mean 8.0

TABLE IV

REPRODUCIBILITY, SENSITIVITY AND RECOVERY OF AFLATOXINS FROM SPIKED SERA SAMPLES USING EXTRACTION METHOD (1)

Aflatoxin	Mean recovery (%)	Limit of detection (pg/ml)	Reproducibility mean variation (%)
B_1	88.9	250	6.7
B_2	79.1	25	5.7
G_1	67.3	500	10.2
G_2	77.0	25	6.3
M_1	76.4	100	8.2
M_2	69.3	25	11.1
Aflatoxicol	81.2	100	9.8

Method (1) was also investigated for the extraction of aflatoxins B_2, G_1, G_2, M_1, M_2, and aflatoxicol from human sera samples spiked with these other aflatoxins. Using replicate analysis of six sera specimens the recoveries, reproducibilities and limits of detection are shown in Table IV. A chromatogram of a serum sample spiked with a mixture of aflatoxins B_1, B_2, G_1, G_2, M_1, M_2 and aflatoxicol is shown in Fig. 1.

DISCUSSION

In the appraisal of the suitability of laboratory techniques for use in clinical practice or in human research, sensitivity and reproducibility of the method and its applicability to small samples of body fluids or tissue are some of the most important considerations.

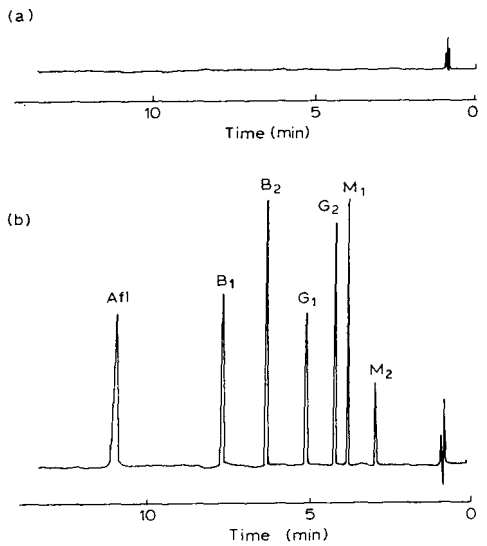

Fig. 1. HPLC chromatograms of human sera extracts. (a) Extract from unspiked serum, (b) extract from 1 ml serum spiked with aflatoxin M_2, 0.3 ng; M_1, 4 ng; G_2, 1.0 ng; G_1, 25 ng; B_2, 1.0 ng; B_1, 13 ng; and aflatoxicol, 2.5 ng. Column: Spherisorb 5 μm ODS; mobile phase: methanol—water (50:50); flow-rate: 2 ml/min; fluorescence detector, excitation 365 nm, emission 418 nm.

Method (1) [11] was found to be more sensitive than the other methods and also gave better recovery of AFB_1 from sera. The mean recovery of AFB_1 with method (1) was 88.9% compared to 71.9% and 52.3% with methods 2 and 3, respectively. It has been demonstrated that aflatoxin B_1 can adhere to the walls of glass vessels [17] and the variation in the recoveries observed could be a reflection of the number of vessels used in processing the sample in each method.

For ease of handling, 1 ml of serum was the preferred volume but all three extraction methods could be performed using as little as 0.1 ml serum.

There is a possibility that some drugs may interfere with this technique and this is at present under investigation.

As a result of these studies method (1) [11] has been adopted as the method of choice in our laboratory for aflatoxin detection in human fluids and tissues and has proved satisfactory also for the detection of aflatoxin B_2, G_1, G_2, M_1, M_2 and aflatoxicol.

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Note

New rapid assay of cimetidine in human plasma by reversed-phase high-performance liquid chromatography

V. NITSCHE* and H. MASCHER

Pharmakologische Untersuchungsgesellschaft m.b.H., Vienna (Austria)

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Cimetidine is a potent inhibitor of gastric acid secretion and is used worldwide with increasing frequency in the treatment of duodenal and stomach ulcers [1, 2]. Earlier dosage directions of 1000 mg of cimetidine per day have now been reduced to values of 400 mg twice daily. It is therefore important to note not only the clinically demonstrable effect, but also the therapeutically effective serum levels. So far, high-performance liquid chromatographic (HPLC) data have been published only for normal-phase chromatography [3—7], three papers on reversed phase [8—10], and one paper using a weak polar phase [11].

The aim of this present publication is the elaboration of a rapid assay for cimetidine, which provides not only an easy procedure and a short analysis time, but also a high recovery rate, making it the method of choice for the determination of cimetidine in biological material, where this is linked with pharmacokinetic examinations.

MATERIAL AND METHODS

Reagents

Methanol, absolute, was from J.T. Baker (Deventer, The Netherlands); ammonium carbamate, for analysis, was from E. Merck, Darmstadt, G.F.R.; Sep-Pak C-18 cartridges were from Waters Assoc. (Milford, MA, U.S.A.); disposable extraction columns were from J.T. Baker (Phillipsburg, NJ, U.S.A.); cimetidine and the internal standard (N-cyano-N'-{2-[5-methyl-1H-imidazol-4-yl)-methylthio] ethyl}-S-methyl-isothiourea), a cimetidine synthesis precursor, were obtained from Gerot Pharmazeutika (Vienna, Austria).

To prepare the internal standard solution for use with Sep-Pak cartridges,

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1 mg of the internal standard was dissolved in 500 ml of water. For use with the disposable columns from Baker, 1 mg of the internal standard was dissolved in 50 ml of water.

HPLC unit

A high-pressure liquid chromatograph Type SP 8000 from Spectra-Physics (Santa Clara, CA, U.S.A.), with a Spectro-Monitor (LDC, FL, U.S.A.) as UV detector, was used to separate the substances. A LiChrosorb RP-18 column (5 μm, 250 × 4 mm; Hibar, Merck) thermostatted at 45°C was used.

Detector operation took place at 0.02 A, at a wavelength of 220 nm. A mixture of 40% of methanol and 60% of 0.01 mol/l ammonium carbamate solution (pH 8.9) was used for the mobile phase, at a flow-rate of 1.2 ml/min. The unit was fitted with a 25-μl loop.

Plasma extraction

To 1 ml of plasma in a small glass tube 1 ml of internal standard and 1 ml of water were added, vortexed for 20 sec, and passed through a conditioned Sep-Pak cartridge by means of a 5-ml syringe. The glass tube and syringe were rinsed afterwards with 2 ml of water and the rinse also passed through the cartridge. After rinsing the cartridge with 9 ml of water, residual water was removed by vigorous shaking. Cimetidine and the internal standard were then eluted with 5 ml of methanol; the eluent was subsequently evaporated until 1 ml of largely aqueous solution remained; 100—200 μl of this solution were used to rinse and fill the 25-μl loop. For conditioning of new or used Sep-Pak cartridges they must first be rinsed with 5 ml of methanol and then with 5 ml of water. For rinsing and elution a constant flow of 1 ml within 2—3 sec should be maintained.

To avoid the large volumes obtained with the above procedure, we used Baker octadecyl extraction columns with encouraging success. A 1-ml aliquot of plasma and 0.1 ml of internal standard solution were thoroughly mixed and put through a Baker C-18 1-ml column by means of short centrifugation. After twice rinsing with 1 ml of water the Baker column was changed to a clean glass tube and the substances eluted with 500 μl of methanol, always with the aid of the centrifuge. The 25-μl loop was rinsed and filled with the solution obtained.

RESULTS

The calibration curve was linear within the concentration range 0.2—15.84 μmol/l ($Y = 0.87326X + 0.0504$; $r = 0.9996$, $n = 16$). The reproducibility of the method has been determined over a period of approx. two years using seven samples with a mean quotient value of 1.64 ± 0.164 (= ± 10%). The recovery rate with Sep-Pak cartridges is shown in Table I. The absolute recovery for cimetidine was 96.2 ± 3.4% and for the internal standard 94.3 ± 4.0%.

TABLE I

RECOVERY OF THE METHOD USING SEP-PAK CARTRIDGES

Quantity added (μmol/l)	Plasma quotient (n = 5)	Water quotient (n = 3)
0.79	0.2231 ± 0.0189 (= ± 8.5%)	0.1626 ± 0.0014 (= ± 0.9%)
3.96	0.9313 ± 0.0395 (= ± 4.2%)	0.8380 ± 0.0160 (= ± 1.9%)
15.84	3.541 ± 0.0735 (= ± 2.1%)	3.525 ± 0.1271 (= ± 3.6%)

Chromatograms of human plasma before and after intake of 600 mg of cimetidine are shown in Fig. 1. Under the above-mentioned conditions, cimetidine showed a retention time of 4 min, the internal standard 6.4 min.

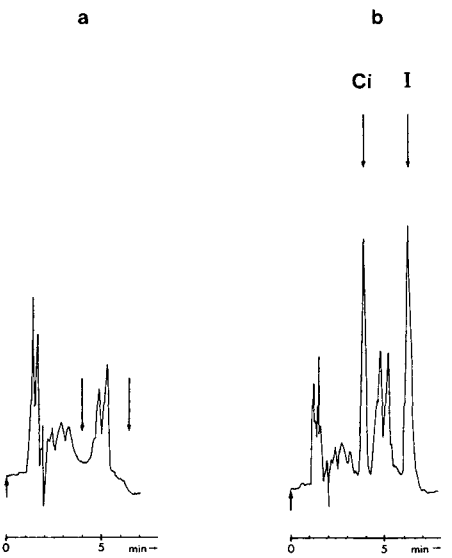

Fig. 1. Chromatograms of extracts of human plasma after oral intake of 600 mg of cimetidine. (a) Blank plasma, (b) 4.12 μmol/l cimetidine (Ci). I = internal standard.

DISCUSSION

The HPLC plasma assay described is characterized by a clearly reduced analysis time thus improving on the methods previously reported [3—11]. Previous cimetidine assays required single or multiple extraction steps prior to chromatography while, in contrast, the assay described involves only passage through an extraction column, thus greatly reducing sample preparation time and manipulation errors.

Naturally, there is always the possibility of working with only 0.1—0.2 ml of plasma, where clinical controls are undertaken in which therapeutic cimetidine concentrations are to be expected, because the sensitivity of the method is very good (0.08 μmol/l). This method can also be used for urine assay although so far no experiments have been undertaken in this direction.

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Note

Determination of metoclopramide in human plasma by high-performance liquid chromatography

G.B. BISHOP-FREUDLING and H. VERGIN*

Biochemical Department, Heumann-Pharma, P.O. Box 2260, D-8500 Nürnberg (G.F.R.)

