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DETERMINATION OF 17-HYDROXYCORTICOSTEROIDS IN URINE BY FLUORESCENCE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING Dns-HYDRAZINE AS A PRE-COLUMN LABELING REAGENT

TAKAO KAWASAKI, MASAKO MAEDA and AKIO TSUJI*

School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo (Japan)

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SUMMARY

A method is described for the determination of urinary 17-hydroxycorticosteroids using fluorescence high-performance liquid chromatography. After enzymatic hydrolysis, 17-hydroxycorticosteroids were extracted using an Extrelut column and then labeled with Dns-hydrazine in hydrochloric acid—ethanol solution. The labeled steroids were chromatographed on a microparticulate silica gel column, the mobile phase was dichloromethane—ethanol—water (900:60:40). The eluate was monitored on a fluorophotometer at 365 nm (excitation) and 505 nm (emission). Linearity of the fluorescence intensities (peak heights) of various 17-hydroxycorticosteroids were obtained between 60 pg and 20 ng. The assay was sensitive, precise and accurate. Comparison with the results obtained by radioimmunoassay gave correlation coefficients of 0.932 for tetrahydrocortisol and 0.930 for tetrahydrocortisone. The proposed method is clinically useful for the routine analysis of urinary 17-hydroxycorticosteroids.

INTRODUCTION

The most important steroid secreted by the human adrenal gland is cortisol. Cortisol in the blood is converted to water-soluble conjugates in the liver; they, along with other metabolites, are excreted as glucosiduronates of tetrahydrocortisol (THF) and tetrahydrocortisone (THE). Measurement of these, the so-called urinary 17-hydroxycorticosteroids (17-OHCS), has been used to screen for abnormalities in adrenocortical function [1]. The Porter—Silber reaction [2, 3], a non-specific color reaction for the estimation of 17,21-dihydroxy-20-oxocorticosteroids, has been widely used in clinical laboratories. However, there is interference by other steroids, non-steroid metabolites and certain drugs [4]. Specific radioimmunoassay (RIA) techniques [5] have been developed and used in routine clinical diagnosis. Although RIA is highly

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sensitive, its specificity is limited because steroid antibodies may cross-react to varying degrees with other steroids. High-performance liquid chromatography (HPLC) offers the desired selectivity and sensitivity for the analysis of steroids such as cortisol [6—13] and, because it does not require the use of radioisotope, HPLC represents a useful, safe alternative to RIA techniques in routine laboratory analysis. However, because tetrahydrocorticosteroids have no strong UV-absorbing group in their structure, no HPLC method for their detection has been reported; instead, urinary steroids have been assayed by gas chromatography [14—18].

We have reported the use of Dns-hydrazine as a fluorescent labeling reagent to increase the detection limit for 17-oxosteroids (17-OS) [19]. We now present a highly sensitive fluorescence HPLC method for the determination of urinary 17-OHCS.

EXPERIMENTAL

Materials

THF, tetrahydro-11-deoxycortisol (THS) and *allo*-tetrahydrocortisol (*allo*-THF) were kindly donated by Dr. D.K. Fukushima. THE, tetrahydrocorticosterone (THB) and Dns-hydrazine (grade II) were from Sigma (St. Louis, MO U.S.A.), and β-D-glucuronidase and β-D-glucuronidase/arylsulphatase from Boehringer Mannheim—Yamanouchi Co. (Tokyo, Japan). The Extrelut Refill Pack was from E. Merck (Darmstadt, G.F.R.). Other steroids, reagents and solvents were from commercial sources.

Instruments and chromatographic conditions

We used an Hitachi Model 635 high-performance liquid chromatograph equipped with a Kyowa Seimitus KHP-UI-130 injection valve, a stainless-steel column (250 × 4.6 mm I.D.) and a Jasco FP-110 fluorescence spectrophotometer equipped with a mercury lamp and a micro flow cell. A Zorbax SIL column (particle size 5—6 μm, 250 × 4.6 mm I.D.) was used. The mobile phase was the organic layer separated from a mixture of dichloromethane—ethanol—water (900:60:40) after 30 min shaking; the flow-rate was 1 ml/min. The detector wavelength was set at 365 nm and 505 nm for excitation and emission, respectively.

Reagent solutions

Dns-hydrazine solution. A 0.05% (w/v) solution was prepared by dissolving 5 mg of Dns-hydrazine in 10 ml of ethanol; it was stored in a refrigerator until use.

Hydrochloric acid—ethanol solution. This was prepared by mixing 2 ml of concentrated hydrochloric acid with 1000 ml of ethanol.

Steroid stock solutions. THF, THE, *allo*-THF, or THS (2 mg) were made up to 10 ml with ethanol and stored in a refrigerator until use.

Buffers. The acetate buffer was 2 *M* (pH 5.2); the phosphate buffer was 0.5 *M* (pH 6.2).

Extrelut column. A 10-ml syringe, closed at the bottom with a Fine Filter F (Ishikawa Manufactory, Tokyo, Japan) was used as a column. It was filled

with 2 g of Extrelut, a large-pore, granular Kieselguhr with high pore volume, and vibrated for 10 sec on a Vortex-type mixer; the surface of the packing was covered with a filter plate.

Procedure

Hydrolysis with β-D-glucuronidase/sulphatase. A mixture consisting of 0.5 ml of urine, 0.5 ml of acetate buffer and 40 μl of enzyme solution was incubated for 24 h at 37°C. Two drops of chloroform were added to avoid bacterial decomposition.

Hydrolysis with β-D-glucuronidase. After adding 0.5 ml of phosphate buffer, 20 μl of enzyme solution and two drops of chloroform to 0.5 ml of urine, the mixture was incubated overnight at 50°C.

Extraction on the Extrelut column. The hydrolysate was diluted to 3 ml with redistilled water, loaded onto a dry Extrelut column (2 g), and allowed to soak in for 10 min to distribute the aqueous phase on the column packing. 17-OHCS were eluted from the column with 6 ml of dichloromethane and the effluent was evaporated to dryness at 40°C under a stream of nitrogen gas. The resultant residue was assayed as described below.

Solvent extraction. After incubation, 6 ml of dichloromethane were added to the mixture; it was stirred vigorously for 1 min with a Vortex-type mixer and centrifuged for 3 min at 1000 *g*. The aqueous layer was removed with a Pasteur pipet. After drying by adding 1 g of anhydrous sodium sulphate, exactly 4 ml of the organic layer were evaporated to dryness at 40°C under a stream of nitrogen gas and the resultant residue was assayed by the following procedure.

Labeling reaction. The residue in a test-tube was dissolved by adding 0.2 ml of hydrochloric acid—ethanol solution, then admixed with 50 μl of Dns-hydrazine solution, left to stand for 10 min at 65°C, and evaporated to dryness under a stream of nitrogen gas. The labeled residue was dissolved in 500 μl of eluent and an aliquot was injected into the chromatograph described above.

RESULTS

Fluorescence spectrum

Fig. 1 shows the excitation and emission spectra of Dns-hydrazone of THF in chloroform. The hydrazone was stable in chloroform for at least one week at 4°C without any change of fluorescence intensity. As shown in Table I, the fluorescence intensity of Dns derivatives was affected by the polarity of the solvent. In polar solvents such as methanol and acetonitrile, it was much lower than in chloroform or dichloromethane. Therefore, normal-phase HPLC on a microparticulate silica gel column was used for the separation of 17-OHCS, using the organic layer of a dichloromethane—ethanol—water mixture as the mobile phase.

Concentration of hydrochloric acid

In acidic media, tetrahydrocorticosteroids reacted with Dns-hydrazine to form hydrazones. Fig. 2 shows the effect of the hydrochloric acid concentration on the fluorescence intensity peak height. The peak height on all three

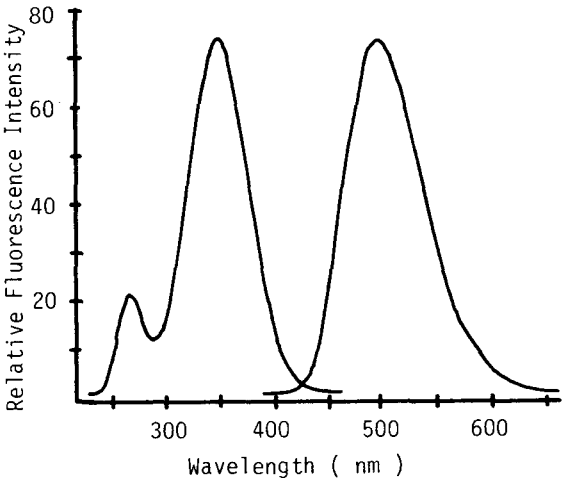

Fig. 1. Excitation and emission spectra of the fluorescent dansyl hydrazone of tetrahydrocortisol in chloroform. Excitation maximum, 350 nm; emission maximum, 505 nm.

TABLE I

RELATIVE FLUORESCENCE INTENSITY (RFI) AND FLUORESCENCE MAXIMUM IN VARIOUS SOLVENTS

Solvent	RFI	Fluorescence maximum (nm)	
		Excitation	Emission
Chloroform	100.0	350	500
Dichloromethane	116.7	350	500
Eluent*	112.0	350	505
Acetonitrile	55.6	350	525
Methanol	44.4	350	525

*Organic layer of dichloromethane—ethanol—water (900:60:40).

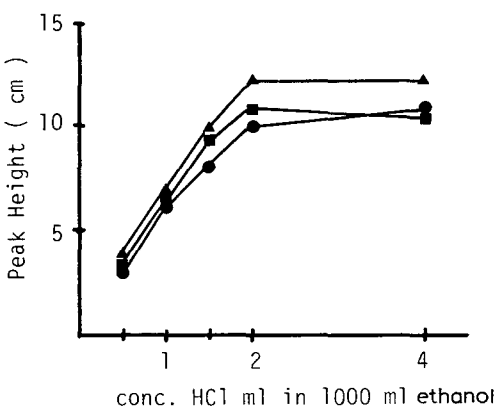

Fig. 2. Effect of hydrochloric acid concentration on fluorescence intensity peak height. (▲) Tetrahydrocortisone, (●) tetrahydrocortisol, (■) tetrahydro-11-deoxycortisol; 20 ng of each steroid were injected.

steroids increases with increasing hydrochloric acid concentration in ethanol up to 2 ml/l; it reached a constant value at 4 ml/l.

Therefore, hydrochloric acid—ethanol solution containing 2 ml of concentrated hydrochloric acid in 1000 ml of ethanol was used for the labeling reaction solvent.

Reaction temperature

As shown in Fig. 3, maximum peak heights were obtained at 65°C; they decreased at higher reaction temperatures. Therefore, the reaction temperature was set at 65°C for the labeling reaction. The reaction time used was 10 min because maximum peak heights were obtained at more than 5 min.

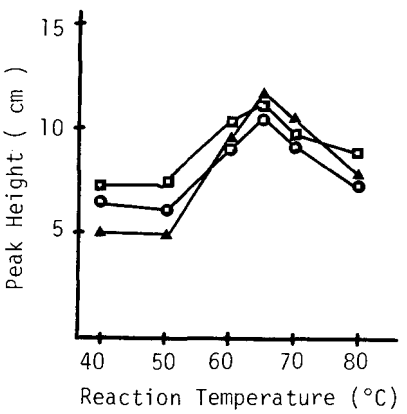

Fig. 3. Effect of reaction temperature on peak height. (▲) Tetrahydrocortisone, (●) tetrahydrocortisol, (■) tetrahydro-11-deoxycortisol; 20 ng of each steroid were injected.

Selection of eluent

Many solvent systems and columns were examined to obtain the complete separation of 17-OHCS. An organic layer consisting of a mixture of dichloromethane—ethanol—water (900:60:40) was found to be suitable when used on a Zorbax SIL column. The chromatograms presented in Fig. 4 show good separation of standard tetrahydrocorticosteroid mixtures including THF, THE, *allo*-THF, THS, and THB. The testing of other silica gel columns showed that urinary 17-OHCS were efficiently separated on various columns (Table II).

Working curves and sensitivities

Linearity of fluorescence (peak heights) intensity with the injected amounts of 17-OHCS (THF, THE, *allo*-THF, THS) were obtained in the range 60 pg—20 ng. When a 0.5-ml urine sample was used for the assay, the detection limit for 17-OHCS was about 3 ng/ml of urine.

Recovery of steroids from Extrelut column

Recovery of radiolabeled steroids upon Extrelut column extraction was 96.8 ± 4.4% (n = 5) and 90.0 ± 5.4% (n = 5) for [1,2-^{3}H]THE and [1,2-^{3}H]-THF, respectively.

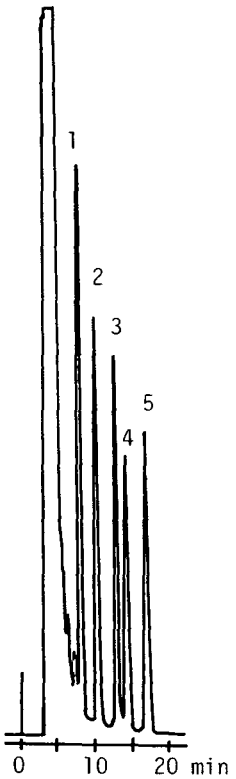

Fig. 4. Separation of fluorescent derivatives of tetrahydrocorticosteroid standard mixtures. Peaks: 1 = tetrahydrocorticosterone, 2 = tetrahydro-11-deoxycortisol, 3 = tetrahydrocortisone, 4 = allo-tetrahydrocortisol, 5 = tetrahydrocortisol. Column: Zorbax SIL column (250 × 4.6 mm I.D.). Mobile phase: dichloromethane—ethanol—water (900:60:40); flow-rate, 1 ml/min.

TABLE II

COMPARISON OF CAPACITY FACTORS (k') OF TETRAHYDROCORTICOSTEROIDS ON DIFFERENT SILICA GEL COLUMNS

Column packing	Producer	Particle size (μm)	Dimensions (length × I.D., mm)	Eluent*	Capacity factor (k')			
					THS	THE	*allo*-THF	THF
Hitachi gel 3042	Hitachi	5	250 × 4.0	I	3.46	5.20	6.06	7.40
Hitachi gel 3043	Hitachi	10	250 × 4.0	II	2.58	3.70	4.15	5.27
LiChrosorb Si 100	Merck	10	250 × 4.0	II	2.47	3.80	4.33	5.28
Zorbax SIL	DuPont	5—6	250 × 4.6	I	2.35	3.15	4.71	5.49
Finepak SIL	Jasco	5	250 × 4.6	I	2.47	3.29	3.81	4.61

*Eluent I: organic layer of dichloromethane—ethanol—water (900:60:40). Eluent II: organic layer of dichloromethane—ethanol—water (920:50:30). Flow-rate: 1 ml/min.

Recovery and reproducibility

An aqueous standard solution (2 ml) containing 0.5 μg each of the three 17-OHCS was added to a urine hydrolysate, and Extrelut column extraction, labeling reaction and HPLC were performed. As shown in Table III, 17-OHCS were recovered in the range 90.8—102.2% with a coefficient of variation (C.V.) range of 3.2—4.3%.

TABLE III

RECOVERY OF TETRAHYDROCORTICOSTEROIDS ADDED TO URINE PRIOR TO EXTRACTION

Urine (0.5 ml) to which 0.5 μg of each of the three 17-OHCS was used.