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Metoclopramide (4-amino-5-chloro-2-methoxy-N-2-diethyl-aminoethylbenzamide) is recommended for use in gastrointestinal diagnostics and in treating various types of vomiting and a variety of functional gastrointestinal disorders. The drug increases the motility of the stomach and gastric-emptying rates. It may provide symptomatic relief in dyspepsia and possibly in vertigo and reflux oesophagitis [1, 2]. Behavioural studies in animals suggest that metoclopramide is a central dopaminergic antagonist exerting its anti-emetic effects by blocking the chemoreceptor trigger zone [3]. When given orally metoclopramide is readily absorbed but shows a wide range in bioavailability which might be due to interindividual first-pass metabolism [4].

Quantitative thin-layer chromatographic methods for the determination of metoclopramide in human plasma are described by Schuppan et al. [5] for the 40—320 ng/ml range and by Berner et al. [6] up to a detection limit of about 4 ng/ml serum using an improved extraction procedure. An electron-capture gas chromatographic assay of the drug in plasma was developed by Ross-Lee et al. [7]. This analytical technique provides sufficient specificity and sensitivity up to a detection limit of 5 ng/ml plasma.

A high-performance liquid chromatographic (HPLC) assay for the analysis of the drug in serum has been published by Block and Pingoud [8]. The procedure, however, requires large sample volumes of 10 ml of whole blood, which are not possible to obtain from patients for routine use in monitoring plasma metoclopramide levels in a clinical laboratory.

This paper describes a newly developed HPLC method which requires only small volumes of plasma (2 ml), and assesses its accuracy, sensitivity and reproducibility in the determination of plasma metoclopramide concentrations.

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EXPERIMENTAL

Materials

All chemicals and solvents were of at least p.a. quality, and water was glass-distilled; all were prefiltered using a GV 100/1 glass filtration apparatus (Ref. No. 392700) and filter-discs, RC 58, 0.2 μm (Ref. No. 371628) both from Schleicher and Schüll (Dassel, G.F.R.). Chloroform, sodium hydroxide, methanol and acetic acid were obtained from E. Merck (Darmstadt, G.F.R.) and acetonitrile HPLC grade S from Rathburn Chemicals (Walkerburn, Great Britain). For preparing standard curves, metoclopramide hydrochloride monohydrate BP 80, charge No. 07581 was used (Heumann-Pharma, Analytical Department).

Extraction procedure

A 2-ml aliquot of plasma was added to 20-ml Sovirel culture test tubes. Sodium hydroxide (1 *N*) was then added up to 20 ml final volume. The resulting solution was carefully mixed and then applied to an Extrelut® column (Merck) of 20 ml bed-volume. Metoclopramide was eluted by rinsing the column with 70 ml of chloroform. The organic eluate was evaporated to dryness. The residue was dissolved in 4 ml of methanol by ultrasonification (Bransonic 221, Branson Europa, Soest, The Netherlands). The solution was transferred to 15-ml Pyrex/Sovirel test tubes and evaporated to dryness again. After redissolving the residue in 0.1 ml of 1% acetic acid, the solution was placed in small reaction vessels of 1.5 ml volume, centrifuged at 6400 *g* (Eppendorf 5412, Eppendorf, Hamburg, G.F.R.) and 10-μl aliquots of the clear supernatant were injected into the chromatograph.

Instrumentation and chromatography

The chromatographic system consisted of a Hewlett-Packard liquid chromatograph 1084 A. A Schoeffel variable-wavelength UV detector SF 770 (Kratos, Karlsruhe, G.F.R.) monitored the effluent at 273 nm. The mobile phase of 32% acetic acid (1%) and of 68% acetonitrile—methanol (3.7:1, v/v) was pumped through a Nucleosil C_{18}, 250 × 4.6 mm, 5 μm particle size, column at a flow-rate of 1.5 ml/min (Macherey and Nagel, Düren, G.F.R.). As a guard column a hyperchrome precolumn cartridge (Bischoff, Analysentechnik, Leonberg, G.F.R.) packed with the same material as the analytical column was used. Chromatography was performed at 50°C.

Calculations

A series of calibration experiments was carried out for the quantitative determination of metoclopramide. Defined amounts (10, 20, 40, 80, 100, 200 and 400 ng/ml) of metoclopramide were added to drug-free human plasma and processed as above. Standard curves were generated daily by plotting peak areas against the known metoclopramide concentrations. The stability of samples was tested from spiked human plasma and from samples obtained after oral metoclopramide administration to healthy subjects. The samples were stored deep-frozen for three months.

RESULTS

Typical chromatograms for blank plasma and for a plasma sample obtained from a volunteer 0.5 h after taking a single oral dose of 20 mg of metoclopramide hydrochloride are presented in Fig. 1. The total elution time per assay is 8 min. As can be seen from Fig. 1A, no plasma constituent peak extracted from the blank interferes with that of metoclopramide; there is merely an increased noise level present, which is well below our detection limit. The plasma standard curves were linear over the range of 10—400 ng/ml. The limit of detection of the method was 8 ng/ml when 2 ml of plasma were used. The between-day precision of the method, expressed as coefficient of variation (C.V.) ranged from 3.8 to 5.8% over the concentration range of 10—400 ng/ml, while the within-day precision ranged from 2.4 to 4.1% over the same concentrations chosen (Table I). The chromatographic procedure is reproducible with retention time of 5.60—5.64 for metoclopramide. This, and the fact that the compound is not destroyed in the columns, make it possible to determine metoclopramide without the use of an internal standard.

Total recovery of metoclopramide was 89.9 ± 1.9% ($\bar{x}$ ± S.D., n = 28) for plasma. Frozen plasma samples remained stable for at least three months.

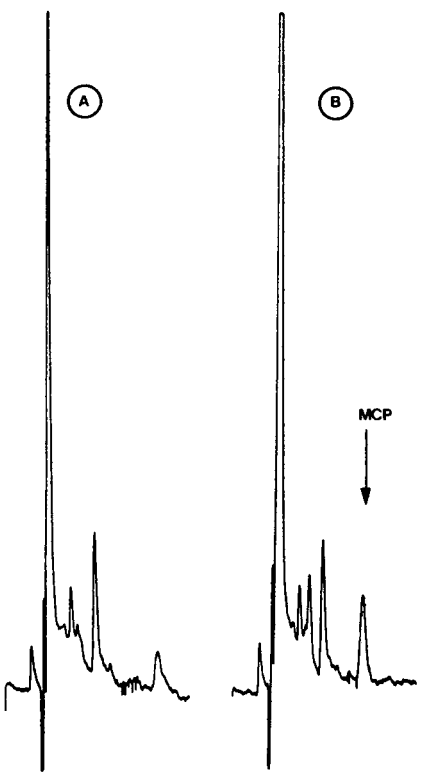

Fig. 1. Chromatograms of extracts from (A) drug-free control plasma and (B) plasma sample obtained from a healthy volunteer 0.5 h after taking a single oral dose of 20 mg of metoclopramide hydrochloride; this corresponds to 24 ng/ml.

TABLE I

VARIATION IN THE ASSAY OF PLASMA METOCLOPRAMIDE

Drug concentration (ng/ml)	Between-day		Within-day	
	n	C.V. (%)	*n*	C.V. (%)
10	8	5.2	5	3.1
20	10	4.7	5	2.9
40	8	5.8	5	3.6
80	6	3.9	4	2.7
100	6	4.4	4	2.4
200	5	4.1	4	4.1
400	5	3.8	4	3.1

DISCUSSION

The wide range in bioavailability makes metoclopramide a difficult drug to use orally. A clinically applicable and rapid procedure for the determination of metoclopramide in small plasma specimens would allow detailed pharmacokinetic studies to be carried out. The assay described in this paper has proved sufficiently sensitive and reliable for research and clinical use. As an example, the results of a bioequivalence study in volunteers who had received single doses of 20 mg of metoclopramide hydrochloride of two different liquid formulations in a cross-over design are shown in Fig. 2. Plasma levels of the unchanged drug and the main pharmacokinetic parameters, half-life of elimination and volume of distribution were in correlation with the literature values [9].

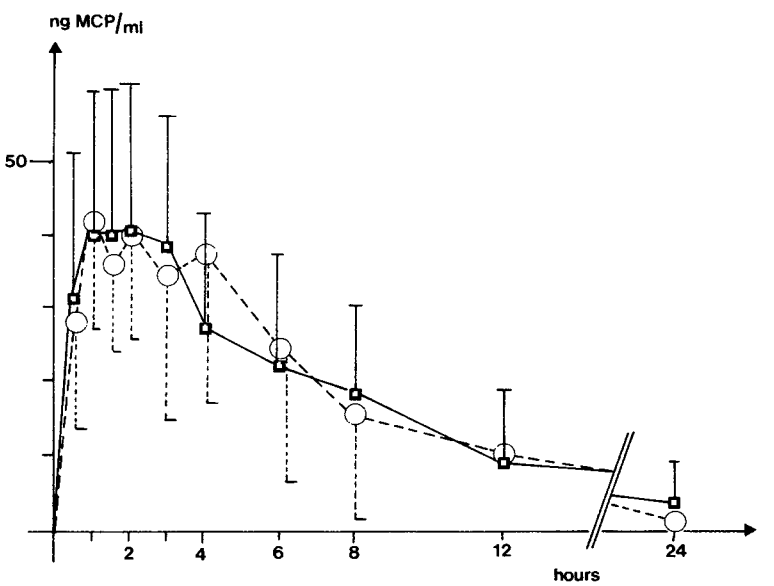

Fig. 2. Metoclopramide (MCP) concentrations in plasma from ten healthy volunteers after oral administration of two different liquid formulations of 20 mg of MCP in a cross-over design. (■), Test formulation; (○) standard formulation.

With respect to sensitivity, the gas chromatographic method of Ross-Lee et al. [7] for determining metoclopramide in serum is slightly more sensitive with a detection limit of 5 ng/ml compared to a detection limit of 8 ng/ml in the HPLC assay presented here. However, the total sample clean-up requires at least 7 h due to a multitude of diverse extraction, reextraction and centrifugation steps. The HPLC method described here involves a single extraction procedure followed by a twofold dissolution and centrifugation step. Therefore the GC assay does not appear to be a suitable method for routine drug monitoring in the clinical laboratory.

Due to the selectivity of the C_{18} 5-μm material used in this investigation, a separation of the drug from the plasma constituents was possible without an additional acidic extraction step as described in the HPLC assay of Block and Pingoud [8].

The present HPLC method demonstrates sensitivity and reproducibility over a wider concentration range than the previous assay mentioned [8]. Our procedure can be recommended for routine drug monitoring and would allow pharmacokinetic studies especially in, for example, patients with renal and/or hepatic failure and infants in whom sampling of large blood volumes would be deleterious.

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Note

High-performance liquid chromatographic determination of cefmenoxime (AB-50912) in human plasma and urine

IRIS A. NOONAN, JOHN G. GAMBERTOGLIO, STEVEN L. BARRIERE, JOHN E. CONTE, Jr. and EMIL T. LIN*

Infectious Disease Research Unit: Division of Clinical Pharmacy, Department of Pharmacy and the Clinical Infectious Diseases Unit, Department of Medicine, University of California, San Francisco, CA 94143 (U.S.A.)

(First received June 30th, 1982; revised manuscript received October 22nd, 1982)

Cefmenoxime (Fig. 1) is a new semisynthetic third generation cephalosporin developed by Takeda Chemical Industries. Currently, it is under clinical evaluation by Abbott Laboratories. Cefmenoxime has a broad antibacterial spectrum against many gram-positive and gram-negative bacteria including *Haemophilus influenzae*, indole-positive *Proteus*, *Serratia marcescens*, *Citrobacter freundii*, *Enterobacter cloacae*, and many strains of *Pseudomonas aeruginosa*. Recently, a high-performance liquid chromatographic (HPLC) procedure for the determination of cefmenoxime in plasma has been developed [1]. This procedure involves special treatment of samples prior to analysis followed by ultrafiltration.

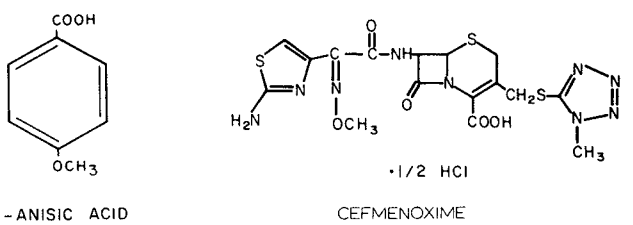

Fig. 1. Chemical structure of cefmenoxime and internal standard *p*-anisic acid.

We report the development of a simple, rapid HPLC assay requiring only deproteination of plasma with addition of acetonitrile containing internal standard, *p*-anisic acid (Fig. 1) prior to injection. In addition, this assay is applicable for analysis of cefmenoxime in urine. This assay has been used to

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analyze plasma and urine samples obtained following pharmacokinetic studies in healthy volunteers and renal failure patients. The stability of cefmenoxime in frozen plasma and urine has also been determined.

MATERIALS AND METHODS

Reagents

Cefmenoxime (Lot No. 17-961-AR) and *p*-anisic acid (Aldrich, Lot No. 092581) were both supplied by Abbott Laboratories (North Chicago, IL, U.S.A.). All organic solvents used were HPLC grade (Alltech, Arlington Heights, IL, U.S.A.). Reagents used were analytical grade. Water was doubly distilled and purified.

Chromatographic system

The chromatographic equipment consisted of an M-45 solvent delivery system connected to a Model 710A WISP sample injector (Waters Assoc., Milford, MA, U.S.A.). A Model 441 fixed-wavelength UV detector, 254 nm (Waters Assoc.) was used at a sensitivity of 0.005 absorbance units full scale (a.u.f.s.) for plasma samples and 0.05 a.u.f.s. for urine samples. The mobile phase consisted of 14% acetonitrile and 0.2% phosphoric acid, which was filtered through a Millipore filter (0.45 μm) and degassed prior to use. Minor changes in the acetonitrile content or the pH of the mobile phase may be necessary due to column efficiency loss or interference from plasma samples. This eluent was pumped through a μBondapak Phenyl column (30 cm × 3.9 mm, particle size 10 μm, Waters Assoc.) at 2 ml/min. Chromatograms were recorded on a Model

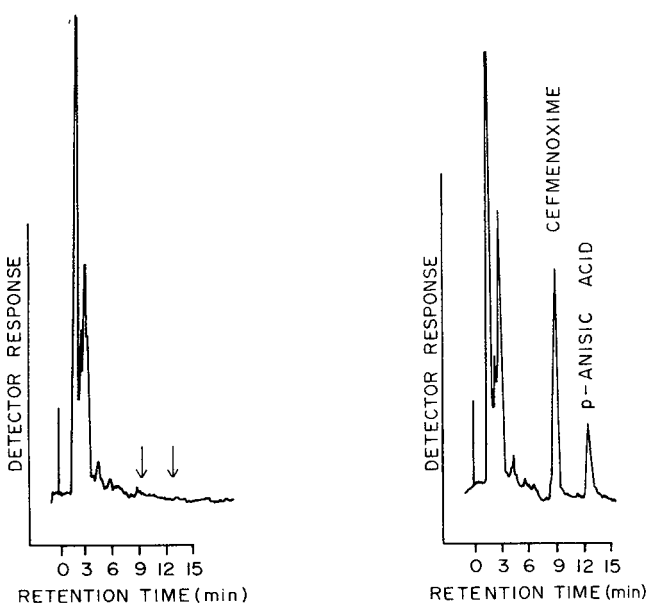


Fig. 2. Typical chromatograms of cefmenoxime and *p*-anisic acid in blank (left) and spiked plasma (right).

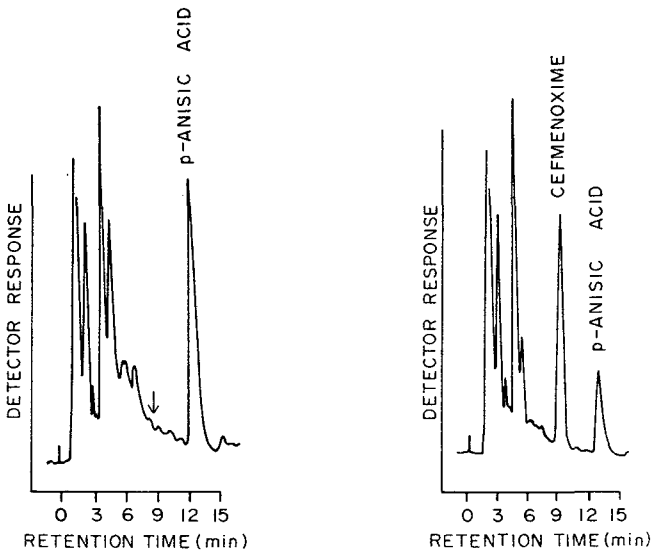


Fig. 3. Typical chromatograms of cefmenoxime and *p*-anisic acid in blank urine with internal standard (left) and spiked urine (right).

37 MR dual-pen strip-chart recorder, 10 mV (Pedersen Instruments, Lafayette, CA, U.S.A.) at a chart speed of 10 in./h. The retention time was 10 min for cefmenoxime and 14 min for the internal standard (Figs. 2 and 3).

Preparation of plasma samples and standards

Plasma samples. A plasma sample, 0.2 ml, was transferred into a disposable culture tube (12 × 75 mm) and deproteinated by adding 0.2 ml of acetonitrile containing the internal standard (0.8 μg/ml, *p*-anisic acid). The mixture was vortexed for 10 sec and centrifuged (700 *g*) for 10 min. The supernatant was decanted into a clean culture tube, evaporated under nitrogen to 0.1 ml, vortexed briefly, and approximately 25—50 μl were injected onto the column. Plasma samples at drug concentrations higher than those used in preparing the standard curve were diluted and evaporated as necessary.

Plasma standards. Cefmenoxime stock solution was prepared by accurately weighing 2—3 mg of cefmenoxime into a 100-ml volumetric flask and dissolving in 0.07 *M* phosphate buffer (pH 7.0). Blank human plasma was spiked with cefmenoxime stock solution to yield concentrations ranging from 0.2—6 μg/ml and prepared and treated identically to that described above. A standard curve was constructed by plotting the peak height ratios of cefmenoxime to *p*-anisic acid against the concentrations of cefmenoxime in plasma.

Preparation of urine samples and standards

Urine samples. Urine samples were diluted to the desired concentration and injected directly onto the column after addition of internal standard (0.2 mg/ml).

Urine standards. Cefmenoxime stock solution was prepared by accurately weighing 8—9 mg of cefmenoxime into a scintillation vial and dissolving with

5 ml of 0.07 *M* phosphate buffer (pH 7.0). Blank human urine was spiked with cefmenoxime stock solution to yield concentrations ranging from 16—210 μg/ml. Urine samples were prepared as described above. A standard curve was constructed by plotting the peak height ratios of cefmenoxime to *p*-anisic acid against the concentrations of cefmenoxime in plasma. Correlation coefficients for 10 plasma standard curves averaged 0.999±0.0004 (±S.D.) and for ten urine standard curves, 0.999±0.001.

RESULTS

Assay precision

Six plasma and six urine samples were prepared, analyzed and compared to the control (spiked) plasma and urine samples. The data are listed in Table I. The assay variability was less than 5% for both concentrations in plasma and urine.

Stability

The stability of cefmenoxime in plasma frozen at −20°C was studied at different concentrations. Duplicate plasma samples containing either 0.7 or 3 μg/ml of cefmenoxime were prepared and assayed. Results are shown in Table II. Duplicate urine samples containing either 44 or 222 μg/ml of cefmenoxime were prepared and assayed. Results are shown in Table III.

TABLE I

ASSAY PRECISION FOR CEFMENOXIME IN PLASMA AND URINE

	Spiked concentration (μg/ml)	Measured concentration (n = 6)	
		Mean ± S.D. (μg/ml)	C.V. (%)
Plasma	0.755	0.804 ± 0.038	4.73
	3.02	3.14 ± 0.054	1.72
Urine	44.45	43.8 ± 1.41	3.22
	222.25	224.72 ± 6.67	2.97

TABLE II

STABILITY OF CEFMENOXIME IN PLASMA FROZEN AT −20°C AT DIFFERENT CONCENTRATIONS

Values are the average of two measurements.

Day	Cefmenoxime concentration (μg/ml)	
	Low (0.7 μg/ml)	High (3.0 μg/ml)
0	0.72	3.07
1	0.76	3.01
2	0.74	2.86
3	0.72	2.83
4	0.79	2.98
7	0.74	2.78
14	0.70	2.69
21	0.73	2.67

TABLE III

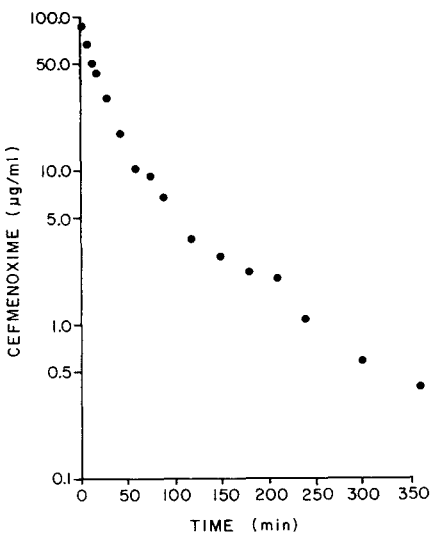
STABILITY OF CEFMENOXIME IN URINE FROZEN AT −20°C AT DIFFERENT CONCENTRATIONS

Values are the average of two measurements.

Day	Cefmenoxime concentration (μg/ml)	
	Low (44.0 μg/ml)	High (222.0 μg/ml)
0	41.5	198.93
1	40.7	186.05
3	42.3	196.86
7	42.1	192.01
14	41.6	187.93

Pharmacokinetic study

Fig. 4 shows a plasma concentration versus time plot following cefmenoxime intravenous infusion (10 mg/kg) to a healthy volunteer. A urine excretion rate curve following a cefmenoxime intravenous infusion (10 mg/kg) to a healthy volunteer is shown in Fig. 5.