Steroid	Recovery (%)	*n*	C.V. (%)
THE	102.2	5	3.2
allo-THF	92.5	5	4.3
THF	90.8	5	4.1

Typical chromatograms of urine samples

Fig. 5 shows typical chromatograms of normal human and patient urine samples; urinary 17-OHCS were clearly separated and identified by comparison with authentic samples.

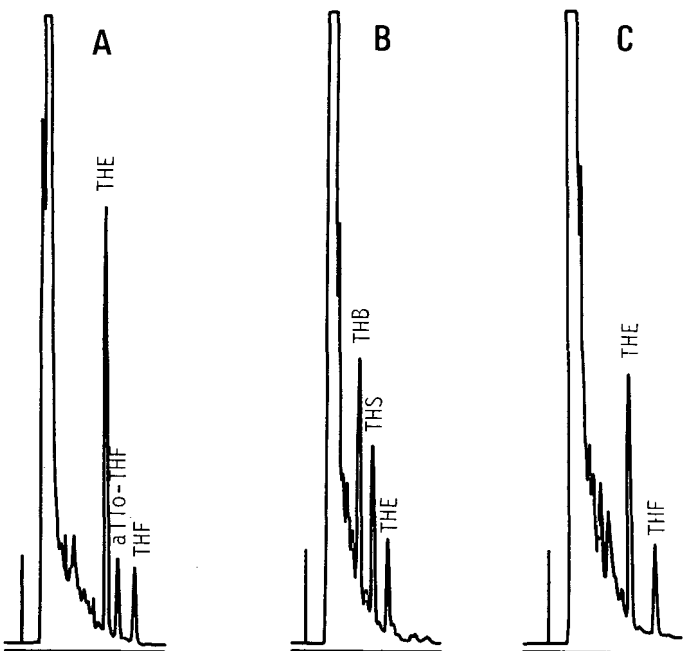

Fig. 5. Typical chromatograms of urine samples. (A) Normal human; (B) pituitary tumor patient; (C) normal human after oral administration of carbamazepine.

Comparison with results obtained by RIA and the colorimetric method

The reliability of the newly devised HPLC method for the determination of urinary 17-OHCS (THF and THE) was assessed by comparing the results with those obtained by RIA. RIA was as described by Kambegawa and Honma [20]; it involves Sephadex LH-20 chromatography for the separation of THF and THE. As illustrated in Fig. 6, the values obtained by both methods were in good agreement; the correlation coefficients were 0.932 and 0.930 for THF and THE, respectively.

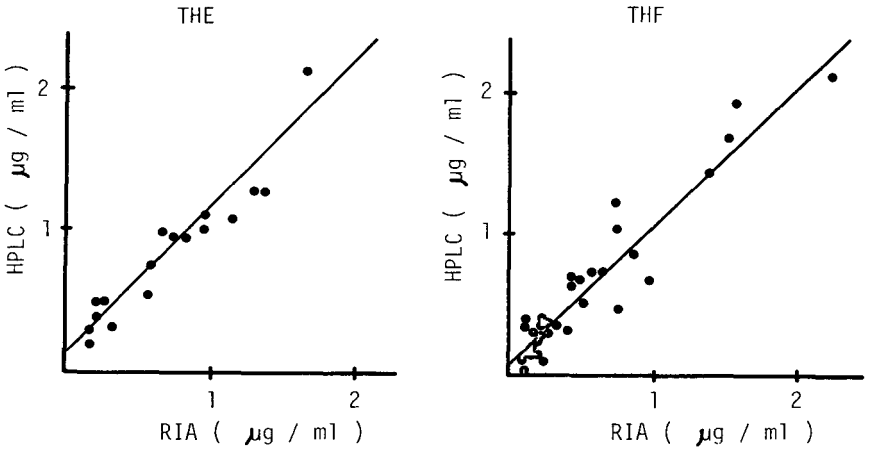

Fig. 6. Correlation between fluorescence HPLC and RIA values of urinary tetrahydrocortisone (THE) and tetrahydrocortisol (THF). THE: $y = 0.98x + 0.14$; $r = 0.930$; $n = 17$. THF: $y = 0.96x + 0.10$; $r = 0.932$; $n = 32$.

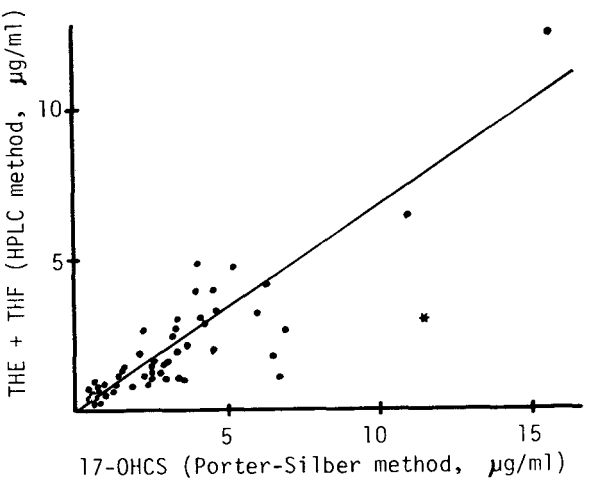

Fig. 7. Comparison of the results obtained with fluorescence HPLC and the colorimetric method for the determination of urinary 17-OHCS. $y = 0.67x - 0.05$; $r = 0.915$; $n = 53$. *, After oral administration of carbamazepine.

Furthermore, the total urinary THF and THE values obtained by the HPLC method were compared with the 17-OHCS values obtained by an ordinary colorimetric method, Porter—Silber reaction, used in routine assay. As shown in Fig. 7, the correlation coefficient was 0.915 and the regression line, $y = 0.67\ x - 0.05$, where x equals the values determined by the colorimetric method.

DISCUSSION

In clinical pathology, fluorometric and colorimetric methods such as the Porter—Silber reaction [3], the tetrazolium reaction [21] and ketogenic steroid

estimation [22], have been routinely used for the determination of 17-OHCS. As these methods are of limited sensitivity and do not have highly specific reactions, there may be some interference by other steroids, non-steroid metabolites and drugs. Sensitive RIA methods for THF [20, 23, 24] and THE [20, 24] have been reported, however, they require chromatographic separation such as Sephadex LH-20 column [20] or paper chromatography [24]. HPLC methods for the determination of steroids in biological fluids have been devised [18, 25]. In our preceding paper [19] we reported the determination of 17-OS in biological fluids by fluorescence HPLC using Dns-hydrazine as the fluorescence labeling reagent. In the present report, we describe a fluorescence HPLC method for the determination of urinary 17-OHCS, THF, THE, THS, *allo*-THF and THB. The Porter—Silber reaction involves the acid-catalyzed rearrangement of the adrenocortical side-chain to a 20,21-glyoxal grouping, followed by the formation of 21-phenylhydrazone [26] which showed a strong band at 1660 cm^{-1} arising from α,β-unsaturated β-hydrazinoketo group. On the other hand, the fluorescent product of THF obtained by the derivatization with Dns-hydrazine showed no band near 1660 cm^{-1}. It seems that the carbonyl group at C-20 position of THF reacted with Dns-hydrazine to yield the Dns-hydrazone of THF. THB, 17-deoxycorticosteroid, reacted with Dns-hydrazine under the same reaction conditions shown in Fig. 4.

We chose chromatographic conditions which, in the shortest possible analysis time, gave acceptable resolution between the Dns-hydrazone of tetrahydrocorticosteroids and the fluorescent coexisting substance in urine samples. As shown in Fig. 4 and Table II, good separation can be achieved on various silica gel columns, using the organic layer of a dichloromethane—ethanol—water mixture as the eluent. The sensitivity of this method is superior to that of other HPLC methods which employ a UV detector. Tetrahydrocorticosteroids have no strong UV-absorbing group in their molecules; therefore, when an refractive index detector is used, their detection limits exceeded 1 μg. When we hydrolyzed urine with β-D-glucuronidase and/or β-D-glucuronidase/sulphatase [27], we obtained equally good results (Table IV). The use of β-D-

TABLE IV

ANALYSIS OF URINARY STEROIDS USING DIFFERENT HYDROLYSIS AND EXTRACTION METHODS

Enzyme	Extraction	Steroid	Peak height (mm)	n	σ_{n-1}	C.V. (%)
Helicase	Mixing with dichloromethane	THE	137.5	4	0.64	4.6
		allo-THF	19.5	4	0.17	5.8
		THF	39.0	4	0.18	4.6
Helicase	Extrelut column	THE	128.5	4	0.36	2.8
		allo-THF	16.2	4	0.06	3.5
		THF	34.5	4	0.17	5.0
β-Glucuronidase	Mixing with dichloromethane	THE	130.0	4	0.45	3.5
		allo-THF	15.8	4	0.10	6.0
		THF	25.0	4	0.07	2.8

glucuronidase/sulphatase makes it possible to obtain simultaneously a hydrolysate for 17-OHCS and 17-OS detection. Although dichloromethane can be used for the extraction of 17-OHCS, this method sometimes produces an emulsion. The Extrelut column extraction method permits quantitation and it is as good as other methods commonly used for recovery. Ende et al. [28] observed contamination when they used the Extrelut column for steroid determination. However, we noted no interference peaks. There was a good correlation between the urinary THF and THE values obtained by our newly devised method and the RIA method, and the total THF and THE values obtained by our method correlated well with those of 17-OHCS obtained by the colorimetric method. A high 17-OHCS value (11.5 μg/ml) was recorded upon application of the Porter—Silber method to a urine sample that was obtained after the oral administration of carbamazepine; our method, used in the same sample, gave a normal (3 μg/ml) summation value for THF and THE. This indicates that urinary 17-OHCS can be determined by our method without interference by a metabolite of carbamazepine [29].

Although the estimation of total urinary neutral 17-OHCS is used in screening for adrenal disease, specific changes will go unnoticed. For example, the THF/THE ratio was increased in patients suffering from a wide range of diseases [30] and a highly significant correlation was obtained between THF-glucuronide and the cortisol secretion rate [24]. THS-glucuronide values were also highly elevated after metyrapone administration to humans [31]. Therefore, to obtain meaningful and accurate information, the determination of individual components of this group of steroids is very important. Our newly developed method may be clinically useful in the routine assay of urinary 17-OHCS.

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STUDIES ON STEROIDS

CLXXIX. DETERMINATION OF ESTRIOL 16- AND 17-GLUCURONIDE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

KAZUTAKE SHIMADA, FUMING XIE and TOSHIO NAMBARA*

Pharmaceutical Institute, Tohoku University, Sendai 980 (Japan)

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SUMMARY

A high-performance liquid chromatographic method for the determination of estriol 16-glucuronide and 17-glucuronide in bile and urine has been developed. The electrochemical detector was found to be more superior than the UV detector with respect to selectivity and sensitivity and therefore more suitable for the determination of estrogen conjugates in biological fluids. The use of the present method revealed that both estriol 16-glucuronide and 17-glucuronide were excreted in rat bile, while only the former was present in human pregnancy urine.

INTRODUCTION

In recent years considerable interest has been focused on the physiological significance of estrogen conjugates in the feto-placental unit. These conjugates are determined by a variety of methods, i.e. spectrophotometry [1], gas chromatography—mass spectrometry [2, 3] and radioimmunoassay [4]. In addition, several papers describe the methods for the determination of estrogens in biological fluids by high-performance liquid chromatography (HPLC) which involve prior hydrolysis and/or solvolysis of the conjugates [5, 6]. Deconjugation, however, has disadvantages with respect to simplicity of the procedure and reliability of the analytical result. In 1978, Van der Wal and Huber reported the HPLC separation of estrogen conjugates employing the authentic samples [7]. In the previous paper we also described the separation of monoglucuronides of estrone, estradiol, estriol and 16-epiestriol on a reversed-phase column by HPLC [8]. This present paper deals with a method for the simultaneous determination of estriol 16-glucuronide and 17-glucuronide in biological fluids by HPLC with electrochemical detection and its application for rat bile [9] and human pregnancy urine.

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EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a Toyo Soda HLC-803A high-performance liquid chromatograph (Toyo Soda Co., Tokyo, Japan) equipped with a Model SF-770 ultraviolet (UV) detector monitoring the absorbance at 280 nm and a Yanagimoto Model VMD 101 electrochemical detector (ECD) (Yanagimoto Co., Kyoto, Japan). The potential of the ECD was set at +1.0 V vs. a Ag/AgCl reference electrode. A TSK GEL LS-410 ODS-SIL (5 μm) (Toyo Soda Co.) column (30 cm × 0.4 cm I.D.) was employed under ambient conditions. The pH of the mobile phase was adjusted using phosphoric acid.

Materials

Estrogen glucuronides were synthesized in these laboratories by known methods [10]. All the reagents used were of analytical reagent grade. Solvents were purified by distillation prior to use.

Bile samples from rats

Male Wistar rats weighing ca. 200 g were used. Rats were anestherized with ether, cannulated to the bile duct with polyethylene tube (PE 10) (Clay Adams, Parsippany, NJ, U.S.A.) by surgical operation and housed in a Bollman cage for collection of bile. All animals were starved overnight prior to administration of estriol. A suspension of estriol (50 mg) in dimethylsulfoxide (0.1 ml) with saline (0.7 ml) and Tween 80 (0.2 ml) was given orally to each rat, and bile was collected every 2 h over a period of 26 h following administration [9].

Urine samples from human pregnancy

Twenty-four-hour urine samples were collected without preservative from pregnant women (30, 34 and 38 weeks of gestation). Each aliquot was taken and stored at −20°C until analysis.

Procedure for determination of conjugated metabolites in biological fluids

To an aliquot of bile or urine sample were added 5 μg of 16-epiestriol 17-glucuronide as internal standard, and the solution was percolated through a column (5 cm × 0.6 cm I.D.) packed with Amberlite XAD-2 resin. After thorough washing with distilled water (5 ml), the conjugate fraction was eluted with 5 ml of methanol (pH 10.0 adjusted by NH_4OH). Evaporation of the solvent under reduced pressure gave a residue which in turn was redissolved in methanol and subjected to a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column (2 cm × 0.6 cm I.D.) [11]. The column was washed with 90% methanol (5 ml) and the estrogen conjugate fraction was then eluted with 5 ml of 2.5% ammonium carbonate in 70% methanol. After evaporation of the solvent under reduced pressure below 45°C, the residue obtained was dissolved in methanol of which an aliquot was subjected to HPLC.

Recovery test for estriol monoglucuronides

One milliliter of control bile or non-pregnancy urine was spiked with known

amounts of estriol 16-glucuronide and 17-glucuronide. The assay was then carried out according to the procedure described above.

Enzymatic hydrolysis of estriol monoglucuronides

The eluate corresponding to the peak on the chromatogram was dissolved in 0.1 *M* acetate buffer (pH 5.4) and incubated with the acetone powder of snail digestive juice at 37°C overnight.

RESULTS AND DISCUSSION

Initially, our effort was directed to the determination of estriol 16-glucuronide and 17-glucuronide in rat bile by using a UV detector. However, numerous interfering peaks appeared on the chromatogram even when several clean-up procedures were carried out (see Fig. 1b). In order to overcome this problem the use of ECD, which is responsive selectively for electrochemically active materials, was undertaken [12]. One-tenth or one-twentieth of a milliliter of rat bile was subjected to chromatography on Amberlite XAD-2 resin, followed by ion-exchange chromatography on PHP-LH-20. As illustrated in Fig. 1a, no interfering peaks were observed on the chromatogram when ECD was used.