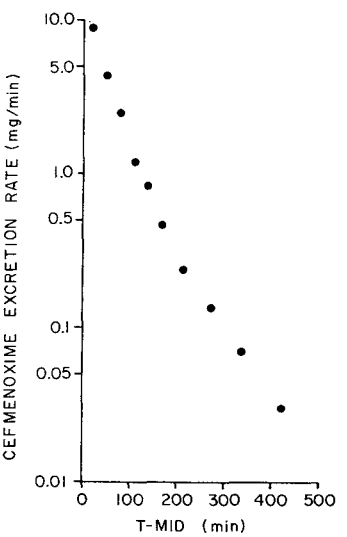


Fig. 4. Plasma concentration versus time plot following the administration of cefmenoxime by intravenous infusion (10 mg/kg) to a healthy volunteer.

Fig. 5. Urine excretion rate curve following the administration of cefmenoxime (10 mg/kg) by intravenous infusion to a healthy volunteer.

DISCUSSION

An HPLC assay has been developed for the analysis of cefmenoxime in human plasma and urine samples. This method which does not require extraction is simple, selective and sensitive and requires only 0.2 ml of biological fluid. Both cefmenoxime and the internal standard, *p*-anisic acid, are resolved

from endogenous plasma and urine components. A previously described HPLC technique for analysis of the drug in plasma requires ultrafiltration of the samples using the Amicon Centriflow system [1]. Our procedure requires only the deproteination of plasma samples by simple addition of acetonitrile prior to HPLC injection. No previous HPLC urine assay has been described. Our procedure utilizes direct injection of urine samples onto the column after addition of internal standard. The assay proved to be quite sensitive with the lower detection limits of 0.2 μg/ml for plasma and 5 μg/ml for urine samples. Assay precision was evaluated and variability was less than 5%. Stability was also determined for cefmenoxime in plasma and urine frozen at −20°C. The results showed less than 10% degradation for plasma samples at one week and for urine samples, two weeks. The HPLC assay described here has shown to be rapid and reproducible and due to its specificity allows cefmenoxime to be analyzed in the presence of other antibiotics. This assay is currently being utilized in pharmacokinetic studies of healthy subjects and renal failure patients.

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Note

High-performance liquid chromatographic method for determination of sulfapyridine in human saliva using post-column, in-line derivatization with fluorescamine

H.S. SISTA, D.M. DYE* and J. LEONARD

ALZA Corporation, 950 Page Mill Road, Palo Alto, CA 94304 (U.S.A.)

(First received July 6th, 1982; revised manuscript received October 7th, 1982)

Sulfasalazine (salicylazosulfapyridine), a drug used to treat ulcerative colitis [1—4] is itself poorly absorbed in the human gastrointestinal tract [5]. It is reduced by bacteria in the colon and cecum to sulfapyridine and 5-amino-salicylic acid [6, 7] which are absorbed.

Numerous analytical methods for analysis of sulfapyridine and the other metabolites of sulfasalazine have been reported. These methods include the Bratton and Marshall-based colorimetric method [8], high-performance liquid chromatography (HPLC) [9—14] and gas—liquid chromatography [15, 16]. These methods employ lengthy extraction procedures, and for the most part suffer from a lack of sensitivity and specificity.

In this paper, an HPLC method with post-column, in-line derivatization of sulfapyridine with fluorescamine followed by measurement of the generated fluorophore with a fluorometric detector is described. Using this method, an increase in sensitivity above that achievable using existing methods was demonstrated. The extraction process is a single-step, rapid process that allows analysis of large numbers of samples quickly.

The use of fluorescamine in post-column, in-line derivatization has presented problems due to the instability of the fluorescamine solution. In this paper, a solution is described in which fluorescamine is stable for up to 48 h.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was assembled from two Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery systems, a Waters Assoc.

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Model 710 B WISP automatic sample processor, a 12.5 cm × 4.6 mm I.D. stainless-steel column packed with RP-18 (5 μm; Brownlee Labs., Santa Clara, CA, U.S.A.), a Schoeffel Instrument (Westwood, NJ, U.S.A.) Model FS 970 spectrofluorometer operated at $\lambda_{ex.}$ = 395 nm and $\lambda_{em.}$ = 470 nm, coiled Polytef tubing (4.8 m × 0.7 mm I.D.) which served as a post-column, in-line reactor, a Thermonix (B. Braun) Model 1420 water bath (60°C) in which the Polytef tubing was immersed, and an Altech (Arlington Heights, IL, U.S.A.) T-fitting which served to connect the reactor, one pump, and the column. The liquid chromatograph was connected to a Spectra Physics (Santa Clara, CA, U.S.A.) Model 4100 integrator-calculator.

Reagents

Sulfapyridine was obtained from Applied Science Labs. (State College, PA, U.S.A.), sulfadiazine from Sigma (St. Louis, MO, U.S.A.), and Fluram from Pierce (Rockford, IL, U.S.A.). The solvents methanol and acetonitrile were analytical-reagent grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). 1-Hexanesulfonate as the sodium salt was from Regis (Morton Grove, IL, U.S.A.), while triethylamine was from Pierce. Reagent grade 2-mercaptoethanol was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and 5-aminosalicylic acid was from Aldrich (Milwaukee, WI, U.S.A.). Sulfasalazine tablets were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.).

Solutions

The mobile phase was composed of 0.05 *M* $NaHPO_4$, 0.01 *M* 1-hexanesulfonate sodium salt, 0.0072 *M* triethylamine, pH 3.0 (using phosphoric acid), and 15% methanol. The volumetric flow-rate of the mobile phase was 1.0 ml/min.

The Fluram solution introduced into the mobile phase, post-column, was prepared as follows: Fluram (400 mg) was dissolved in methanol (250 ml) followed by addition of 2-mercaptoethanol (1 ml), and mobile phase (250 ml). Volumetric flow-rate of this solution was 0.3 ml/min.

Stock solutions of sulfapyridine and internal standard, sulfadiazine, 1 mg/ml in methanol were prepared and stored at 4°C. A standard curve was generated by spiking blank saliva samples (1 ml) with varying amounts of sulfapyridine and a constant amount of internal standard. Microliter aliquots of the stock solutions or a 1:10 dilution of the stock solutions in water were added to the saliva. The internal standard concentration in saliva was 148 ng/ml. The sulfapyridine concentration range in saliva was 20.0—497.7 ng/ml. These samples were treated according to the extraction procedure described.

Analytical procedure

Acetonitrile (1 ml), solid potassium carbonate (approximately 400 mg), and internal standard, sulfadiazine (148 ng/ml saliva) were added to saliva samples (1 ml) in 15-ml glass stoppered centrifuge tubes. The tubes were then shaken on a vortex mixer for 1 min. After centrifugation ($\geqslant$1000 *g*) for 10 min, the upper acetonitrile layer was transferred to a second centrifuge tube followed by evaporation to dryness under nitrogen in a 60°C water bath.

The residues were then dissolved in mobile phase (200 μl), and mixed in the vortex mixer. Aliquots (40 μl) of these solutions were then injected into the chromatographic system.

RESULTS AND DISCUSSION

Plots of the peak area ratios (sulfapyridine to internal standard) against the respective sulfapyridine concentrations were linear. Typical standard curves for sulfapyridine in saliva had correlation coefficients of 0.9998, over a range of 20 to 498 ng/ml. The coefficients of variation for five replicate assays at 20.0, 49.8, 97.4, 248.9 and 497.7 ng/ml of saliva were ±6.6, ±3.7, ±8.4, ±6.4 and ±6.4%, respectively. A typical chromatogram for a saliva extract is shown in Fig. 1.

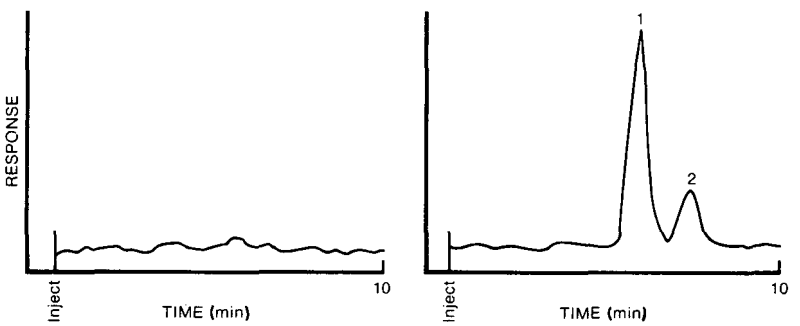

Fig. 1. Chromatograms of an analysis of a saliva blank (left) and of a saliva sample with added internal standard and sulfapyridine (right) carried through the procedure (148 and 20 ng/ml, respectively). Peaks: 1 = internal standard, sulfadiazine (retention time = 5.66 min); 2 = sulfapyridine (retention time = 7.32 min).

The achievable detection limit for sulfapyridine in saliva using the method described is 5 ng/ml (1 ml of saliva volume). The method is more sensitive than existing methods utilizing HPLC [9—14]. The high sensitivity of the method is due to the specificity of the fluorescence detection step for sulfapyridine relative to the low biological background. The extraction procedure is rapid, allowing analysis of up to 100 samples per day.

Acetonitrile has been reported to be preferable to methanol both as an organic modifier in the mobile phase and as a solvent for the fluorescamine reagent. This is presumably due to an increase in reaction time between fluorescamine and primary amines caused by a reversible hydrolysis of fluorescamine by alcohols [17, 18]. In addition, hydrolysis of fluorescamine has been reported to result in the formation of a yellow (fluorescent) end-product [18, 19], causing a rising baseline during the chromatographic step. We have found that the addition of 2-mercaptoethanol to the Fluram solution prevents baseline drift for up to 48 h. After this addition, there were no differences between acetonitrile and methanol in terms of obtainable detection limits for sulfapyridine or stability problems of the Fluram solution for up to 48 h.

The effect of pH on fluorescence intensity was studied. There was little dif-

ference in intensity between pH 2.5 and 4.0, except that at pH 4.0 the Fluram solution became yellow at a faster rate. At pH 7.3, the Fluram solution turned yellow within minutes of being made up, and neither sulfapyridine nor sulfadiazine could be detected using this solution in the chromatographic system. Sulfanilamides are reported to react most favorably at pH 3.0—4.5, and have maximum fluorescence intensity at pH 3.0—4.0 [20].

No interference to either sulfapyridine or sulfadiazine was found from the following compounds which are either primary amines or naturally fluorescent; amphetamine, 2-amino-3-phenyl-1-propanol, furosamide, salicylic acid, 5-aminosalicylic acid, sulfasalazine, N-acetylsulfapyridine, viloxazine, levallorphan, metoprolol and riboflavin.