Known amounts of estriol 16-glucuronide and 17-glucuronide were added to control bile samples, and their recovery rates were then determined. As listed in

TABLE I

RECOVERY OF ESTRIOL MONOGLUCURONIDES ADDED TO RAT BILE

Amount added (μg/ml)	Percentage recovery (mean ± S.D., n = 5)	
	Estriol 16-glucuronide	Estriol 17-glucuronide
20	78.6 ± 1.6	78.6 ± 2.1
40	78.8 ± 2.1	79.0 ± 2.4
80	81.0 ± 1.6	81.4 ± 1.2

TABLE II

ACCURACY AND PRECISION OF THE PROPOSED METHOD FOR DETERMINATION OF ESTRIOL MONOGLUCURONIDES IN RAT BILE

Amount added (μg/ml)	Amount found (μg/ml) (mean ± S.D., n = 10)	
	Estriol 16-glucuronide	Estriol 17-glucuronide
20	20.0 ± 0.5	20.0 ± 0.3
100	102.4 ± 3.1	100.5 ± 3.3

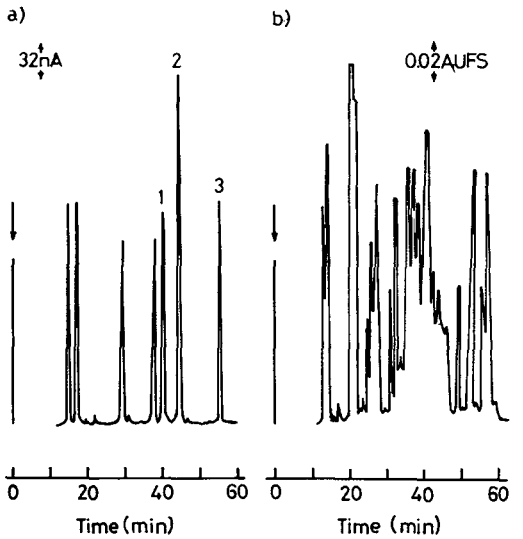

Fig. 1. Separation of estriol monoglucuronides in rat bile by HPLC. 1 = estriol 17-glucuronide, 2 = estriol 16-glucuronide, 3 = internal standard. Conditions: column, TSK GEL LS-410 ODS-SIL; mobile phase, 0.7% Na_2HPO_4 (pH 3.0)—tetrahydrofuran (6:1), 1 ml/min; detection, (a) electrochemical detector, (b) UV detector at 280 nm.

Table I, the two compounds spiked to rat bile at three levels were recovered at a rate of more than 78%. Estriol monoglucuronides added to non-pregnancy urine were similarly recovered at a satisfactory rate. 16-Epiestriol 17-glucuronide was chosen as internal standard for the determination because it was absent in the bile of rats administered estriol. The recovery rate of the internal standard was determined to be 71.4 ± 2.1%. A calibration graph was constructed by plotting the ratio of the peak height of estriol 16-glucuronide or 17-glucuronide to that of the internal standard, dissolved in control bile or non-pregnancy urine, against the amount of the glucuronide, a linear response to each glucuronide being observed in the range 0—10 μg. It is evident from the data in Table II that the proposed method was satisfactory in accuracy and precision. The detection limit of these compounds was 5 ng per injection (signal-to-noise ratio = 2 at 2 nA full scale). A typical chromatogram illustrated in Fig. 1a demonstrates unequivocally the excretion of the two isomeric estriol monoglucuronides in rat bile.

Identification of the two peaks corresponding to estriol 16-glucuronide and 17-glucuronide was then carried out. These compounds showed chromatographic behaviors identical with those of the respective authentic samples, along with the change in pH of the mobile phase [8]. Upon methylation with diazomethane, the peaks of estriol monoglucuronides disappeared from the chromatogram. In addition, when treated with β-glucuronidase from snail digestive juice, both peaks were changed into a peak corresponding to estriol.

The excreted amounts of estriol 16-glucuronide and 17-glucuronide in rat bile following oral administration of estriol were determined. As shown in Fig. 2, the concentration of both conjugated metabolites in bile reached the maximum value at 22 h after administration.

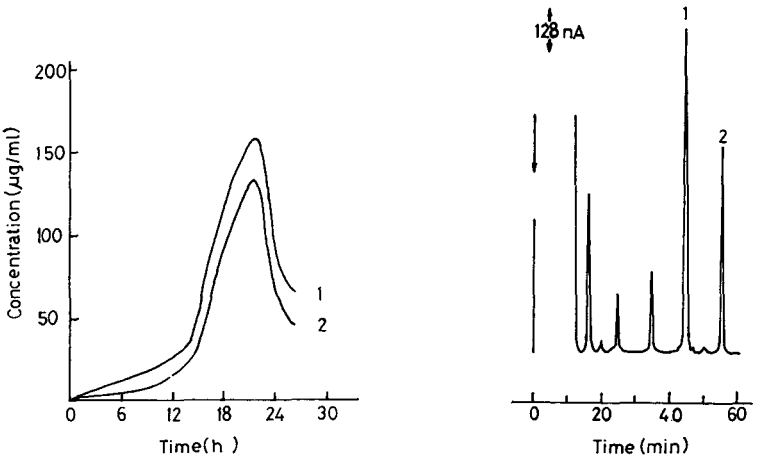

Fig. 2. Biliary excretion of estriol monoglucuronides in the rat after oral administration of estriol. 1 = estriol 16-glucuronide, 2 = estriol 17-glucuronide.

Fig. 3. Separation of estriol 16-glucuronide in pregnancy urine by HPLC. 1 = estriol 16-glucuronide, 2 = internal standard. Conditions as in Fig. 1a.

The application of the present method for the analysis of estriol monoglucuronides in pregnancy urine was undertaken. One-tenth of a milliliter of urine specimen was treated in a similar fashion as that described for rat bile. A typical chromatogram is shown in Fig. 3. Only one peak corresponding to estriol 16-glucuronide was observed on the chromatogram and its structure was unequivocally characterized in the manner described above.

The separation of isomeric monoglucuronides of estriol and 16-epiestriol has been previously attained on a reversed-phase column using an acidic mobile phase [8]. It has been demonstrated that ECD is much superior in selectivity and sensitivity than the UV detector for the determination of estrogen monoglucuronides in biological fluids. The excretion of estriol 17-glucuronide in pregnancy urine has been ambiguous since the reports by Hashimoto and Neeman [13] and Carpenter and Kellie [14]. The present study has revealed that both estriol 16-glucuronide and 17-glucuronide are excreted in rat bile while only the former is present in human pregnancy urine. The multiplicity of glucuronyltransferase, which catalyzes the formation of estrogen glucuronides, appears to be of interest.

The application of the present method to the quantitation of other estrogen conjugates in pregnancy urine and blood is being conducted in these laboratories and the details will be reported elsewhere in the near future.

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RAPID SEPARATION OF GANGLIOSIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SAMAR K. KUNDU* and DEBORAH DUNN SCOTT

Department of Medicine, Baylor College of Medicine, 1200 Moursund Avenue, Houston, TX 77030 (U.S.A.)

(Received March 17th, 1982)

SUMMARY

We have developed a high-performance liquid chromatographic (HPLC) procedure for the rapid separation of individual ganglioside components on a 5-μm porous silica gel column using a mixture of isopropanol—hexane—water with increasing water content and decreasing hexane content. Total ganglioside mixtures were first fractionated on DEAE-silica gel according to the number of sialic acid residues. Each fraction was then separated into individual ganglioside components by HPLC. Total elution time was less than 2 h. This procedure has been applied for the separation of major ganglioside components of human erythrocytes and beef brain and is highly reproducible.

INTRODUCTION

In recent years, high-performance liquid chromatography (HPLC) has been used as an analytical tool for the quantitation of glycolipids from animal tissues or cells [1—11]. All these procedures involved derivatization to introduce an aromatic chromophore for detection in the standard ultraviolet detector system. Unfortunately, it has not been possible to regenerate the native glycolipids from derivatized components. Thus the available methods could not be utilized for isolation of intact glycolipids. Gangliosides have been separated without derivatization using HPLC with a chloroform- methanol—hydrochloric acid system, equipped with moving-wire flame ionization detector [12]. Due to the non-availability of this type of the detector system and the danger of using hydrochloric acid to isolate labile gangliosides, this solvent system does not seem to be appropriate. Recently, Watanabe and Arao [13] have used a mixture of isopropanol—hexane—water as a solvent system and were able to separate underivatized neutral glycosphingolipids (GSLs) from

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human erythrocytes. In this present paper, we report a HPLC procedure for purification of underivatized ganglioside components from human erhythrocytes and beef brain using isopropanol—hexane—water as a solvent system as described [13]. The procedure is highly reproducible and the separation can be achieved in a much shorter time in comparison to the conventional column chromatographic procedures [14—20].

EXPERIMENTAL

The gangliosides from human erythrocytes and beef brain were isolated as described previously [21, 22]. The erythrocyte ganglioside mixtures were further fractionated on DEAE-silica gel into mono and disialo species [19]. The beef brain ganglioside mixtures were similarly separated into mono-, di-, tri-

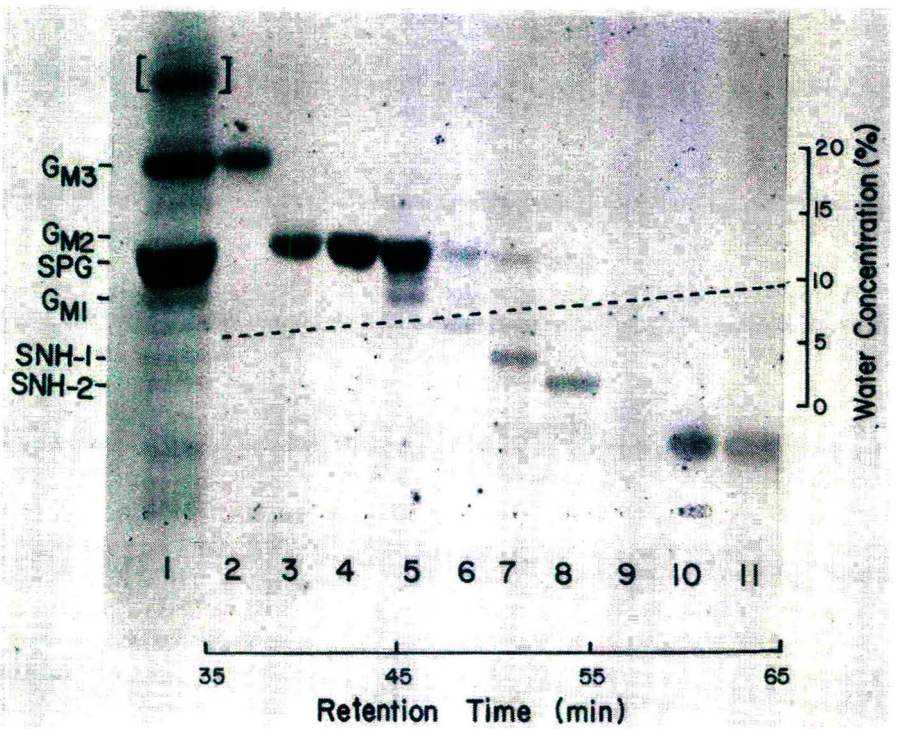

Fig. 1. Elution profile of the monosialoganglioside fraction of human erythrocytes separated by HPLC as monitored by TLC. HPLC column: Zorbax SIL (5 μm, 25 cm × 4.6 mm); sample applied, 500 μg. The column was eluted with a linear gradient of a mixture of isopropanol—hexane—water from a ratio of 55:42:3 to 55:25:20 during a period of 2 h. Fractions of 1 ml of effluent were collected and aliquots of 200 μl were spotted after evaporation and redissolving in 20 μl of chloroform—methanol—2.5 *N* ammonium hydroxide (50:50:10) on a precoated silica gel 60 plate. Lane 1 contains total monosialoganglioside fraction and lanes 2—11 contain eluted fractions. Solvent system: chloroform—methanol—water (55:45:10) containing 0.02% (w/v) $CaCl_2 \cdot 2H_2O$. All bands were purple after detection by resorcinol spray [26]. The faint band in lane 1 above G_{M_3} contained non-ganglioside impurities indicated by a bracket and was yellow in color. The dotted line indicates the concentration of water in the elution mixture. Faint ganglioside bands eluting in water concentrations between 10—15% were visible (data not shown).

and tetrasialo species [19]. For purification of individual ganglioside components of human erythrocytes by HPLC, mono- and disialoganglioside fractions were separately used. Similarly, for purification of individual ganglioside components of beef brain, mono-, di-, and trisialoganglioside fractions were separately employed. Total beef brain ganglioside mixtures were also used for purification of individual ganglioside components by HPLC. Standard gangliosides used in this study have been described previously [23, 24].

High-performance liquid chromatography

The apparatus included Altex Model 100 A pumps and a Model 420 microprocessor (Altex Scientific, Berkeley, CA, U.S.A.). A Zorbax Sil column (5 μm, 25 cm × 4.6 mm I.D., DuPont, Wilmington, DE, U.S.A.) was used and separation was carried out by programmed gradient elution. A linear gradient of isopropanol—hexane—water from 55:42:3 (solvent A) to 55:25:20 (solvent B) was used during a period of 2 h. The column was equilibrated with solvent A prior to injection of sample and after use the column was regenerated with the initial solvent for 15 min. The flow-rate was adjusted to 0.5 ml/min and the

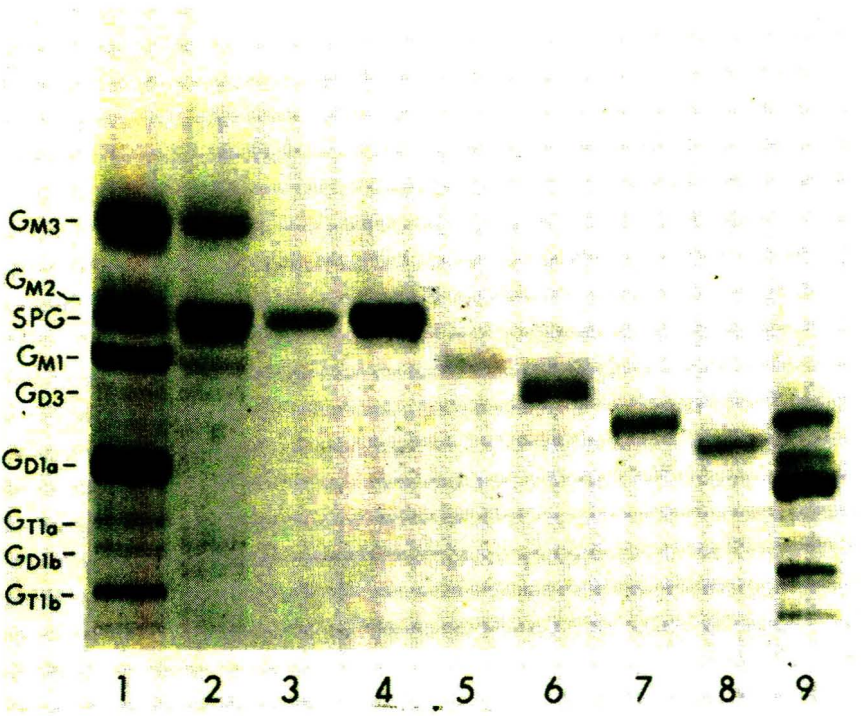

Fig. 2. TLC of purified monosialogangliosides of human erythrocytes separated by HPLC. Lane 1 contains standard mixtures of G_{M3}, G_{M2}, SPG and beef brain gangliosides. Lane 2 contains total monosialoganglioside fraction. Lane 9 contains total disialoganglioside fraction of human erythrocytes. Lanes 3—8 are purified monosialogangliosides of human erythrocytes. Lane 3 was identified as G_{M2}; lane 4 as SPG; lane 5 as G_{M1}; lane 7 as SNH-1 containing C_{22} and C_{24} fatty acid and lane 8 as SNH-2 containing C_{16} fatty acid [27]; lane 6 was not identified. Solvent system and spray reagent as in Fig. 1.

ganglioside sample (up to 500 μg) dissolved in 50 μl of isopropanol—hexane—water (55:30:15) by mild sonication was injected and gradient elution started. Fractions of 1 ml were collected by a fraction collector. The elution profile of gangliosides was monitored by thin-layer chromatography (TLC).