To demonstrate the usefulness of this method, results from the analysis of saliva samples obtained from a healthy volunteer who had ingested a 500-mg Azulfidine tablet (Pharmacia) are shown in Table I.

TABLE I

SALIVA CONCENTRATIONS OF SULFAPYRIDINE IN A HUMAN AFTER A SINGLE 500-mg ORAL DOSE OF AZULFIDINE

One subject was studied.

Time after dose (h)	Saliva sulfapyridine (ng/ml as free base)
4.0	<20
5.0	<20
6.0	<20
7.0	25
7.5	208
8.0	423
8.5	682
9.0	955
10.5	1376
12.5	102
21.5	212

The method has been used to analyze sulfapyridine in over 1500 saliva samples. No deterioration of column performance or of the assay as a whole was observed.

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Note

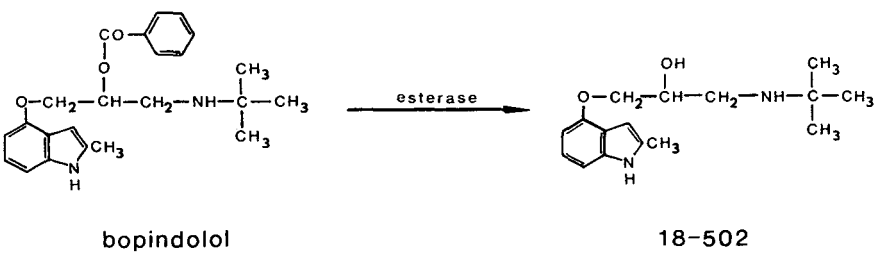
Analysis of bopindolol and its active metabolite 18-502 in human plasma by high-performance liquid chromatography

C.J. ODDIE*, G.P. JACKMAN and A. BOBIK

Clinical Research Unit, Alfred Hospital, Prahran, Victoria 3181 (Australia)

(First received July 12th, 1982; revised manuscript received October 15th, 1982)

Bopindolol [4-(2-benzoyloxy-3-*tert.*-butylaminopropoxy)-2-methylindole-hydrogenmalonate, LT 31-200] (Fig. 1) is a new, potent and long-acting β-adrenoceptor antagonist [1]. Preliminary clinical trials have shown that the antihypertensive activity of the drug is similar to other currently used β-blocking drugs. This activity, however, is of greater magnitude in regard to blood pressure reduction and duration of action per mg dose than that seen with other β-blocking agents [2].



bopindolol 18-502

Fig. 1. Formulae of bopindolol and its metabolite 18-502.

In vitro pharmacological experiments indicate that bopindolol itself has no β-adrenoceptor blocking activity and that hydrolysis by endogenous esterases to an active metabolite [4-(2-hydroxy-3-*tert.*-butylaminopropoxy)-2-methylindole, 18-502] (Fig. 1) is necessary for activity.

The aim of the present study was to develop an assay to measure the concentration of the active metabolite 18-502 and the parent compound bopindolol in human plasma. The assay is based on previously developed techniques from this laboratory for measuring indoles utilising high-performance liquid chromatography (HPLC) combined with fluorescence detection [3, 4].

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EXPERIMENTAL

Reagents

Bopindolol (LT 31-200, MW 484) and 18-502 (MW 276) were obtained from Sandoz Australia (North Ryde, Australia). Stock solutions (1 mg/ml) were prepared in 0.1 *M* phosphoric acid adjusted to pH 3.0 with sodium hydroxide, and stored at 4°C. Dilutions of the stock solutions in 0.01 *M* phosphate solution pH 3.0 were freshly prepared for each assay and stored on ice. Diethyl ether (analytical grade, Ajax Chemicals, Sydney, Australia) was washed with 1 *M* sodium hydroxide, 1 *M* hydrochloric acid and distilled water before use. Methanol (205 nm liquid chromatography grade) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC was redistilled from alkaline potassium permanganate. Esterase (100 U/mg, Boehringer, Mannheim, G.F.R.) was diluted prior to use to a concentration of 50 U/ml in 0.1 *M* boric acid adjusted to pH 8.0 with sodium hydroxide. All other reagents were of analytical grade.

Chromatographic system

A 5000 series liquid chromatograph fitted with a universal loop injector (Varian Assoc., Palo Alto, CA, U.S.A.) was used with a Zorbax 15 cm × 4.6 mm I.D. column packed with 5 μm cyano-bonded reversed-phase material (Du Pont, Wilmington, DE, U.S.A.). The mobile phase was 0.01 *M* perchloric acid—methanol (11:9) at a flow-rate of 1 ml/min. Detection was by means of a Schoeffel FS-970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. The metabolite 18-502 was detected by excitation at 220 nm and its fluorescence emission selected by a Corning 7-60 glass filter with bandpass 320—400 nm. Retention time of 18-502 was 5.5 min.

Plasma samples

Drug-free venous blood was obtained from healthy human subjects. Blood from patients in a clinical study investigating the pharmacokinetics and therapeutic efficacy of bopindolol was obtained following an oral dose of 1 mg or 8 mg bopindolol. Blood was collected into heparinised tubes on ice and, without delay, centrifuged 10 min at 1000 *g* and 4°C. Plasma was immediately separated and frozen, and stored at −20°C until assayed for 18-502 and bopindolol.

Extraction and HPLC estimation of 18-502

Plasma (1 ml or 2 ml) was placed into 25-ml stoppered glass tubes on ice and 100 μl 2 *M* sodium carbonate per ml plasma and 10 ml diethyl ether added. Extraction was by 2 min mixing using a vortex mixer. The phases were separated by centrifugation for 3 min at 1000 *g* and 4°C. The aqueous phase was then frozen by immersion of the tubes into a dry ice—ethanol mixture and the ethereal extract transferred to 15-ml stoppered glass tubes on ice and containing 200 μl 0.1 *M* phosphate pH 3.0. 18-502 was extracted into the aqueous phase by 2 min mixing using a vortex mixer and the phases separated by centrifugation and freezing as described above. The ethereal phase was aspirated and discarded. Samples and standards were maintained on ice prior to

chromatography. Accurate aliquots (100 μl) of the acidic phase were injected directly on to the HPLC column. A calibration curve was prepared by the simultaneous assay of known amounts of 18-502 in the range 1.25—200 ng/ml added to drug-free or patient control plasma.

Enzyme hydrolysis of bopindolol

The levels of bopindolol in plasma were determined by enzymatic conversion to 18-502 and subsequent analysis of 18-502 according to the method described above. The amount of bopindolol in the plasma could then be calculated from the difference between 18-502 levels in the enzyme hydrolysed and non-hydrolysed samples.

Plasma (1 ml) was placed into 25-ml stoppered glass tubes on ice, 50 μl (2.5 U) esterase in borate pH 8.0 added and hydrolysis allowed to proceed by incubation for 30 min at 37°C. Reaction was stopped by removal of the tubes on to ice and addition of 10 ml diethyl ether. Sodium carbonate (2 *M*, 100 μl) was then added and extraction of plasma and all subsequent steps of the 18-502 method carried out. Calibration curves of bopindolol and 18-502 were prepared by subjecting known amounts of the respective compounds to the same procedures of enzyme hydrolysis and 18-502 analysis. The bopindolol calibration curve was in the range 12.5—400 ng/ml.

Plasma concentration of 18-502 and bopindolol in human subjects

Plasma concentrations of the active metabolite and parent compound were measured in five informed volunteer patients (three male, two female) with essential hypertension, who were part of a study investigating the therapeutic efficacy and pharmacokinetics of bopindolol. The patients were on no other medication during the study. Bopindolol was administered as oral doses of 1 mg and 8 mg and plasma concentrations were monitored over 8—10 h.

RESULTS AND DISCUSSION

The overall recovery of 18-502 through the assay procedure was 78 ± 7% (mean ± S.D., n = 18). Precautions to keep plasma samples cold during collection and analysis were necessary to minimise losses due to endogenous esterase activity and an instability of 18-502 at room temperature. 18-502 was stable when stored in plasma at −20°C for periods checked up to one month. No hydrolysis of bopindolol to 18-502 when similarly stored was seen; however, at 37°C for 60 min a 5% conversion of bopindolol to 18-502 in plasma occurred, presumably due to the presence of endogenous esterases. 18-502 shows a 20—30% loss in detectable fluorescence when allowed to stand at room temperature in 0.1 *M* phosphoric acid pH 3.0 over periods of up to 6 h. Maintaining samples on ice during analysis reduced this decay to 4%.

18-502 isolated from plasma chromatographed with a retention time of 5.5 min and was resolved from other peaks present in plasma (Fig. 2). The mean assay blank for 18-502 in drug-free and patient control plasma was 0.019 ± 0.094 ng/ml, n = 24.

The calibration curve for the assay related fluorescence (y) expressed as nA of detector current to plasma concentration of 18-502 (x) in ng/ml according

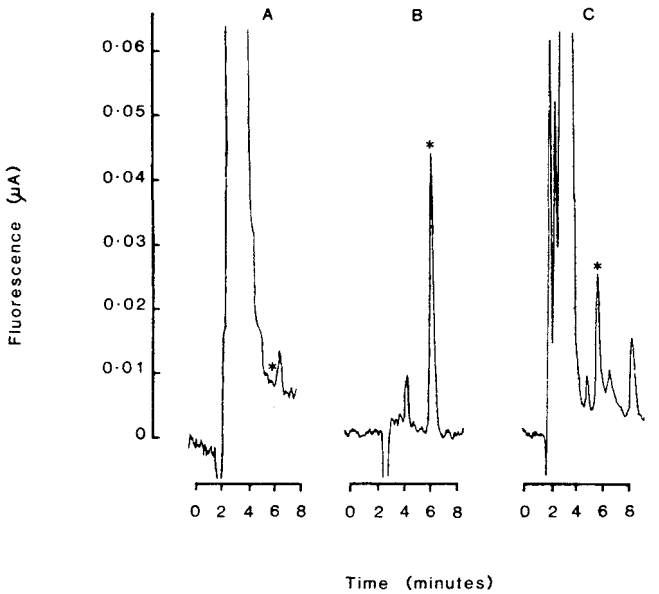

Fig. 2. Chromatographic traces of 18-502: (A) extract of control drug-free plasma shown at 2 × scale; (B) 5 ng 18-502 standard (*); (C) plasma extract from patient No. 3, 2 h following 1 mg bopindolol administered orally, equivalent to 2.6 ng/ml.

to the equation $y = 8.55x + 0.99$ ($r^2 = 0.996$). The calibration curve was linear over the range 0—200 ng/ml.

The precision and accuracy of the method were determined by replicate analysis of known amounts of 18-502 added to 1 ml drug-free plasma. The results are shown in Table I. The regression line for 18-502 added versus 18-502 obtained, corrected for the mean recovery (79%), has the formula $y = 1.057x - 0.509$ with a correlation coefficient of 0.999.

From these data, from the assay blank and from the chromatography of 18-502, it can be deduced that the sensitivity of the assay is approximately 0.5 ng/ml.

TABLE I

PRECISION FOR THE 18-502 ASSAY

The values for 18-502 obtained have not been corrected for recovery. In all cases $n = 6$.

18-502 added (ng/ml)	18-502 obtained (ng/ml)		
	Mean ± S.D.	C.V. (%)	Mean recovery (%)
1.25	0.97 ± 0.10	10.2	78
2.5	1.93 ± 0.12	6.3	77
5	3.78 ± 0.12	3.1	76
10	8.00 ± 0.39	4.8	80
20	15.4 ± 0.51	3.3	77
40	33.4 ± 0.86	2.6	84
80	66.7 ± 2.50	3.7	83

It was not possible to measure bopindolol using a direct procedure similar to that for 18-502. Analysis of the excitation and emission spectra of bopindolol showed that it was weakly fluorescent and would therefore not be detectable with adequate sensitivity. By converting bopindolol to 18-502 using enzyme hydrolysis, the necessary sensitivity could be obtained.

Following enzyme hydrolysis, 93% of standard bopindolol could be recovered as 18-502. Increasing enzyme concentration and incubation time did not increase this conversion showing that optimum conditions were being employed and suggesting therefore, that the 7% apparent loss in conversion is probably due to incomplete hydrolysis. The calibration curve of bopindolol enzymatically hydrolysed to 18-502 and calculated as 18-502 equivalents was $y = 7.19x - 22.2$, $r^2 = 0.996$. This was similar to the calibration curve for 18-502 obtained in the same experiments, $y = 7.51x - 7.44$, $r^2 = 0.998$. Under the conditions used for enzyme hydrolysis, there was no significant effect on concentrations of 18-502 present in standards or samples or on recovery (79 ± 8%, $n = 6$) or assay blank (0.008 ± 0.027 ng/ml, $n = 13$). The limit of sensitivity for bopindolol can be calculated to be 1 ng/ml.

Internal standards are not necessary in the 18-502 assay or for the enzyme hydrolysis step because of the good reproducibility and recovery of the procedures.

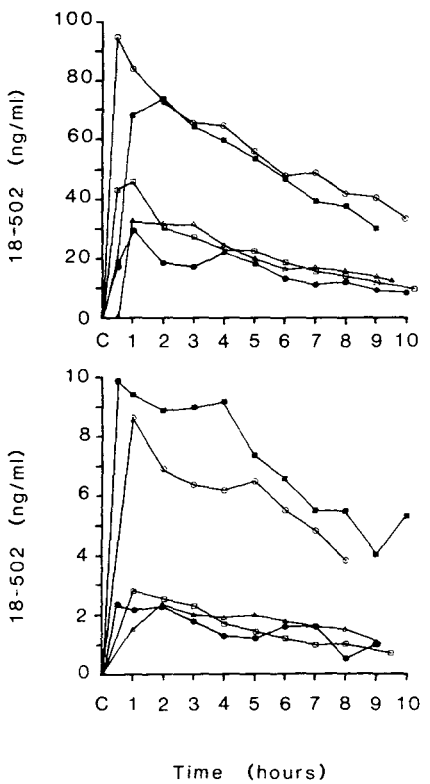

Fig. 3. Plasma levels of 18-502 in five patients with essential hypertension given 1 mg orally (lower panel) and 8 mg orally (upper panel) bopindolol, plotted against time.

Interference from other drugs in the assay was not specifically checked, firstly because the patients studied were on no other medication and secondly because previous experience with similar extraction and HPLC procedures has shown that of the many drugs commonly used in treating hypertension and related cardiovascular disorders, only quinidine and prazosin could be detected [4].

The plasma concentrations of bopindolol and 18-502 were measured in five patients with essential hypertension who were given 1-mg and 8-mg oral doses of bopindolol (Fig. 3). Maximum 18-502 concentrations occurred at 0.5—2 h following administration then declined over the ensuing 8—9 h.

At both doses, 18-502 concentrations in the five patients varied over a three-fold range. Whether this variation represents differences in the bioavailability of 18-502 or in its apparent volume of distribution is not known. At both doses, however, the same two patients (female) had consistently higher plasma concentrations than the three male patients.

No significant difference in 18-502 concentration in enzyme hydrolysed samples compared to non-enzyme hydrolysed samples in the five patients was found, suggesting that bopindolol is rapidly converted to its metabolite following oral administration.

In summary, the HPLC method using fluorescence detection developed for the measurement of 18-502 in plasma and for bopindolol following enzyme hydrolysis to 18-502 shows adequate sensitivity, specificity, precision and accuracy. It has the advantage of being a relatively simple and quick assay. It has been successfully applied to the measurement of 18-502 concentrations in patients with essential hypertension. 18-502 was readily detected following oral administration of therapeutic doses of bopindolol. Bopindolol, however, was not detected suggesting that the pro-drug is rapidly hydrolysed to its active metabolite in vivo.

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Note

Measurement of ornidazole by high-performance liquid chromatography

H. MERDJAN, C. BONNAT, E. SINGLAS* and B. DIQUET

Clinical Pharmacy Unit, CHU Bicêtre, 78 rue du Général Leclerc, 94270 - Le Kremlin Bicêtre (France)

(First received July 27th, 1982; revised manuscript received September 24th, 1982)

Ornidazole [α-(chloromethyl-2-methyl-5-nitroimidazole-1-ethanol] is a derivative of nitroimidazole, with antiprotozoal and antibacterial properties. It is used in the prevention and treatment of infections due to anaerobic germs, such as *Bacteroides fragilis*. The drug is especially useful in abdominal or gynecological surgery.

The measurement of plasma ornidazole allows the dosage to be adjusted in patients suffering from malabsorption or pathological conditions which modify excretion of the drug.

Nitroimidazole derivatives in general, and metronidazole in particular, can be measured by several methods: polarography [1], spectrophotometry [2], microbiology [3, 4], thin-layer chromatography [5, 6], gas—liquid chromatography [7, 8] or high-performance liquid chromatography [9—12]. Not all of these methods are capable of measuring the concentration of ornidazole.

Two types of pharmacokinetic ornidazole studies have been carried out: after administration of radiolabeled product, by separation of ornidazole and its metabolites using thin-layer chromatography [13, 14], and by microbiological assay [15]. Radioactive assay is not a feasible method in common medical practice, and the microbiological assay techniques are long and not very specific.

We have developed a method of measuring ornidazole by high-performance liquid chromatography. The method is rapid, selective and reproducible, by using an internal standard. Furthermore, the method allows metabolites to be separated and measured.

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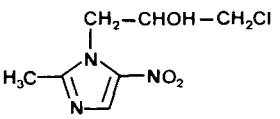
EXPERIMENTAL

Standards and solvents

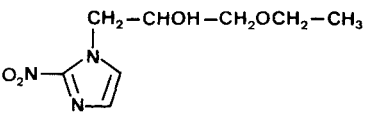
Ornidazole, the internal standard and the metabolites (Fig. 1) were kindly supplied to us by Roche, Neuilly/Seine, France. Ornidazole (Ro 07-0207) is α-(chloromethyl)-2-methyl-5-nitroimidazole-1-ethanol. Internal standard (Ro 07-0913) is α-(ethoxymethyl)-2-nitroimidazole-1-ethanol. Metabolite 1 (Ro 11-4791) is α-(chloromethyl)-2-hydoxymethyl-5-nitroimidazole-1-ethanol. Metabolite 4 (Ro 11-2616) is 3-(2-methyl-5-nitroimidazole 1-yl)-1,2-propane diol. Metabolites M_1 and M_4 are quantitatively the most important metabolites [13, 14, 16].

The aqueous reagents, i.e. $HClO_4$ 0.7 mol/l (Prolabo, Paris, France), NaOH 1 mol/l (Prolabo), buffer Titrisol* citrate—HCl pH 4 (Merck, Darmstadt, G.F.R. Réf. 9884), are all prepared using distilled water.

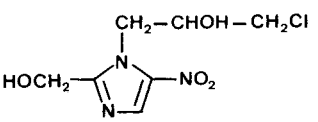
The mobile phase, water—ethanol (1:9, v/v), is filtered through a Whatman No. 2 filter and degassed by ultrasound (Bromsonic 52, Bioblock Apparatus, Strasbourg, France) before use. The flow-rate is 2 ml/min, corresponding to a pressure of 140 bars (2000 p.s.i.)



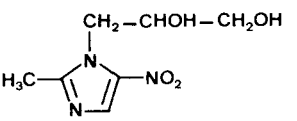
ORNIDAZOLE
Ro 07-0207



INTERNAL STANDARD
Ro 07-0913



METABOLITE M_1
Ro 11-4791



METABOLITE M_4
Ro 11-2616

Fig. 1. Chemical structures of compounds used.

Chromatographic conditions

The high-performance liquid chromatograph used is a Du Pont de Nemours (Paris, France) Model 870, coupled to a spectrophotometric detector, which is itself linked to a recording scope, both by Du Pont de Nemours.

Absorption is measured at 318 nm; the sensitivity is 0.02 absorbance units over the full scale. The products are injected with a 50-μl loop injector (Rheodyne 7125, Berkeley, CA, U.S.A.). The column used is a μBondapak C_{18} reversed-phase 30 cm × 4.6 mm column. The size of the particles is 10 μm (Waters, Paris, France). The peak areas are calculated using an integrating microprocessor ICAP 10 (Delsi, Suresnes, France).

Extraction

A 1-ml volume of plasma (sample to be assayed or standard) is added to 50 μl of an aqueous solution of 100 μg/ml internal standard and 1 ml of 0.7 mol/l perchloric acid. The mixture is agitated for 5 min using an alternating agitator (Realis, Villejuif, France) and then the solution is centrifuged for 10 min at 1000 *g*. Then 1 ml of the supernatant is taken off, neutralized with 0.3 ml of 1 mol/l sodium hydroxide and buffered with 0.2 ml of citrate—HCl buffer. After shaking, 50 μl are injected into the chromatograph.

Calibration

A standard concentration curve is obtained by adding ornidazole at concentrations of 0.2, 0.5, 1, 2, 5, 10, 15 and 20 μg/ml to control plasma samples under the same experimental conditions. The ratios of the peak areas of ornidazole and internal standard are plotted.

The precision of the method is estimated by determining the coefficient of variation over three concentrations.