Thin-layer chromatography

TLC was performed on precoated silica gel 60 plates (E. Merck, Darmstadt, G.F.R.) with chloroform—methanol—water (55:45:10) containing 0.02% (w/v) $CaCl_2 \cdot 2H_2O$ (solvent 1) or chloroform—methanol—2.5 *N* ammonium hydroxide (60:40:9) (solvent 2) as solvent systems [25]. Gangliosides were revealed by spraying the plate with resorcinol reagent [26] and heating the plate at 100° C for 10 min. Fractions were pooled after TLC examination.

RESULTS*

The elution profile of the monosialoganglioside fraction from human erythrocytes by the HPLC procedure is presented in Fig. 1. The elution was complete in approximately 1 h and gangliosides were eluted in a water concentration of between 5 and 9%. The three gangliosides viz. G_{M3} (lane 2), G_{M2} (lane 3) and sialosylparagloboside (SPG, lane 4) that comprise more than 90% of the monosialoganglioside fraction were well separated. For the isolation of other minor ganglioside components, the partially purified fractions in lanes 5, 6, 7 and 8 were separately reloaded on the HPLC column and eluted exactly under identical conditions as described for the total mixture but fractions of 250 μl of eluate were collected. After TLC examination, the pure fractions were pooled. Fig. 2 shows a thin-layer chromatogram of purified monosialogangliosides from human erhythrocytes (G_{M3} not shown). The purity of each ganglioside component was established by running duplicate TLC plates in solvent systems 1 and 2.

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*Abbreviations of the ganglio series gangliosides are according to the nomenclature of Svennerholm [29]. G_{M_3} = NeuAc(α2-3)Gal(β1-4)Glc-Cer; G_{M_2} = GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; G_{M_1} = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; G_{D_3} = NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc-Cer; $G_{D_{1a}}$ = NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; $G_{D_{1b}}$ = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(1-4)Glc-Cer; $G_{T_{1a}}$ = NeuAc(α2-8)NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; $G_{T_{1b}}$ = NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer; SPG (sialosylparagloboside) = NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)-Gal(β1-4)Glc-Cer; DPG (disialosylparagloboside) = NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc-NAc(β1-3)Gal(β1-4)Glc-Cer; SNH (sialosylnorhexaosylceramide) = NeuAc(α2-3)Gal(β1-4)Glc-NAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer; DPGC (disialosylpentaglycosylceramide) = $\begin{matrix}\text{NeuAc}(\alpha 2\text{—}6)\\ \text{NeuAc}(\alpha 2\text{—}3)\end{matrix}$Gal($\beta$1-3)GalNAc(1-3)Gal(1-4)Gal(β1-4)Glc-Cer; DL10C (disialosyllacto-N-isooctaosylceramide) = $\begin{matrix}\text{NeuAc}(\alpha 2\text{-}3)\text{Gal}(\beta 1\text{-}4)\text{GlcNAc}(\beta 1\text{—}6)\\ \text{NeuAc}(\alpha 2\text{-}3)\text{Gal}(\beta 1\text{-}4)\text{GlcNAc}(\beta 1\text{—}3)\end{matrix}$Gal($\beta$1-4)GlcNAc($\beta$1-3)-Gal($\beta$1-4)Glc-Cer; where Gal = D-galactose; Glc = D-glucose; GalNAc = N-acetyl-D-galactosamine; GlcNac = N-Acetyl-D-glucosamine; NeuAc = N-acetylneuraminic acid; Cer(ceramide) = N-acylsphingosine.

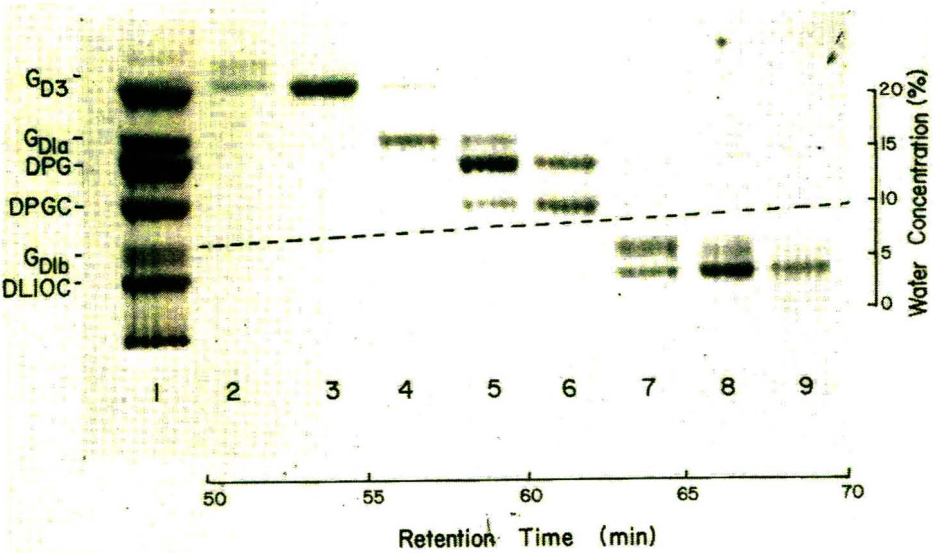

Fig. 3. Elution profile of the disialoganglioside fraction of human erythrocytes separated by HPLC as monitored by TLC. Solvent system: chloroform—methanol—2.5 *N* ammonium hydroxide (60:40:9). All bands were purple after detection by resorcinol spray. Lane 1 contains total disialoganglioside fraction and lanes 2—9 contain eluted fractions. Faint ganglioside bands eluting in water concentrations between 10—16% were visible (data not shown). Other details as in Fig. 1.

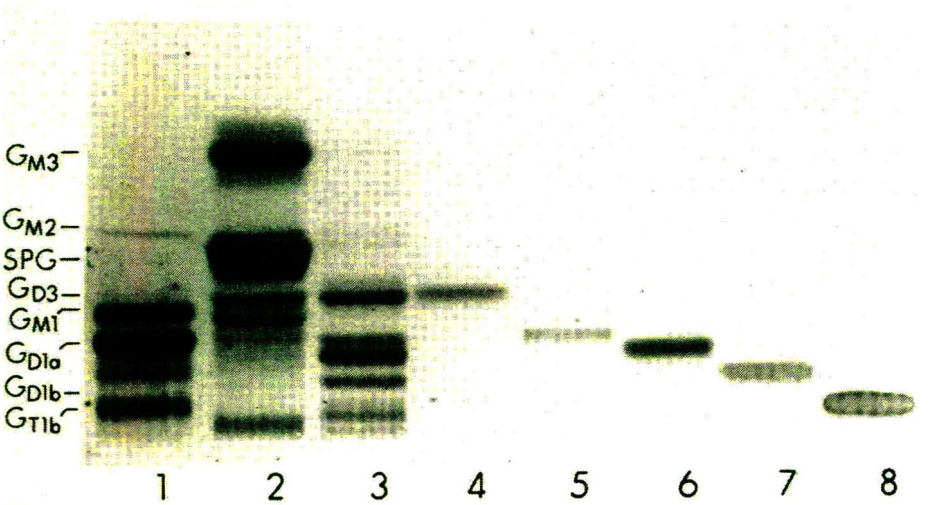

Fig. 4. TLC of purified disialogangliosides of human erythrocytes separated by HPLC. Lane 1 contain beef brain ganglioside mixture. Lane 2 contains monosialoganglioside fraction of human erythrocytes. Lane 3 contains disialoganglioside fraction of human erythrocytes. Lane 4—8 are purified disialogangliosides of human erythrocytes. Lane 4 was identified as G_{D_3}; lane 5 as $G_{D_{1a}}$; lane 6 as disialosylparagloboside; lane 7 was identified as a new ganglioside that has disialosylpentaglycosylceramide structure [28]. Ganglioside $G_{D_{1b}}$ which runs in between bands 7 and 8 is not shown. Solvent system and spray reagent as in Fig. 3.

The elution pattern of the disialoganglioside fraction from human erythrocytes which comprises about 15% of the total ganglioside [19, 22] is presented in Fig. 3. The majority of the gangliosides were eluted off the column in 1 h 15 min in a water concentration of between 7 and 10%. Ganglioside G_{D3} (lane 2, two bands and lane 3) was obtained in sufficient purity but for purification of other gangliosides, lanes 4, 5, 6, 7 and 8 were pooled together and reloaded on the HPLC column as described for monosialoganglioside fraction. The ganglioside fraction in lane 9 was separately reloaded as above. The disialogangliosides of human erythrocytes purified by HPLC procedure are presented in Fig. 4 (G_{D1b} not shown). The purity of each ganglioside was checked by running duplicate TLC plates in both solvent systems.

For HPLC purification of individual ganglioside components from beef brain, mono-, di- and trisialoganglioside fractions were employed. Excellent separation of G_{M3}, G_{M2} and G_{M1} gangliosides was achieved from the mono-

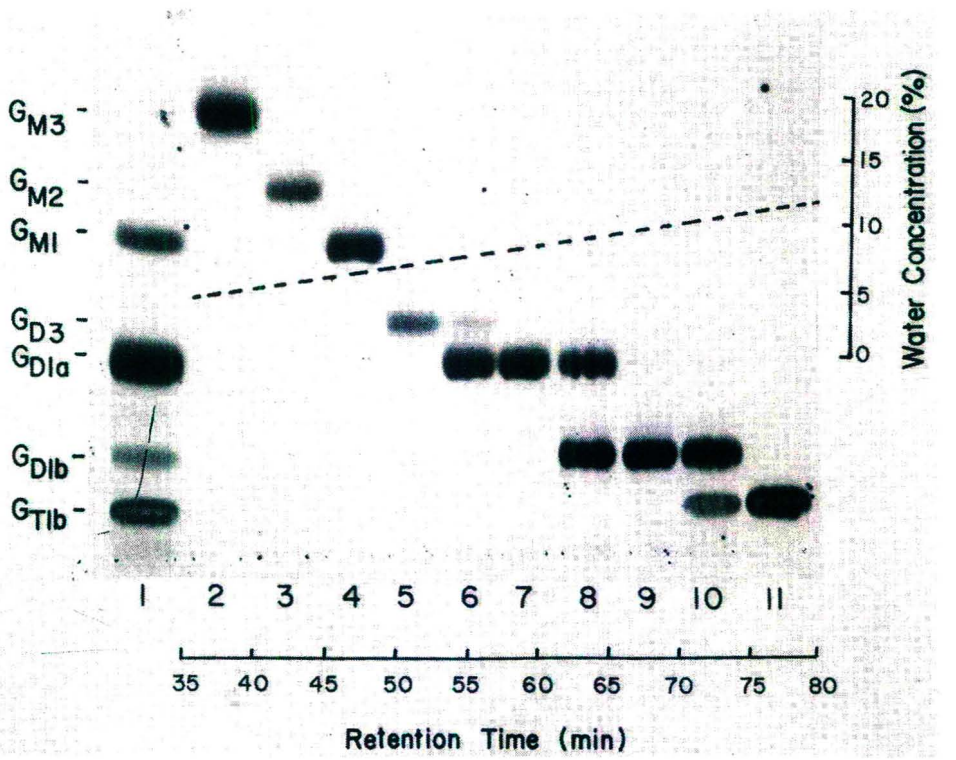

Fig. 5. Elution profile of the total beef brain ganglioside mixture separated by HPLC as monitored by TLC. Solvent system: chloroform—methanol—water (55:45:10) containing 0.02% (w/v) $CaCl_2 \cdot 2H_2O$. All bands were purple by resorcinol spray. Lane 1 contains total beef brain ganglioside mixture and lanes 2—11 contain eluted fractions. Faint ganglioside bands eluting in water concentrations of between 14—20% were visible (data not shown). Other details as in Fig. 1.

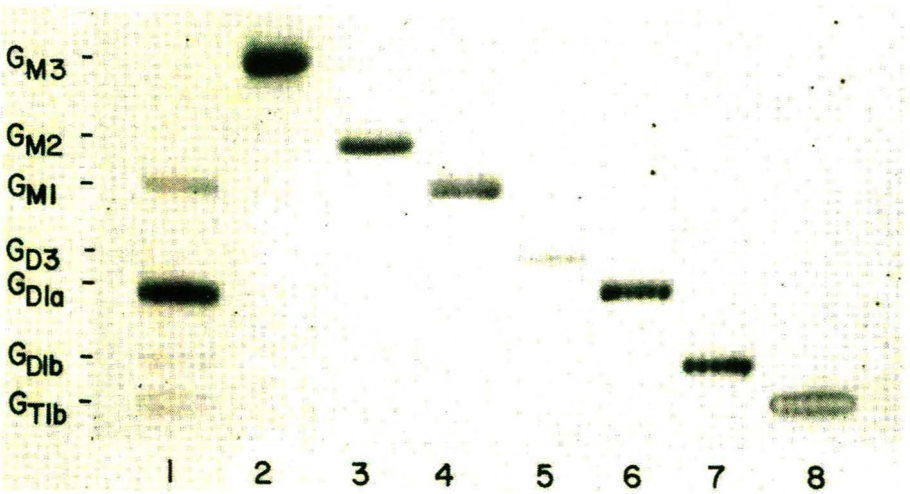

Fig. 6. TLC of purified gangliosides of beef brain separated by HPLC. Lane 1 contains total beef brain ganglioside mixture. Lanes 2—8 are purified gangliosides of beef brain. Lane 2 was identified as G_{M_3}; lane 3 as G_{M_2}; lane 4 as G_{M_1}; lane 5 as G_{D_3}; lane 6 as $G_{D_{1a}}$; lane 7 as $G_{D_{1b}}$ and lane 8 as $G_{T_{1b}}$. Attempts were not made to isolate other minor gangliosides. Solvent system and spray ragent as in Fig. 5.

sialoganglioside fraction. The elution was complete in 1 h and gangliosides were eluted in a water concentration of between 5 and 7.6%. From the disialoganglioside fraction, G_{D3}, G_{D1a} and G_{D1b} were well separated in a water concentration of between 10 and 11.5%. The major gangliosides of beef brain were also purified by the HPLC procedure with the total ganglioside mixture. The elution profile of the gangliosides from total beef brain ganglioside mixture is shown in Fig. 5. The elution was complete within 1 h 30 min and gangliosides were eluted in a water concentration of between 5 and 12%. Excellent separation of G_{M3} (lane 2), G_{M2} (lane 3), G_{M1} (lane 4) and G_{D3} (lane 5) gangliosides was achieved. The other major gangliosides viz. G_{D1a} (lane 7), G_{D1b} (lane 9) and G_{T1b} (lane 11) were also obtained in pure forms although the gangliosides in lanes 6, 8 and 10 were not homogeneous. These fractions were separated into pure components by reloading the partially purified fractions on to the HPLC column and eluting under identical conditions as described for the total mixture but fractions of 250 μl were collected. The gangliosides purified from total beef brain ganglioside mixture are shown in Fig. 6. The purity of each ganglioside component was established by running duplicate TLC plates in both solvent systems.

DISCUSSION

For the purification of gangliosides, silica gel column chromatographic procedures have been most frequently used [14—20]. Because of the complexity of these compounds, gradient elution and repeated chromatographic separation are often needed to purify the ganglioside components. Preparative TLC procedures commonly used in many cases [15], are not feasible for larger

amounts of gangliosides. A major disadvantage of the preparative TLC procedure is due to the poor recovery of the gangliosides from the silica gel scrapings. In recent years, HPLC procedures which utilize silica gel of controlled pore diameter have been found to be extremely useful for the quantitative separation of derivatized (aromatic chromophore) neutral GSLs [1—11]. More recently, Watanabe and Arao [13] have been able to separate underivatized neutral GSLs containing mono to dodecasaccharides on HPLC using a mixture of isopropanol—hexane—water as an eluting solvent. An advantage of this solvent system is its non-toxicity and better resolution power compared to the conventionally employed chloroform—methanol—water system. We have shown in this report that complex gangliosides from human erythrocytes and beef brain can be separated in pure forms using HPLC on a silica gel column and similar solvent mixtures as was employed by Watanabe and Arao [13]. The separation pattern of ganglioside components is highly reproducible because the gradient elution solvent could be strictly controlled with the HPLC system. Moreover, the gangliosides could be separated in a matter of hours compared to a few days in conventional column chromatography. We have used from 50—500 μg of gangliosides in our system and have been able to reproduce the results. We have been able to regenerate the same column without loss of any resolving power even after 50 applications. We plan to exploit this procedure on a much larger scale utilizing a preparative silica gel column. Therefore, this HPLC procedure provides a highly efficient method for purification of individual ganglioside components on micro or macro scale in many other tisues or cells.

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DETERMINATION OF NOREPINEPHRINE IN BRAIN PERFUSATES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

STEVEN K. SALZMAN* and MICHAEL S. SELLERS

Research Department, Alfred I. du Pont Institute of the Nemours Foundation, P.O. Box 269, Wilmington, DE 19899 (U.S.A.)

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SUMMARY

A procedure for determining catecholamine levels in brain perfusates is described. A single-step extraction on alumina followed by reversed-phase ion-pair chromatography and electrochemical detection was employed. Levels of norepinephrine are reported in push-pull perfusates of the rat hypothalamus under baseline and various ion substitution conditions. The resulting estimated norepinephrine release values are correlated with the behavior of the animal. The data are discussed in terms of the validity of the measures and the noradrenergic mediation of behavior.

INTRODUCTION

The catecholamines, norepinephrine, epinephrine and dopamine, are of considerable importance in clinical as well as basic neurobiological research [1]. The development of sensitive assays for these compounds and their metabolites [2] has led to renewed interest in their role in neurological diseases [3], sympathetic neuronal function, and development [4, 5], and in the transmission and modulation of central neural information [6]. We were interested in adapting a single assay technique for the catecholamines in a variety of tissues and physiological fluids. Of the available methods, high-performance liquid chromatography (HPLC) with electrochemical detection (ElCD) is the method of choice because of its resolution, selectivity, sensitivity and simplicity [7].

Our interest in the function of catecholamines in the central nervous system has led us to develop a highly specific and sensitive assay for norepinephrine, epinephrine and dopamine in brain perfusates. Other HPLC—ElCD methods have been successfully applied to the measurement of catecholamines in brain

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perfusates without a pre-purification step [8]. The procedure presented here utilizes a well-documented single-step extraction procedure [9] with an improved chromatographic step resulting in a relatively high capacity factor for norepinephrine as well as the resolution required for determination of the catecholamines in a variety of samples. We have been able to successfully apply this method to brain perfusates, spinal fluid and plasma. In this report, we present the data obtained from the measurement of norepinephrine in push-pull perfusates from the medial preoptic/anterior hypothalamus (POAH) of the rat. Data on spinal fluid and plasma catecholamines will be presented elsewhere.

EXPERIMENTAL

Reagents

Norepinephrine (NE), epinephrine (E) and dopamine (DA) (Sigma, St. Louis, MO, U.S.A.), 3,4-dihydroxybenzylamine (DHBA) (Aldrich, St. Regis, WI, U.S.A.), and sodium octyl sulfate (Eastman, Rochester, NY, U.S.A.) were used. Acid-washed activated alumina was purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.) but was customarily re-activated according to the method of Anton and Sayre [9]. All other chemicals were of reagent grade and all solutions were prepared from HPLC-grade water obtained from Fisher (Fairlawn, NJ, U.S.A.). All solutions were filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) before use.

Stock solutions of catecholamines and DHBA, 1 mg/ml in 0.1 *M* perchloric acid containing 1.3 m*M* EDTA and 7.9 m*M* sodium metabisulfite, were prepared bimonthly and stored at 4°C. Working standards were prepared daily by making appropriate dilutions of the stock solutions in perchloric acid. Solutions of 1.5 *M* Tris, (pH 8.6) and 0.1 *M* phosphate buffer, pH 7.4, both containing 52 m*M* EDTA, were prepared monthly.

In vivo brain perfusates

Fifteen rats were implanted with push-pull cannulae constructed according to the method of Sparber [10] in the right medial POAH. After 1 week post-operative recovery and habituation to the perfusion chamber, experiments were begun. Rats were perfused with filtered Krebs—bicarbonate solution, pH 7.4, aerated with oxygen—carbon dioxide (95:5) at a constant flow-rate of 20.6 μl/min by means of a Harvard syringe pump for a 30-min washout period followed by a 1-h baseline period. Solutions with altered ion concentrations (0.75 m*M* cobalt replacing calcium in five rats or 30 m*M* potassium replacing equimolar amounts of sodium in three rats) were then switched into the push flow remotely through 3-way valves in eight rats, while in seven others, a second hour of baseline measures were taken. In all rats, an attempt was made to correlate behavior with NE release. Their behavior was classified on a minute-by-minute basis by observation as waking, waking-with-movement (includes locomotion and grooming) and sleeping. Samples were collected in 20-min intervals by means of 3-way valves into tubes on ice containing 600 μl 0.1 *M* perchloric acid. The perchloric acid was partially replaced by 412 μl of perfusate in each 20-min interval. The perfusate took exactly 9.5 min to reach

the collection tube from the brain. Thus, no pharmacological measures were employed to limit NE oxidation. While some auto- and/or enzymatic oxidation of NE probably took place, we were more interested in maintaining a preparation free of artificial pharmacological intervention. After collection, the sample was stored at −80°C until analysis. Cannula placements were verified histologically; only those placements in the medial preoptic/anterior hypothalamus are included in the data presented here.

Extraction of catechols

Brain perfusate (0.6 ml) and 4 ng DHBA in 20 μl 0.1 *M* perchloric acid were added to a 5-ml reaction vial containing 50 mg acid washed alumina (baked at 180°C for 3 h prior to use). A 0.5-ml aliquot of 1.5 *M* Tris, pH 8.6 was added and the vial quickly capped, vortexed briefly, and shaken for 5 min on a reciprocal shaker.

The liquid in the vials was then aspirated and the alumina then washed with 0.5—1.0 ml of water, which was aspirated again. The alumina was then transferred as a water slurry in a Pasteur pipet to a centrifugal microfilter (Bioanalytical Systems) containing a 0.2-μm filter. The alumina was dried by centrifugation at 1000 *g* for 1 min.

The catechol compounds were then eluted by adding 100 μl of 0.1 *M* perchloric acid and vortexing for 1 min. This slurry was then centrifuged at 100 *g* for 1 min yielding the final injectate.

Chromatography

A Hewlett-Packard 1084B system was used (Hewlett-Packard, King of Prussia, PA, U.S.A.); it included two reciprocating diaphragm pumps, a continuously adjustable injection system, a Supelco LC-18 column (5 μm, C_{18}-bonded silica, 250 × 4.6 mm I.D.) and precolumn (50 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) maintained at a constant temperature of 30°C, and a Bioanalytical Systems TL5 glassy carbon or TL3 oil-based carbon paste electrode and LC4 amperometric controller. Chromatograms were recorded on both the Hewlett-Packard graphic-integrator and a Houston Omniscribe potentiometric recorder (Houston Instruments, Austin, TX, U.S.A.).

The mobile phase was composed of 14.5 m*M* citric acid, 71.0 m*M* dibasic sodium phosphate, 1.3 m*M* EDTA and $1.7 \cdot 10^{-4}$ *M* sodium octyl sulfate (pH 6.5, using 2 *M* sodium hydroxide) and was pumped at a flow-rate of 1.0 ml/min. The mobile phase was filtered through a 0.22-μm filter and degassed in vacuo and by helium purging. The working electrode potential was + 0.9 V vs. a Ag/AgCl reference electrode. An 85-μl aliquot of the final 100-μl alumina extract was injected onto the column.

Quantitation of catecholamine concentrations

The concentration of NE in each sample, was calculated by determining its peak height ratio relative to DHBA and comparing this ratio to that obtained with synthetic standards prepared in Krebs—bicarbonate solution, pH 7.4 (the perfusion medium). These synthetic standards were prepared in three different concentrations in the range of expected sample values and carried through the same procedure as the samples. In addition, the relative recovery of catechol-

amines was determined using perfusate pools with known amounts of standards added.

RESULTS

Sample chromatograms

Fig. 1 illustrates chromatograms of a synthetic standard and a 0.6-ml brain perfusate sample taken during perfusion with 30 m*M* potassium solution. All samples were spiked with 4 ng of DHBA. Each run was done at a temperature of 30°C in order to obtain the maximum capacity factor for NE. Higher temperatures can be used for DA determinations but will decrease the retention of NE.

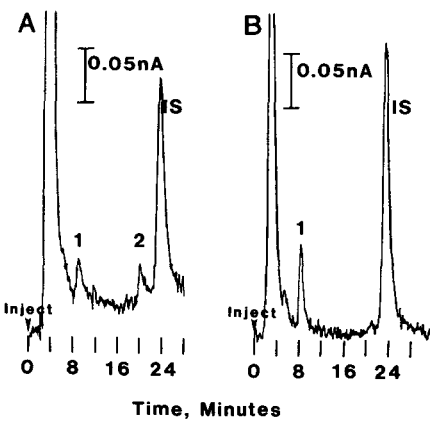

Fig. 1. Sample chromatograms obtained by the present HPLC—ElCD method. (A) Synthetic standard composed of 1 ml Krebs—bicarbonate buffer with 60 pg NE, 100 pg E and 4 ng DHBA (internal standard) added prior to alumina extraction; (B) 0.6 ml rat brain perfusate sample taken during perfusion with normal Krebs—bicarbonate solution and containing 215.8 pg/ml NE, corresponding to 129.5 pg NE released during that 20-min period. The rat was exhibiting waking-without-movement during the period corresponding to this release. A column temperature of 30°C was employed for each run. The glassy carbon electrode, at +0.9 V vs. Ag/AgCl, was used in each case. Peaks: 1 = NE; 2 = E; IS = DHBA (internal standard).

Linear range and limits of detection

The linear range of the method for perfusates was determined by adding various amounts of the catecholamines to 1-ml aliquots of a perfusate pool. The assay was linear in the range 60 pg/ml—20 ng/ml for NE, 100 pg/ml—20 ng/ml for E, and 80 pg/ml—10 ng/ml for DA. The limit of detection in all cases was determined at a minimum signal-to-noise ratio of 3:1. Since the minimum amplitude of the noise encountered was 0.01 nA, a signal of 0.03 nA corresponded to the minimum detectable amount injected which was typically 30 pg of NE, 45 pg of E and 39 pg of DA.

Precision

Repeated determinations (n = 20) of NE, E and DA in pooled 1-ml perfusate

samples gave the following coefficients of variation (C.V.): NE, C.V. = 3.2% at a concentration of 333 pg/ml; E, C.V. = 4.4% at a concentration of 167 pg/ml; DA, C.V. = 5.2% at a concentration of 80 pg/ml. The relative recovery of catecholamines from pooled perfusates relative to the synthetic Krebs—bicarbonate standard was 92.1% based on sixteen determinations.

Capacity factor for NE

The capacity factor (k'), calculated as $(t_R - t_0)/t_0$, where t_R and t_0 are the analyte and void volume retention times, respectively, varied between 2.80 and 3.50 for NE, depending on the age of the column. Thus, as can be seen in Fig. 1, at a column temperature of 30°C, NE is completely resolved from the elution front, allowing for accurate quantitation.

Interferences

The following compounds were found not to interfere with the assay: α-methyl-DA, α-methyl-NE, *l*-dopa, dihydroxyphenylacetic acid, 4-hydroxy-3-methoxy-phenylglycol, normetanephrine, ascorbic acid and uric acid. A contaminant, present on the alumina that is eluted with perchloric acid, has been seen occasionally. This unknown component has a retention time near NE in this system. Although it has not been a problem in our studies, it could possibly interfere with the determination of NE if it is present in large quantities and if NE is near the detection limit. We have found that the most satisfactory solution to this problem has been to pre-elute the alumina several times with 100 μl of perchloric acid and to run reagent blanks each day.