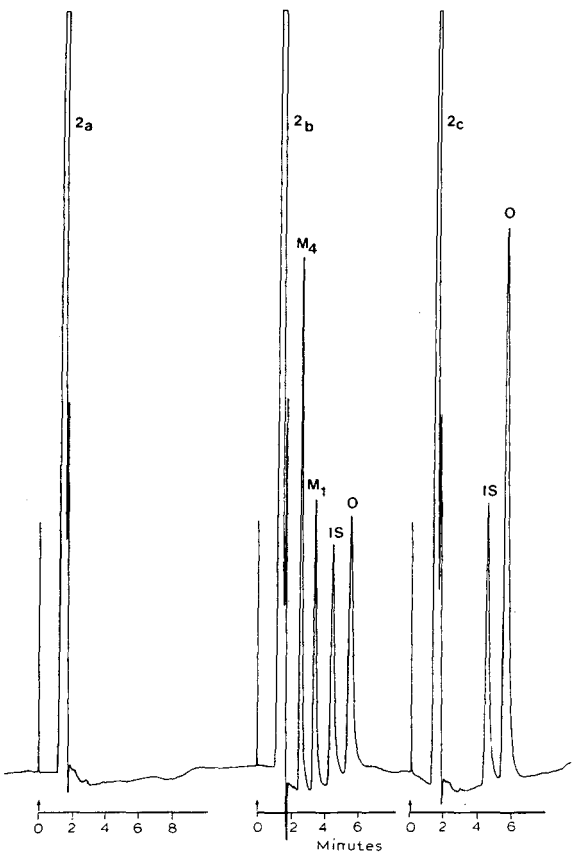


Fig. 2. Chromatograms of extracts from blank human plasma (a), standard plasma containing 5 μg/ml ornidazole (O), metabolites M_1 and M_4 and 5 μg internal standard (b), and patient plasma (c). The retention times are 2.6 (M_4), 3.4 (M_1), 4.5 (internal standard, IS) and 5.6 (O) min.

RESULTS

Fig. 2 shows the chromatograms obtained with 1 ml of control plasma, 1 ml of standard plasma (containing 5 μg/ml ornidazole, metabolites M_1 and M_4 and 5 μg of internal standard), and 1 ml of plasma obtained from a patient 1 h after the slow intravenous injection of 500 mg of ornidazole. The different substances are well separated and there is no interference between the peaks.

The retention times of metabolites M_4 and M_1, internal standard and ornidazole are, respectively: 2.6, 3.4, 4.5 and 5.6 min, (capacity coefficient k': 0.9, 1.4, 2.2 and 3.0 respectively).

Fig. 3 shows the standard concentration curve from 0.2 to 20 μg/ml. Each point repesents the mean ± 2 S.E.M. over five measurements. Under the present experimental conditions, the limits of detection of M_4, M_1 and ornidazole are 0.1, 0.2 and 0.2 μg/ml, respectively.

Reproducibility

The reproducibility of the method was checked for three plasma concentrations (1, 10 and 20 μg/ml). Twelve measurements were made at each concentration. The coefficients of variation ($\sigma/m \times 100$) were 4.7, 1.7, 1.8%, respectively, for the three concentrations.

Selectivity

The quantitatively more important metabolites of ornidazole do not interfere with the measurement of plasma concentration. Firstly, their retention time is different from that of ornidazole and the internal standard, and secondly, their plasma concentrations are low, much lower than ornidazole, especially when the drug is given by a single intravenous injection.

Neither was there any interference with the other derivatives of nitroimidazole (tinidazole, metronidazole, misonidazole). The different peaks were well apart.

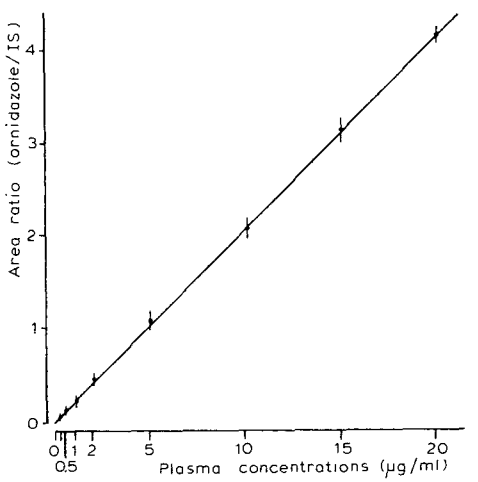


Fig. 3. Area ratio (ornidazole/IS) versus plasma concentrations of added ornidazole. Each point corresponds to the mean ± 2 S.E.M. of five analytical determinations.

Recovery

The absolute percentage of extraction could not be calculated because we were not able to obtain radioactive ornidazole. But we were able to make an estimation by comparing the peak areas obtained after the direct injection of a pure aqueous solution and after the injection of plasma containing the same concentration of ornidazole (5 μg/ml). By this method, the percentage extraction was greater than 98%.

DISCUSSION AND CONCLUSION

Several proportions of the ethanol—water mixture were tried at different input rates. The ratio 1:9 (v/v) at an input rate of 2 ml/min gave the best results with an acceptable analysis time.

Similarly, we also tested several columns (LiChrosorb RP-2 15 cm, LiChrosorb RP-8 15 and 20 cm, LiChrosorb RP-18 15 and 25 cm; Merck, France). The best separations were obtained with the column we suggested above. The technique is easy. However, one step must be conducted with extreme care: this is the neutralization and then buffering of the acid phase, because at pH above 5.5—6.0, ornidazole is degraded and a double peak is obtained. So the addition of sodium hydroxide and buffer should be very exact and the proportions properly respected.

Although metabolites M_4 and M_1 are quantitatively the most important metabolites of ornidazole (they account for 70% of total urinary metabolites [17]), they do not appear on the chromatogram presented on Fig. 2c. The reason is that they are slowly produced and rapidly eliminated from the blood stream. This leads to very low plasma concentrations after a single dose. They should become detectable after repeated administrations [16].

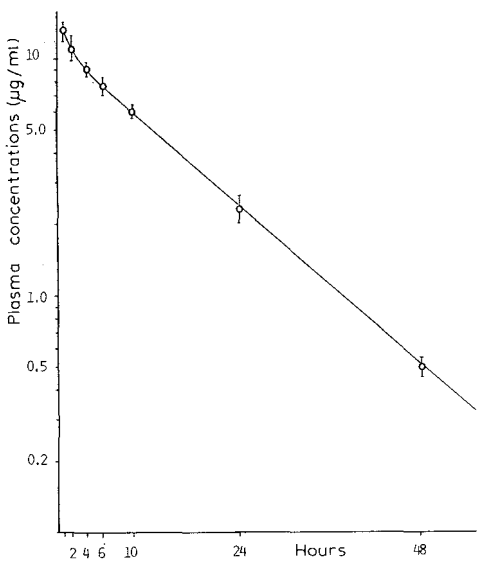


Fig. 4. Mean plasma levels ± S.E.M. of ornidazole after intravenous injection of 500 mg in eight patients.

To demonstrate the usefulness of this method, Fig. 4 represents the mean pharmacokinetic profile of ornidazole in plasma after an intravenous injection of 500 mg.

This method is rapid and reproducible. The selectivity is good and its sensitivity is sufficient for pharmacokinetic studies and therapeutic drug monitoring.

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Note

Determination of colterol in human plasma and urine by reversed-phase chromatography with amperometric detection

GEORGE B. PARK, RAYMOND F. KOSS, JULIE UTTER and JEROME EDELSON*

Department of Drug Metabolism and Disposition, Sterling-Winthrop Research Institute, Rensselaer, NY 12144 (U.S.A.)

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Bitolterol, the di-*p*-toluate ester of colterol (Fig. 1) is an experimental bronchodilator currently undergoing clinical evaluation in both oral and aerosol formulations. Bitolterol is readily hydrolyzed in vivo to colterol, a catecholamine, which is the active moiety. Colterol is further metabolized to N-*tert.*-butylmetarterenol through the action of enzyme catechol-O-methyl transferase. Both colterol and N-*tert.*-butylmetarterenol are conjugated as the glucuronide [1] and possibly as the sulfate.

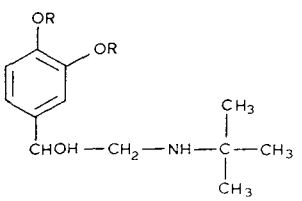

Fig. 1. Structural formulae of colterol (R=H) and bitolterol (R = —CO—C_6H_4—*p*-CH_3).

Most sample preparation techniques in catecholamine assays are based on the method of Anton and Sayre [2] in which the compound of interest is adsorbed to alumina through the catechol moiety. The use of di-(2-ethylhexyl)phosphoric acid (DEHPA) as an ion-pairing agent in the extraction of catecholamines has been reported [3, 4]. Cation-exchange resins have also been used to isolate catecholamines from biological specimens [5, 6]. Extraction of the ion-exchange eluate with a combination containing DEHPA and an organic solvent offers a convenient concentration step, with a resulting increase in sensitivity.

This report describes methods for the analysis of colterol in human plasma

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and urine by the ion-pair extraction of the catecholamine followed by high-performance liquid chromatography (HPLC) with amperometric detection.

EXPERIMENTAL

Materials

Colterol and the internal standard, the hydrochloride salt of 1-(3,4-dihydroxyphenyl)-2-cyclopentylaminoethanol, were synthesized at the Sterling-Winthrop Research Institute. Nanograde benzene (Mallinckrodt, St. Louis, MO, U.S.A.) and HPLC grade methanol were used. DEHPA was obtained from Sigma (St. Louis, MO, U.S.A.). Water was purified by the Barnstead ORGANICpure^{TM} system. All other reagents were analytical reagent grade, from commercial sources, and were used without additional purification. All glassware was silanized by treatment with 5% dimethyldichlorosilane in toluene prior to use.

Preparation of standards and samples

Stock solutions of colterol and the internal standard were prepared in 0.05 *N* sulfuric acid. Standards were prepared by adding appropriate aliquots from the stock solution to 1.0 ml of control human plasma (oxalate anticoagulant) or to 1.0 ml of control human urine. Fresh standards were prepared on the day of analysis of each set of samples.

Spiked samples were prepared for single-blind analysis in the same manner as the standards. Two sets of triplicate samples were prepared in plasma at concentrations of 0, 0.35, 0.6, 1.1 and 3.3 ng/ml. One set was analyzed upon preparation, the remaining set was stored for one week at −70°C prior to analysis. Spiked urine samples (in triplicate) were prepared in a similar manner, at concentrations of 0, 80, 180, 540 and 800 ng/ml.

Plasma assay

To 1.0 ml of plasma in a 20-ml silanized tube were added 100 μl of the internal standard solution (5 ng per 100 μl), 2.0 ml of 0.2 *M* phosphate buffer (pH 6.9), and 10 ml of 1.5% (v/v) DEHPA in benzene. The samples were mixed by shaking mechanically for 10 min, then centrifuged at 1400 *g* for 10 min. The organic phase was transferred to silanized 15-ml conical tubes, and extracted with 130 μl of 0.2 *N* sulfuric acid. The samples were shaken for 10 min followed by centrifuging at 500 *g*. The upper organic layer was carefully removed and discarded. Residual benzene vapors were evaporated under nitrogen. A 100-μl aliquot of the acid layer was injected into the HPLC system described below.