Experimental applications

Fig. 2 shows the effects of perfusion with solutions of altered ion con-

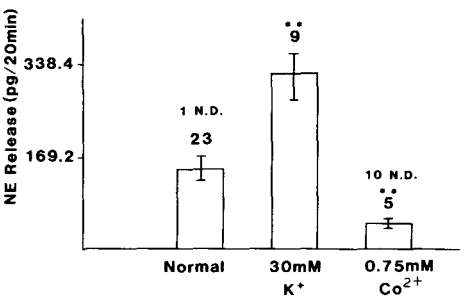

Fig. 2. Estimation of the ion dependent in vivo release of rat hypothalamic NE in pg/20 min ± S.E.M. using the present HPLC—ElCD method. Rats were perfused with normal Krebs—bicarbonate solution at a rate of 20.6 μl/min for 60 min, followed by a Krebs solution containing elevated levels of potassium (30 m*M* K^+) replacing sodium, or with a Krebs solution containing cobalt (0.75 m*M* Co^{2+}) to replace calcium for an additional 60 min. The addition of depolarizing concentrations of K^+ significantly elevated NE release as would be expected if NE release was truly being measured. Furthermore, the significant inhibition of spontaneous NE release due to the elimination of calcium, indicates that this release is calcium sensitive. The numbers above the standard error bars indicate the number of determinations where detectable levels of NE were found. The numbers above these indicate the number of samples in which NE could not be detected. ** = $P < 0.01$ by two-tailed *t*-test, vs. normal.

stituents on hypothalamic NE release in eight rats. The baseline values are composed of sixteen samples in which the rats exhibited waking-without-movement, seven samples in which there was waking-with-movement, and one sample during waking-without-movement in which the levels of NE were below the detection limit. The resulting average NE release value is thus at a level associated with waking-without-movement (Fig. 3). In three rats, a perfusate with elevated (30 m*M*) potassium concentration replacing sodium was then switched into the push flow for 1 h. An increase in locomotor activity, and in some cases, vigorous grooming, accompanied the potassium elevation in all rats and persisted for the entire hour. The level of NE release was at a value associated with waking-with-movement (Fig. 3) and was significantly different from baseline ($P < 0.01$). In the remaining five rats, an alternate perfusate containing 0.75 m*M* $CoCl_2$ replacing $CaCl_2$ was perfused for 1 h. This perfusion was accompanied by behavioral sedation in all rats, but not sleep, and persisted for the entire hour. The average NE release value was at a level below that associated with behavioral sleep and in fact, could not be detected in ten of the fifteen samples collected. The average value of the five measurable samples was significantly lower than baseline ($P < 0.01$).

Fig. 3 shows the spontaneous release of NE from the medial POAH as correlated with the behavior (arousal state) of the rat. Neither E nor DA were consistently detected in these samples. The release of hypothalamic NE correlated with movement, as the waking-with-movement values were significant-

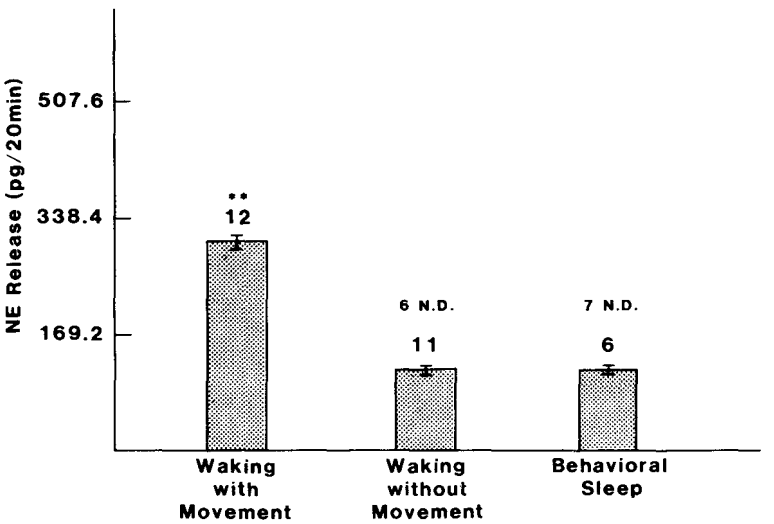

Fig. 3. Estimation of the spontaneous in vivo release of rat hypothalamic NE in pg/20 min ± S.E.M. as correlated with behavior using the present HPLC—ElCD method. Rats were perfused with normal Krebs—bicarbonate solution at a rate of 20.6 μl/min for 120 min. The rat's behavior was determined min-by-min and classified into the three categories shown (see text for details). The spontaneous release of NE was significantly greater during waking-with-movement than during waking-without-movement or behavioral sleep. The numbers above the standard error bars indicate the number of determinations where detectable levels of NE were found. The numbers above these indicate the number of samples in which NE could not be detected. ** = $P < 0.01$ by two-tailed *t*-test vs. waking-without-movement and behavioral sleep.

ly greater than either those during waking-without-movement ($P < 0.01$, two-tailed t-test) or behavioral sleep ($P < 0.01$). NE could not be detected in six waking-without-movement samples and seven behavioral-sleep samples. Since these values could not be included in the statistical measures, the aforementioned differences are biased towards less significance.

DISCUSSION

The chromatographic system employed in this study was found to be free of interferences from other acidic catechol species that may be co-extracted on alumina. Furthermore, the increased retention of NE at the high mobile phase pH employed (6.5), afforded a good separation of NE from uric acid; a constituent found in high concentrations in plasma [11] and most likely present as an extracellular constituent in brain. At this pH, protonation of acidic species is decreased, whereas protonation of basic catecholamines is unaffected. Thus, ion-pair formation of the acidic species is decreased resulting in a selective decrease in their capacity factors. An additional benefit of using a higher mobile phase pH is an improved detector response [12]. The high degree of resolution in this assay was achieved without excessive concentrations of the ion-pairing reagent, which decreases column life-time [13]. It was found that effective column life could be extended by monthly flushing with a 30% methanol in water mixture. Column viability could also be prolonged by lowering the column temperature a few degrees when retention times began decreasing.

The perfusion of solutions with altered ion concentrations yielded the results one would expect if, indeed, NE release (and not leakage) was being predominantly measured. Elevation of the extracellular concentration of potassium ion should cause neuronal depolarization and an enhancement of neurotransmitter release. By raising the concentration of potassium ion to 30 mM in our perfusing solution, we were able to more than double the amount of NE collected in the perfusate as compared to the amount of NE collected under normal ionic conditions. This indicates that the ability of the noradrenergic terminals, at the perfusion site, to respond to depolarizing stimuli was intact. Similarly, substitution of calcium ion by cobalt ion in the perfusing medium should depress neurotransmitter release, since extracellular calcium ion is an important concomitant to the release of neurotransmitter. By substituting 0.75 mM cobalt ion for calcium in our perfusions, we were able to lower the amount of NE collected in five perfusates to roughly one-third of that collected under normal ionic conditions. Furthermore, the levels of NE in ten perfusate samples were below the limits of detection (36 pg/20 min), whereas only 1 of 24 samples under normal ionic conditions had non-detectable levels of NE. These data indicate that at least two-thirds of the spontaneous release of NE measured under normal ionic conditions was calcium dependent. The remaining one-third, that is, the release of NE that persisted during cobalt perfusion, could be attributed to at least three factors. A percentage of the noradrenergic terminals being perfused could have been damaged, allowing NE to leak out. Alternatively, calcium ion was not completely eliminated, and the remaining calcium then, was sufficient to allow the level

of NE release that was measured during cobalt perfusion. Finally, it is possible that neurotransmitter release is not totally calcium dependent and that some physiological release of neurotransmitter can occur by alternative mechanisms [14]. The data from the ion substitution experiments indicate that the noradrenergic terminals under study, were, for the most part, functional and that measurement of spontaneous NE release by push-pull perfusion is a valid approach to the study of neurotransmission.

The alteration of the ionic composition of the perfusion medium not only resulted in changes in the release of NE in the POAH, but also in the behavior of the animals. Potassium-evoked NE release was accompanied by increased movement (locomotion and grooming) in all animals. The increase in movement persisted as long as extracellular concentrations of potassium were elevated. In no case was there a dissociation between elevated potassium concentrations, enhanced NE release and the increase in movement. Similarly, the attenuation of NE release by cobalt was accompanied by behavioral quieting, marked by absence of locomotion or grooming, in all animals. Once again, the behavioral and neurochemical effects persisted as long as cobalt was perfused. While these data indicate that the release of NE in the medial POAH of the rat is correlated with movement, it is possible that the ion substitutions affected one or more neurotransmitter systems in this brain area, which in turn mediated the behavioral effects. The experiments which examined the correlation of spontaneous NE release with the animals' behavior were performed in order to test the hypothesis that POAH NE release is correlated with movement. The spontaneous release of NE from the POAH, measured during the waking states, is in excellent agreement with previously reported values in the hypothalamus [15]. Furthermore, the spontaneous release of NE was significantly greater during waking-with-movement (locomotion and grooming) than during waking-without-movement or sleep. This is in general agreement with the findings of Van der Gugten and Slangen [15] who found that the spontaneous release of NE from anterolateral hypothalamic areas correlated with locomotion.

These data, indicate that POAH noradrenergic transmission is, at least in part, movement related. Whether the release of NE in the POAH actually mediates the movement, or is a consequence of it, is difficult to determine. Noradrenergic transmission in the POAH may mediate other physiological functions ascribed to this brain region. For instance, the POAH is important for the central integration of body temperature [16], and NE appears to be involved in this function [17]. Since increased motor activity is generally accompanied by increases in body temperature, it is possible that the increases in spontaneous NE release seen during movement, mediate any accompanying thermal changes that might occur. We are currently investigating this possibility.

The noradrenergic projections to the POAH originate from the ventral noradrenergic system [18, 19]. The ventral system has not been studied as extensively as the dorsal noradrenergic pathway originating from nucleus locus coeruleus. Recent evidence indicates that the activity of the dorsal pathway, is correlated with level of arousal, rather than movement [20]. Our data indicate, that the activity of the ventral pathway is not identical to that of the

dorsal system, and is not related to arousal per se, but to specific behaviors that accompany arousal responses.

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CHROMATOGRAPHIC RESOLUTION OF MOLECULAR SPECIES OF PHOSPHATIDYLSERINES AND PHOSPHATIDYLETHANOLAMINES AS THEIR N-TRIFLUOROACETYL-O-METHYL AND N-ACETYL-O-METHYL DERIVATIVES

KRISTIAN S. BJERVE

Institute of Clinical Biochemistry, University of Oslo, Rikshospitalet, Oslo 1 (Norway)

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SUMMARY

A new technique is described for preparing the N-trifluoroacetyl-O-methyl derivatives of phosphatidylserine and phosphatidylethanolamine. It is rapid, and the derivatives are well separated into the following main molecular species by argentation thin-layer chromatography: saturated-monoene, saturated-dienoic, monoene-dienoic, diene-dienoic, saturated-trienoic, saturated-tetraenoic, saturated-pentaenoic and saturated-hexaenoic. The procedure gave full recovery of phosphatidylethanolamine, while phosphatidylserine in addition gave varying amounts of another derivative, probably a cyclic azlactone. The N-acetyl-O-methyl derivatives were therefore also prepared by a similar procedure. These derivatives separated similarly well on argentation chromatography, and both phosphatidylserine and phosphatidylethanolamine gave full recoveries. The two techniques have been used to analyze rat liver phosphatidylserine and phosphatidylethanolamine.

INTRODUCTION

Several systems for the chromatographic separation of intact phospholipids into subclasses according to fatty acid composition have been described for lecithins [1], phosphatidylethanolamines [1—4], phosphatidylinositols [5] and phosphatidylserines [3, 5]. The best resolution has in general been obtained when the phospholipids were converted to apolar diacylacetyl- or diacylglycerols [7, 8], or dimethylphosphatidates [9]. Part of the molecule is thereby lost, which is a disadvantage in biological studies. However, blocking of the polar groups of the phospholipids can give comparable resolution [2].

Separation of intact phosphatidylserine gives a very poor separation [6]. The N-dinitrobenzamide-O-methyl derivative gave a good resolution, but gave

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low recoveries [10]. The preparation of the trifluoroacetamides gave a relatively good separation, although the carboxyl and hydroxyl groups were not derivatized [3]. However, the authors did not state the recovery of their procedure, and also stated that varying amounts of a azlactone derivative was formed together with the trifluoroacetamides [3]. In our hands, this procedure gave very low recoveries using small amounts of phosphatidylserine, and we therefore investigated the possibility of preparing N-acyl-O-methyl derivatives with high recovery.

MATERIALS AND METHODS

Preparation of rat liver phosphatidylethanolamine and phosphatidylserine

Male Wistar rats weighing 180—250 g were decapitated, the livers rapidly removed, weighed and homogenized in 30 ml of ice-cold methanol containing 1.5 mg of 2.6-di-*tert.*-butyl-*p*-cresol (BHT) as antioxidant. Lipids were extracted from this homogenate according to the method of Bligh and Dyer [11]. Phosphatidylethanolamine and phosphatidylserine were isolated by thin-layer chromatography (TLC) on 0.5 mm silica gel H (Merck, Darmstadt, G.F.R.) layers containing Na_2CO_3 [12], using chloroform—methanol—acetic acid—water (50:25:7:3) as developing solvent. The fractions were located by spraying with 0.02% dichlorofluorescein in ethanol, and eluted [1]. The phosphatidylserine fraction was further purified by TLC on Na_2CO_3-impregnated silica gel H using chloroform—methanol—acetic acid—water (50:15:12:6) as solvent. The purity was finally checked by TLC on silica gel H using chloroform—methanol—conc. ammonia—water (60 : 35 : 2.5 : 2.5) as solvent, and was better than 98% calculated from phosphorus content. The phospholipids were stored at −20°C under nitrogen in chloroform containing 50 mg/l BHT.

Preparation of N-trifluoroacetyl-O-methyl derivatives

Phosphatidylserine or phosphatidylethanolamine (0.03—1.5 μmoles) was dried under nitrogen in a 5-ml reagent tube, and dissolved in 1 ml of freshly distilled chloroform containing 20 μl of trifluoroacetic anhydride (Fluka, Buchs, Switzerland). The tube was flushed with nitrogen and closed with a Teflon-lined stopper. After 5 min at room temperature, excess trifluoroacetic anhydride was removed by dropwise addition of a 0.2—0.3 mol/l solution of freshly prepared diazomethane in diethyl ether [13]. Diazomethane was added in slight excess as indicated by a persisting light yellow colour of the reaction mixture. After 20 min at room temperature the reaction mixture was taken to dryness under nitrogen and dissolved in a small volume of chloroform. The derivatives were isolated by TLC on silica gel H using chloroform—isopropanol (96 : 5) as solvent, and located by spraying with 0.02% dichlorofluorescein in ethanol before elution from the gel [1].

Preparation of N-acetyl-O-methyl derivatives

Phosphatidylserine or phosphatidylethanolamine (0.03—1.5 μmoles) was dried under N_2 and dissolved in 1 ml of freshly distilled chloroform containing 25 μl of acetic anhydride. After 30 min at room temperature, 1.5 ml of freshly distilled diethyl ether was added, and then freshly prepared 0.2—0.3 mol/l

diazomethane in diethyl ether [13] was added dropwise in slight excess as indicated by a persisting light yellow colour. After 20 min at room temperature, the reaction mixture was evaporated at 40°C under nitrogen until all traces of acetic anhydride had been removed. The derivatives were isolated by TLC as described for the N-trifluoroacetyl-O-methyl derivatives.

Argentation chromatography

Thin-layer plates (20 × 20 cm, 0.5 mm thickness) containing 12 g of silver nitrate per 40 g of silica gel H were activated at 180°C for 2 h, and stored over phosphorus pentoxide until used. The solvent systems are described in the figures. Fractions were visualized after spraying with 0.002% dichlorofluorescein in ethanol, viewed under ultraviolet light, and eluted [1] and washed with 0.45% sodium chloride in water—methanol (1:1). Aliquots were taken for phosphorus and fatty acid analysis.

Other methods

The relative abundance of fatty acids in the lipid fractions was determined after transmethylation with 14% boron trifluoride in methanol at 90°C for 10 min in a nitrogen atmosphere [14]. The methyl esters were chromatographed on a SP-2330 capillary column (30 m × 0.25 mm I.D.) using a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector, an injector split ratio of 1:30, and argon as carrier gas. Column temperature was 195°C, injector and detector temperatures were both 250°C. The fatty acids were identified by comparing retention times with authentic standards. Phosphorus was determined according to the method of Bartlett [15], and liquid scintillation counting was performed in a Packard TriCarb 3385 instrument using Insta gel II (Packard) as the scintillation fluid.

RESULTS AND DISCUSSION

The efficacy of the two different derivatisation procedures was checked both by phosphorus analysis of the derivatives and by preparing ^{14}C-labeled phosphatidylserines and -ethanolamines isolated from rat liver cells incubated with L-[^{14}C]serine [16]. N-Trifluoroacetyl-O-methyl-phosphatidylserines and -ethanolamines were both rapidly formed, and the derivatives separated well on silica gel H using chloroform—isopropanol (96:5) as solvent (R_F values 0.45 and 0.30, respectively). The N-acetyl derivatives formed more slowly, and had R_F values of 0.55 and 0.40, respectively. No traces of dimethylphosphatidates [17] could be detected using the present, low concentrations of diazomethane. Phosphatidylinositols did not produce any interfering derivatives during these procedures, as all material remained at the origin.

Recovery of the N-trifluoroacetyl-O-methyl-phosphatidylethanolamines was 90—98% based on phosphorus, while recovery of the corresponding N-acetyl derivatives was 99—100% based on phosphorus and 93—97% based on radioactivity. Recovery of the N-acetyl-O-methyl-phosphatidylserines was 93—102% based on phosphorus and 95—98% based on radioactivity, while the corresponding N-trifluoroacetyl derivatives gave a varying recovery from 45 to 93% by both phosphorus and radioactivity analysis. The remainder was found in

TABLE I

MOLAR FATTY ACID COMPOSITION OF RAT LIVER PHOSPHATIDYLSERINES AND PHOSPHATIDYLETHANOLAMINES AND THEIR N-TRIFLUOROACETYL-O-METHYL AND N-ACETYL-O-METHYL DERIVATIVES

In each experiment, phospholipids from one rat liver were isolated and derivatized as described in Methods.

	Moles per cent							
	Experiment I		Experiment II		Experiment I		Experiment II	
	Original PS*	N-TFA-O-Me-PS*	Original PS	N-Ac-O-Me-PS*	Original PE*	N-TFA-O-Me-PE*	Original PE	N-Ac-O-Me-PE*
16:0	3.1	3.1	4.3	3.0	21.5	21.2	16.7	18.1
16:1	0.1	0.3	0.1	0.2	0.3	0.3	0.5	0.5
18:0	46.6	47.3	47.0	50.2	24.4	24.3	28.1	27.8
18:1	2.2	2.3	3.3	3.7	5.0	4.8	6.0	6.3
18:2	3.6	3.4	3.2	2.7	7.0	7.1	6.4	6.4
18:3	—	—	—	—	0.5	0.4	0.2	0.2
20:1	—	—	—	—	0.3	0.3	—	—
20:2**	—	—	—	—	0.3	0.3	0.2	0.2
20:3	1.3	1.0	0.7	0.8	0.6	0.6	0.7	0.6
20:4	21.9	20.2	17.5	16.8	19.5	19.3	19.1	18.0
20:5	0.7	0.6	1.4	0.9	1.1	1.4	1.7	1.7
22:4**	—	1.0	0.4	0.4	—	—	0.3	0.3
22:5	1.9	1.8	1.9	1.8	2.9	2.9	2.2	2.2
22:6	18.3	18.5	20.2	19.5	16.3	16.9	17.9	17.7

*PS = phosphatidylserine; PE = phosphatidylethanolamine; N-TFA-O-Me- = N-trifluoroacetyl-O-methyl-; N-Ac-O-Me- = N-acetyl-O-methyl-.
**Tentatively identified.

another, more polar, derivative, which was tentatively identified as the cyclic azlactone [3]. No azlactones were formed during N-acetylation.

The fatty acid composition of both the N-trifluoroacetyl and the N-acetyl derivatives of phosphatidylserines and phosphatidylethanolamines was identical to that of the intact phospholipids (Table I). The azlactones formed during trifluoroacetylation of phosphatidylserine also had the same fatty acid composition (not shown). These results indicate that no selective loss of any particular molecular phospholipid species occurred during any of the two derivatisation procedures, although the recovery of the N-trifluoroacetyl-O-methyl-phosphatidylserines was variable.

Argentation chromatography of the phospholipid derivatives is shown in Figs. 1 and 2. The rat liver phosphatidylserines were clearly separated into the following fractions: saturated-hexanoic, saturated-pentanoic, saturated-tetraenoic, saturated-trienoic, diene-dienoic, monoene-dienoic, saturated-dienoic and saturated-monoenic. The saturated-trienoic fraction was very low in rat liver, and was therefore included in the tetraenoic fraction. Similarly, the diene-dienoic fraction from rat liver was combined with the monoene-dienoic fraction. The N-trifluoroacetyl-O-methyl-phosphatidylethanolamines separated into similar fractions as previously reported for the corresponding N-acetyl-O-

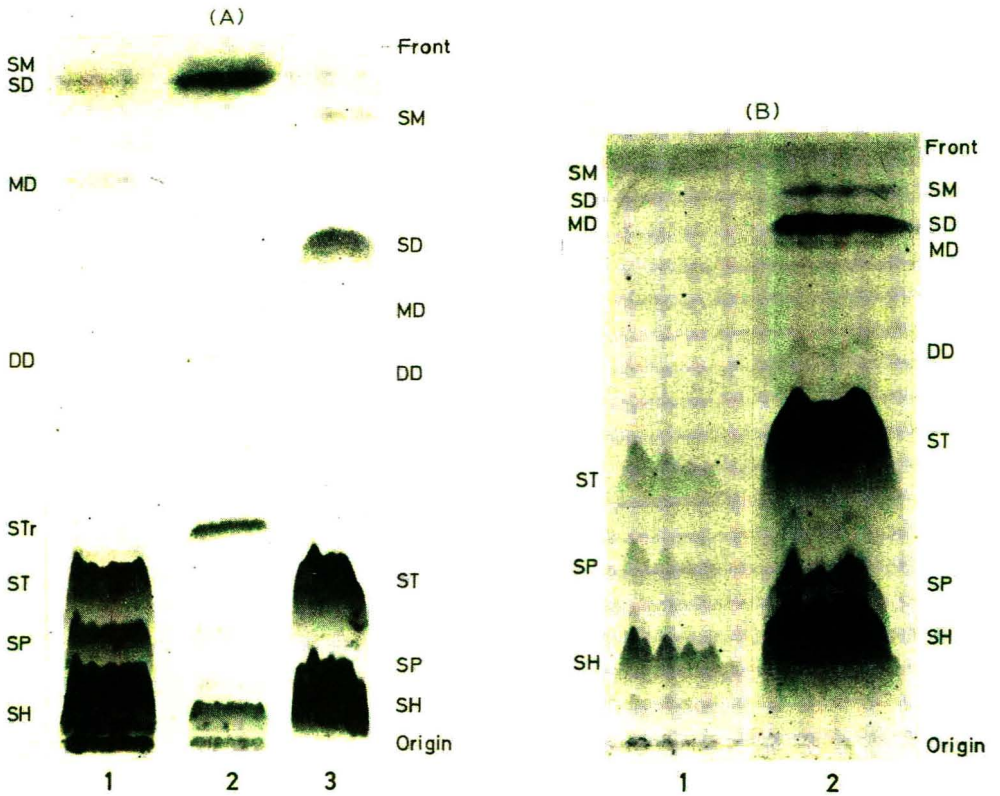

Fig. 1. Separation of N-trifluoroacetyl-O-methyl derivatives of phosphatidylserine and phosphatidylethanolamine. (A) 1 = Rat liver phosphatidylserine, 0.3 μmole; 2 = ox brain phosphatidylserine (Sigma, St. Louis, MO, U.S.A.), 0.4 μmole; 3 = rat liver phosphatidylethanolamine, 0.4 μmole. The plate was first developed for 10 cm with chloroform—methanol—water (80:15:2), briefly dried, and then developed to 1 cm from the top with chloroform—methanol (97:3). (B) 1 = Rat liver phosphatidylserines, 0.3 μmole; 2 = rat liver phosphatidylethanolamines, 1.2 μmole. The first solvent (chloroform—methanol—water, 80:20:3) was run for 10 cm, the plate briefly dried and then developed in chloroform—methanol (99:1). Spots were visualized by charring after spraying with 48% sulfuric acid. The fatty acid composition of the fractions is indicated along the side of the chromatograms. Abbreviations: S = saturated, M = monoene, D = diene, Tr = triene, T = tetraene, P = pentaene, H = hexaene.

methyl derivative [2]. The N-acyl-O-methyl derivatives thus seem to give a better chromatographic resolution than reported for the trifluoroacetamides of phosphatidylserine and -ethanolamine, or for the azlactone of phosphatidylserines [3].

The fatty acid composition of isolated subfractions of N-acetyl-O-methyl-phosphatidylserines and N-trifluoroacetyl-O-methyl-phosphatidylethanolamines is shown in Tables II and III. The phosphatidylethanolamine fractions were similar to that reported previously [2]. The saturated-hexaenoic, saturated-tetraenoic and saturated-dienoic fractions of rat liver phosphatidylserines seem to be nearly unimolecular, with stearic acid as the dominating saturated fatty acid. The monoene-dienoic fraction is the most inhomogeneous fraction and

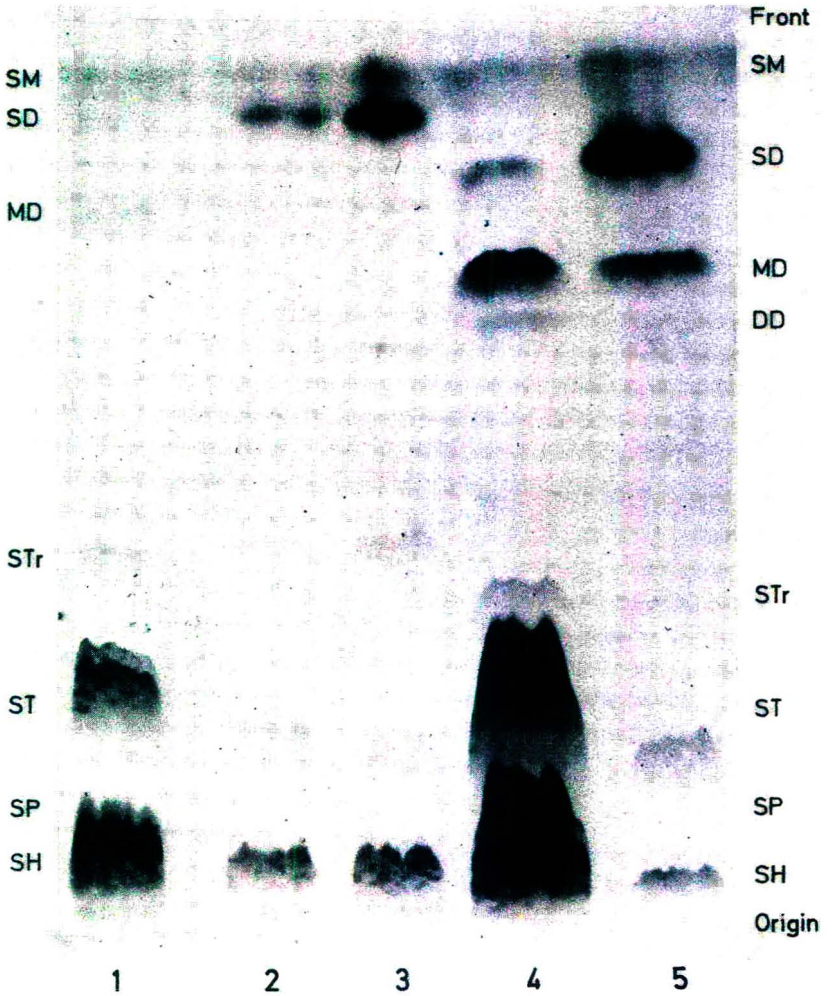

Fig. 2. Separation of N-acetyl-O-methyl derivatives of phosphatidylserines and phosphatidylethanolamines. 1 = Rat liver phosphatidylserines, 0.1 μmole; 2 = ox brain phosphatidylserines (Sigma), 0.05 μmole; 3 = ox brain phosphatidylserines (Koch-Light, Colnbrook, Great Britain), 0.1 μmole; 4 = rat liver phosphatidylethanolamines, 0.3 μmole; 5 = egg phosphatidylethanolamines, 0.1 μmole. The first solvent was chloroform—methanol—water (80 : 20 : 3) which was run for 10 cm. After a brief drying, the plate was run to 1 cm from the top with chloroform— methanol (97 : 3). Abbreviations and visualization as in Fig. 1.

could be seen to consist of several small fractions on the $AgNO_3$ plates. The saturated-monoene fraction seems to contain some disaturated species, and a rather high concentration of lignoceric acid was found in this fraction. For both the N-trifluoroacetyl-O-methyl-phosphatidylethanolamines and the N-acetyl-O-methyl-phosphatidylserines, summing the individual fatty acids according to the relative abundance of each fraction gives a calculated fatty acid composition of the total phosphatidylserines and phosphatidylethanolamines which agrees closely with that found in the intact phospholipids.

The present work is the first report on argentation chromatography of

TABLE II

FATTY ACID COMPOSITION OF N-ACETYL-O-METHYL-PHOSPHATIDYLSERINE MOLECULAR SPECIES ISOLATED FROM RAT LIVER

Abbreviations as in Fig. 1.

	Fatty acid composition (moles per cent)							
	Fraction (relative amount)						Total PS measured	Total PS calculated*
	SH (48.5%)	SP (6.3%)	ST (35.7%)	MD (1.0%)	SD (4.6%)	SM (3.9%)		
14:0	0.2	0.8	0.2	2.6	0.8	2.0	0.2	0.3
14:1	0.8	0.3	1.3	0.4	0.2	0.3	0.5	0.9
16:0	2.6	5.2	2.9	16.0	6.3	7.3	2.9	3.3
16:1	0.1	0.3	0.1	4.3	0.2	4.3	0.2	0.3
16:2**	1.2	1.1	1.2	2.7	1.1	1.0	1.4	1.2
18:0	48.4	45.2	49.0	43.6	45.2	32.3	48.9	47.7
18:1	1.3	4.2	0.8	12.0	2.9	34.6	3.1	2.8
18:2	0.1	0.7	0.4	5.6	42.8	2.3	2.5	2.4
20:0	—	—	—	—	—	1.0	0.2	—
20:1	—	—	—	—	—	1.0	—	—
20:2***	—	—	—	9.5	—	—	0.2	0.1
20:3	0.1	—	1.9	0.7	—	—	0.7	0.7
20:4	1.9	12.0	41.3	0.6	—	—	16.4	16.4
20:5	0.7	9.6	—	—	—	—	0.9	0.9
22:0	—	—	—	—	—	1.4	—	0.1
22:1	—	—	—	—	—	0.2	—	—
22:4***	—	0.9	0.9	—	—	—	0.3	0.4
22:5	1.5	18.1	—	—	—	—	1.8	1.9
22:6	41.1	1.2	—	—	—	—	19.4	20.0
24:0	—	0.4	—	2.0	0.5	12.3	0.4	0.6

*Calculated from the relative amount of each subfraction and its fatty acid composition.
**Includes dimethylacetals.
***Tentatively identified.