Urine assay

Cation-exchange columns were prepared by adding a slurry of resin (Bio-Rex 70, 50—100 mesh) in water to glass columns (8 mm I.D.) fitted with glass-wool plugs and PTFE stopcocks. The settled resin bed height was 4 cm. It was washed with 10 ml of water followed by 20 ml of 0.2 *M* phosphate buffer (pH 6.9).

To 1.0 ml of human urine were added 100 μl of the internal standard solu-

tion (50 ng per 100 μl) and 1.0 ml of 0.2 *M* phosphate buffer (pH 6.9). The buffered urine was transferred to the cation-exchange column and allowed to flow through the column. Following elution of the urine sample the column was rinsed with 30 ml of distilled water and drained. The catecholamines were then eluted with 4.0 ml of a 2% boric acid solution. The eluate was collected in a 20-ml silanized tube. Ten ml of a 1% DEHPA—benzene solution were added and the sample was mixed and centrifuged. The organic layer was then extracted with 1.0 ml of 0.05 *N* sulfuric acid. A 100-μl aliquot of the acid phase was chromatographed.

Acid hydrolysis

To 1.0 ml of urine, 0.5 ml of 1 *N* hydrochloric acid was added. The sample was placed in a 90°C water bath for about 30 min. The mixture was neutralized with 1 *N* sodium hydroxide (approx. 0.5 ml) before the addition of 1.0 ml of 0.2 *M* phosphate buffer (pH 6.9). The hydrolyzed sample was processed as described above.

Chromatography

Chromatographic separation was performed at ambient temperature on a modular system composed of a Dupont Model 870 pump, a Rheodyne Model 7125 sample injector, and an Altex Ultrasphere ODS reversed-phase 5-μm column (25 cm × 4.6 mm). A Bioanalytical Systems Model LC-4 amperometric detector with a TL-5 glassy carbon electrode containing a 0.002-in. spacer was used. The applied potential was +0.60 V vs. Ag/AgCl (3 *M* sodium chloride). The flow-rate was 1.0 ml/min at 125 bar.

Mobile phase

The chromatographic mobile phase consisted of 88 parts (v/v) of 0.1 *M* sodium sulfate (containing 100 mg of disodium ethylenediaminetetraacetate (EDTA) per liter adjusted to pH 2.8 with phosphoric acid, and then brought to a final pH of 3.0 with 1 *N* sodium hydroxide) and 12 parts of methanol. The mobile phase was filtered through 0.45-μm filters, and purged with helium before use.

Animal study

To demonstrate the biological applicability of the method, rats were medicated orally with 25 mg of bitolterol. Blood was drawn from the aorta and placed in tubes containing oxalate as the anticoagulant, 60 min after medication. Glass syringes were used when drawing blood samples. (A large peak which interfered with the chromatography of colterol was observed when plastic syringes were used.) Plasma was processed as described above. Urine was collected from a similarly dosed rat over a 0—6 h period and treated as described above; an aliquot of the urine was acid-hydrolyzed before analysis.

Calculations

A least-squares regression analysis of the peak height ratios (colterol:internal standard) obtained for the standards was performed. Linearity of response was evaluated on the basis of visual examination of the plot, examination of the

residuals, and an F-test for lack of fit. From the slope and y-intercept of the regression line, the concentrations of colterol in the samples were determined. The minimum quantifiable limit of the assay, defined as the concentration whose lower 80% confidence limit just encompasses zero, was estimated from the least-squares regression analysis [7].

The assayed levels for the validation samples were expressed as percent differences from the nominal values and analyzed by a two-way analysis of variance with replication to test for a concentration effect, a time effect, and a concentration-times-time effect (fresh vs. frozen). The accuracy of the assay was estimated from the mean percent differences at each concentration. The precision of the assay was estimated from the derived standard deviation of the percent differences.

RESULTS AND DISCUSSION

Chromatograms of extracted plasma and urine samples from rats medicated orally with bitolterol are shown in Fig. 2. Retention times for colterol and the internal standard are 8.5 min and 13.5 min, respectively. Regression analysis on each set of standards demonstrated a linear relationship between peak height ratio and concentration over the range of 0 and 0.25 to 5.0 ng/ml for plasma and 0 and 25 to 1000 ng/ml for urine. The concentration of each of the prepared plasma and urine specimens was estimated by inverse prediction [8] from the appropriate equation which was generated from the linear regression analysis; the results of these calculations for plasma and urine samples are summarized in Tables I and II, respectively.

A two-way analysis of variance of the nominal values for the plasma samples indicated no significant sources (either concentration, time, or concentration-times-time) of variation at $p \leqslant 0.01$. An overall estimate of the precision of the plasma method, based on the variance of the replicate determinations at each

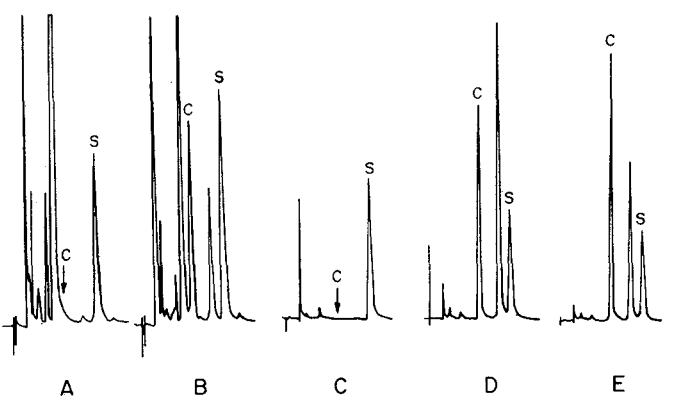

Fig. 2. Chromatograms of (A) processed rat plasma containing only the internal standard (s); (B) processed rat plasma sample taken 1.0 h after oral bitolterol dosing at 25 mg, showing colterol (c); (C) processed rat urine containing only the internal standard (s); (D) processed rat urine sample collected 0—6 h after bitolterol dosing at 25 mg; (E) same as (D), subjected to acid hydrolysis before processing. Attenuation settings: plasma samples 5 nA; urine samples 50 nA.

TABLE I

CONCENTRATION OF COLTEROL (FREE BASE) FOUND IN SPIKED PLASMA SAMPLES

Nominal concentration (ng/ml)	Mean concentration found (n = 3) (ng/ml)		
		Non-frozen	Frozen
0		<MQL*	<MQL**
0.35	Mean	0.32	0.37
	% S.E.M.***	1.03	5.43
	Mean % difference§	−7.62	6.67
	% C.V.§§	1.79	9.41
0.60	Mean	0.59	0.61
	% S.E.M.	2.48	4.64
	Mean % difference	−2.22	2.22
	% C.V.	4.29	8.04
1.1	Mean	1.12	1.06
	% S.E.M.	2.39	3.64
	Mean % difference	1.52	−3.94
	% C.V.	4.14	6.30
3.3	Mean	3.25	3.19
	% S.E.M.	2.65	0.69
	Mean % difference	−1.52	−3.43
	% C.V.	4.59	1.19

*Minimum quantifiable limit = 0.09 ng/ml.
**Minimum quantifiable limit = 0.14 ng/ml.
***% S.E.M. = S.E.M./mean × 100.
§Mean % difference = assayed mean/(nominal level − 1) × 100.
§§% Coefficient of variation (C.V.) = S.D./mean.

concentration level, was 5.8%. The accuracy of the analysis, defined by the ranges of the mean percent differences from the nominal concentrations, varied from −7.6% to +5.7%. The mean (±S.E.) minimum quantifiable level of the plasma assay was 0.11 (±0.02) ng/ml, n = 2.

An overall estimate of the precision of the urine assay, based on the variance of the repeat determinations, was 3.8%. The accuracy of the urine method, defined by the mean percent difference of the assayed value from the nominal value, ranged from −0.2% to +2.6%. The minimum quantifiable level was 5 ng/ml. Omission of the step involving isolation on the ion-exchange resin reduced the sensitivity to 19 ng/ml; since the ion-exchange procedure removes much endogenous material, the background noise level is reduced.

The extraction efficiencies of colterol and the internal standard were based on a comparison of peak heights of extracted versus direct standards and were independent of the concentration. From plasma the mean efficiency was 61% for colterol and 59% for the internal standard; from urine, the percent recovery was 75% for both colterol and the internal standard.

The concentration of colterol in rat plasma 1 h after a 25-mg oral dose of bitolterol was 36 ng/ml. The concentration of colterol in unhydrolyzed urine was 240 ng/ml; hydrolysis increased the concentration to 930 ng/ml.

In conclusion, an accurate, sensitive and reproducible HPLC assay with

TABLE II

CONCENTRATION OF COLTEROL (FREE BASE) FOUND IN SPIKED URINE SAMPLES

Nominal concentration (μg/ml)		Mean concentration found, n = 3 (μg/ml)
0		<MQL*
0.08	Mean	0.081
	S.E.M. (%)	2.14
	Mean percent difference	1.25
	C.V. (%)	3.70
0.18	Mean	0.18
	S.E.M. (%)	0.18
	Mean percent difference	0.19
	C.V. (%)	0.32
0.54	Mean	0.55
	S.E.M. (%)	2.20
	Mean percent difference	0.99
	C.V. (%)	3.80
0.80	Mean	0.82
	S.E.M. (%)	3.00
	Mean percent difference	2.63
	C.V. (%)	5.20

*Less than the minimum quantifiable level, 5 ng/ml.

amperometric detection has been developed for the determination of colterol in both plasma and urine; its utility in biological specimens has been demonstrated.

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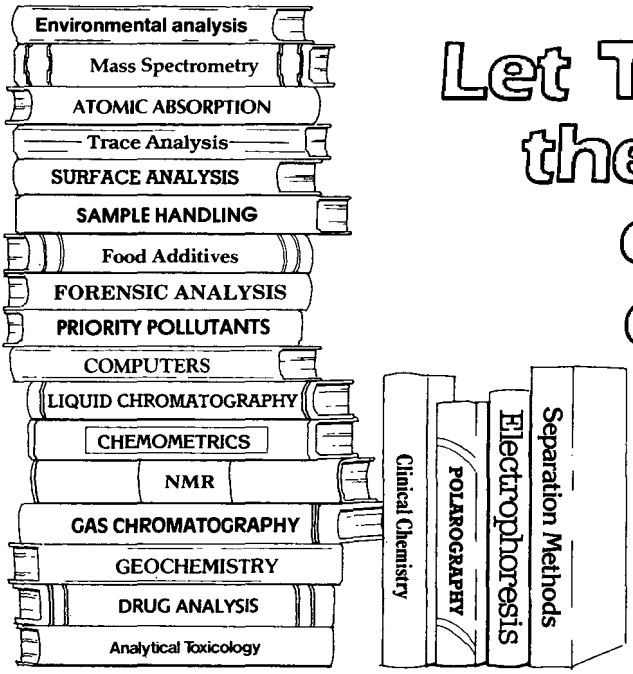

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