N-trifluoroacetyl-O-methyl and N-acetyl-O-methyl derivatives of phosphatidylserines. It also for the first time describes the separation of N-trifluoroacetyl-O-methyl-phosphatidylethanolamines. The O-methylation gives better chromatographic resolution compared to trifluoroacetylation only. Further, the present method is considerably more rapid than those published previously [2, 3]. The most important point, however, is that the present procedure for preparing N-acetyl-O-methyl-phosphatidylserines gives a complete recovery of the derivatives, also when using very small amounts of the phospholipid.

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TABLE III

FATTY ACID COMPOSITION OF N-ACETYL-O-METHYL-PHOSPHATIDYLETHANOLAMINE MOLECULAR SPECIES ISOLATED FROM RAT LIVER

Abbreviations as in Fig. 1.

	Fatty acid composition (moles per cent)								
	Fraction (relative amount)							Total PE measured	Total PE calculated*
	SH (24.0%)	SP (7.7%)	ST (45.8%)	DD (0.7%)	MD (4.6%)	SD (14.2%)	SM (3.1%)		
14:0	0.2	—	—	1.4	0.3	0.3	—	0.3	0.1
14:1	0.5	—	—	0.2	—	—	—	0.1	0.1
16:0	33.4	21.8	12.2	13.9	9.2	25.8	25.9	22.6	19.7
16:1	0.9	1.1	0.3	3.0	1.8	0.6	—	0.4	0.6
18:0	11.6	15.0	28.5	18.2	2.5	20.6	10.3	23.0	20.5
18:1	5.1	6.8	6.3	9.9	37.0	4.8	33.6	9.0	8.1
18:2	0.7	4.5	1.9	24.5	49.2	46.2	—	9.9	10.4
18:3	—	—	0.5	5.9	—	0.8	—	0.4	0.4
20:1	—	—	—	6.3	—	1.1	30.2	0.2	1.0
20:2**	—	—	—	16.9	—	—	—	0.4	0.1
20:3	0.8	3.2	1.6	—	—	—	—	0.8	1.2
20:4	0.6	11.6	47.7	—	—	—	—	20.0	22.9
20:5	1.0	11.9	0.2	—	—	—	—	1.4	1.3
22:3**	—	0.2	0.6	—	—	—	—	0.2	0.3
22:4**	—	0.4	—	—	—	—	—	—	—
22:5	2.4	23.2	0.1	—	—	—	—	2.4	2.4
22:6	43.5	0.4	0.1	—	—	—	—	8.9	10.5

*Calculated from the relative amount of each fraction and its fatty acid composition.
**Tentatively identified.

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PHOSPHOLIPID AND NEUTRAL LIPID SEPARATION BY ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

KLAUS KORTE* and M. LINETTE CASEY

The Cecil H. and Ida Green Center for Reproductive Biology Sciences and the Departments of Obstetrics and Gynecology and Biochemistry, The University of Texas Southwestern Medical School, 5323 Harry Hines Boulevard, Dallas, TX 75235 (U.S.A.)

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SUMMARY

A simple and rapid method for separation of six major phospholipids and four major neutral lipids from cell extracts by one-dimensional preadsorbant thin-layer chromatography was developed. Due to the inert characteristics of the preadsorbent layer (Celite) no separation occurs until the sample reaches the preadsorbent (Celite)—silica gel junction. The compounds were applied to the preadsorbent (Celite) area (625 mm^2) in 10-μl aliquots (total volume of 0.15 ml). By this method, samples can be applied rapidly in large volumes without respotting any area of the preadsorbent layer. The time required to apply one sample was reduced considerably (2 min) compared to conventional methods (10 min). Since all the compounds move with the solvent front as a sharp, narrow band to the preadsorbent (Celite)—silica gel boundary, excellent separation was achieved when up to 650 μg of lipid material was applied on each lane (25 mm wide). Thus, this method is suitable for the separation of relatively large amounts of radiolabeled and non-radiolabeled lipids and free fatty acids from extracts of biological fluids, tissues, or cells maintained in monolayer culture.

INTRODUCTION

In investigations of the metabolism of phospholipids, neutral lipids and free fatty acids, a method(s) is required for the separation of a number of compounds with similar mobility in various chromatographic systems. Thin-layer chromatography (TLC) on silica gel is used widely for the separation and identification of lipids extracted from tissue fragments, cells maintained in monolayer culture or biological fluids [1]. However, many of the separation procedures presently employed are tedious and require chromatography in more than one solvent system [1, 2]. Often the capacity of the thin-layer

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system is small and localization of the separated lipids must be accomplished by processes that destroy the original compounds. Recently Touchstone et al. [3] described a method that was suitable for the separation of six major phospholipids by one-dimensional TLC. However, the total amount of lipids that can be separated using this TLC system is small (0.2 μg) and therefore charring of the chromatograms is necessary to visualize the separated lipids. The charring process is a distinct disadvantage since it may bring about destruction of the original lipid and thereby preclude further purification and identification of the compound. In addition, the quantification of radiolabeled lipids is hampered by charring since this process causes severe quenching of β-emitting radioisotopes [4].

According to conventional methods for TLC, samples are applied to the silica gel in a successive series of small aliquots (5—10 μl) overlaid at the origin. This process is very time consuming since the solvent must be removed completely between each application of sample. In the present investigation we developed a method for the complete separation of six major phospholipids as well as the separation of the phospholipids from free fatty acids and neutral lipids. In addition a method for the separation of four major neutral lipids from free fatty acids and phospholipids is described. We have employed these methods to monitor the incorporation of radiolabeled fatty acids into the various lipids of cells maintained in monolayer culture. By use of this method up to 650 μg total lipid can be applied on each TLC lane (25 mm wide) without the necessity of respotting any area of the origin, thereby reducing considerably the time required for application of the sample.

EXPERIMENTAL

Standards

Phospholipid and neutral lipid standards were obtained from Serdary Research Laboratories (London, Canada). The standard-mix solution used routinely for our experiments for phospholipid TLC was prepared in chloroform and consisted of L-3-phosphatidylcholine dioleoyl, 0.5 mg ml^{-1}; phosphatidylserine, pig brain, 1.5 mg ml^{-1}; phosphatidylinositol, pig liver, 1.5 mg ml^{-1}; DL-phosphatidylethanolamine dioleoyl, 1.5 mg ml^{-1}; oleic acid, 1 mg ml^{-1}; triolein, 0.5 mg ml^{-1}. A 100-μl volume of this mixture was applied to each TLC lane. The standard-mix solution for neutral lipid TLC was prepared in hexane and contained 1-monoolein, 0.3 mg ml^{-1}; 1,3-diolein, 2 mg ml^{-1}; oleic acid, 0.3 mg ml^{-1}; triolein, 0.3 mg ml^{-1}; cholesteryl ester, 0.3 mg ml^{-1}. A 50-μl volume of the mixture was applied to each lane of the TLC plate.

Chromatography

Silica gel G preadsorbent thin-layer plates were purchased from Analtech (Newark, DE, U.S.A.), and were used without an activation or washing procedure. The important feature of the preadsorbent TLC plate is the sharp demarcation between the inert preadsorbent (Celite) and the silica gel layer that facilitates high resolution of the compounds to be separated. The silica gel and preadsorbent (Celite) zone on each plate were scored into seven lanes

(25 mm each). Samples were applied with a microselectapette (Clay Adams, Parsipanny, NJ, U.S.A.) on an area (625 mm^2) at a spot 5 mm above the lower edge and 5 mm below the preadsorbent (Celite)—silica gel limit of the plate.

Solvents

All solvents were the best analytical grade available from scientific supply houses. The following solvent systems were used: system A (phospholipid TLC), chloroform—ethanol—water—triethylamine (30:34:8:35); system B (neutral lipid TLC), *n*-heptane—diethyl ether—acetic acid (75:25:4).

Procedure

In a typical experiment human endometrial stromal cells were maintained in monolayer culture in the presence of [^{14}C]arachidonic acid (Amersham, Arlington Heights, IL, U.S.A.). Cells were extracted and washed according to the method of Folch et al. [5]. After concentration of the extract to 2 ml by evaporation under nitrogen, aliquots for phospholipid (10%) and neutral lipid (10%) TLC were transferred into 75 × 12 mm tubes and the appropriate standard-mix solution was added. The extracts were reduced to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 0.15 ml chloroform—methanol (2:1), and spotted on the preadsorbent (Celite) area of the chromatogram under an atmosphere of nitrogen by use of a device supplied by Sindco (Miami, FL, U.S.A.). After equilibration with the solvent system to be used in a plate conditioning apparatus (Analtech) for 15 min, plates were lowered into the solvent and developed to a height of 16 cm above the preadsorbent (Celite)—silica gel boundary. After removal from the chamber, the solvent was removed under a stream of air or nitrogen and the chromatogram was exposed to iodine vapor to visualize standards. After evaporation of the iodine, the silica gel was scraped from the areas of the chromatogram that corresponded to the various lipids. The lipids were eluted from the silica gel with 6 ml chloroform—methanol (2:1). The extracts were evaporated under a stream of nitrogen at room temperature and radioactivity was assayed in 16 ml Liquiscint (National Diagnostics, Somerville, NJ, U.S.A.) by liquid scintillation spectrometry.

RESULTS

The chromatograms that are illustrative of the separation of phospholipids, neutral lipids and free fatty acid are shown in Figs. 1 and 2. The standards were applied on TLC lanes either separately or as the standard-mix in 0.15 ml chloroform—methanol (2:1). When solvent system A was employed (phospholipid TLC), six major phospholipids were separated. In addition free fatty acids and neutral lipids (monoacylglycerols, diacylglycerols, triacylglycerols, cholesteryl esters) that migrate near the solvent front were separated from the phospholipids. With solvent system B (neutral lipid TLC) the separation of four major neutral lipids, free fatty acids and phospholipids was achieved. By use of this solvent system (B), the phospholipids migrate only to the preadsorbent (Celite)—silica gel boundary. The mobilities of the various compounds tested, expressed as R_F values, are given in Table I. The preadsorbent (Celite)—silica

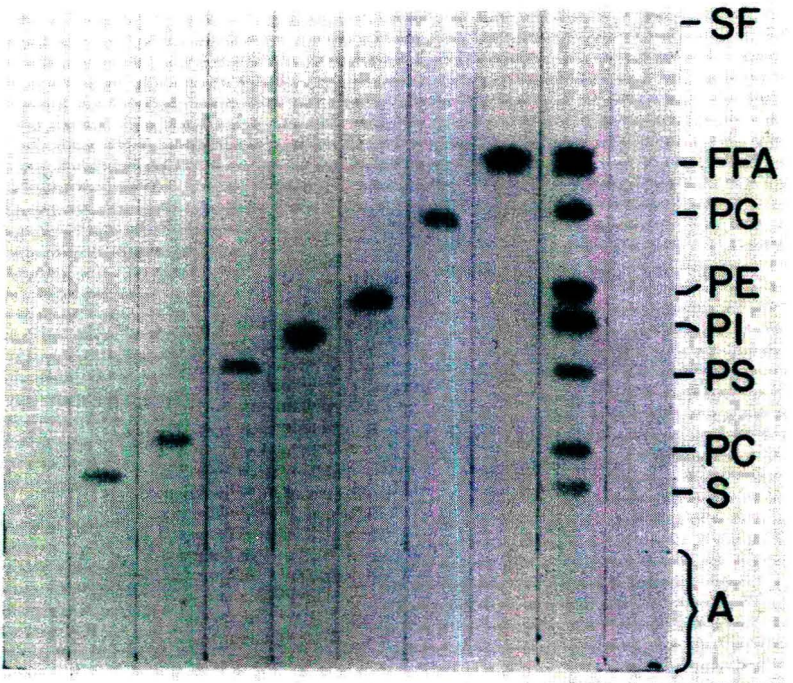

Fig. 1. Phospholipid TLC chromatogram (solvent system A) after iodine staining. The compounds were applied either separately or as a mixture. A = preadsorbent (Celite) layer; S = sphingomyelin (100 μg); PC = phosphatidylcholine (50 μg); PS = phosphatidylserine (150 μg); PI = phosphatidylinositol (150 μg); PE = phosphatidylethanolamine (150 μg); PG = phosphatidylglycerol (100 μg); FFA = free fatty acid (100 μg); SF = solvent front. For purpose of illustration neutral lipids were not included.

TABLE I

R_F VALUES OF SEPARATED COMPOUNDS IN SOLVENT SYSTEM A (PHOSPHOLIPID TLC) AND SOLVENT SYSTEM B (NEUTRAL LIPID TLC)

Compound	Solvent system	
	A	B
Sphingomyelin*	0.13	0
Lysophosphatidylcholine*	0.14	0
Phosphatidylcholine	0.21	0
Lysophosphatidylethanolamine*	0.32	0
Phosphatidylserine	0.37	0
Phosphatidylinositol	0.42	0
Phosphatidylethanolamine	0.49	0
Phosphatidylglycerol*	0.62	0
Free fatty acid	0.73	0.59
Monoacylglycerol	0.96	0.03
Diacylglycerol	0.97	0.31
Cholesterol*	0.97	0.35
Triacylglycerol	0.98	0.68
Cholesteryl ester	0.97	0.91

*These compounds were not included routinely in our experiments.

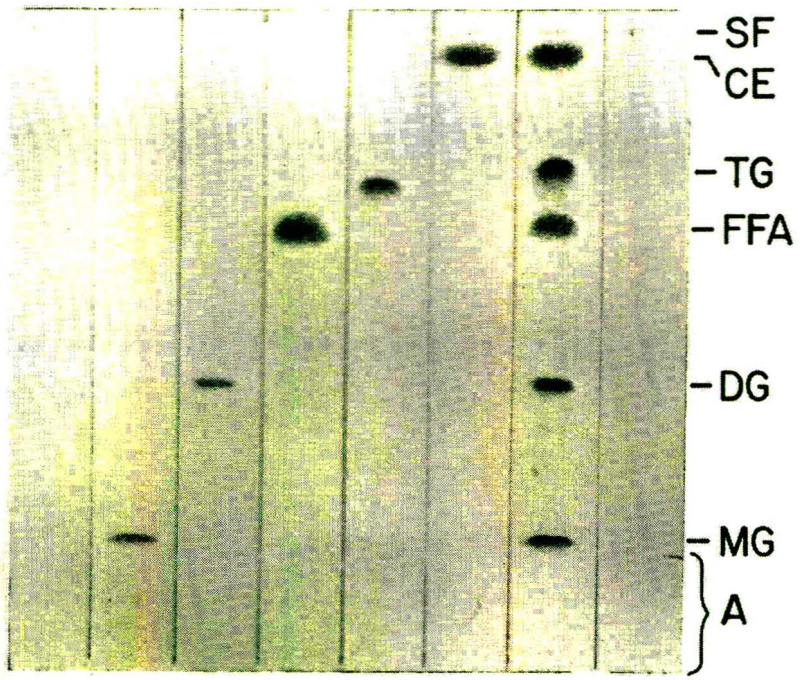

Fig. 2. Neutral lipid TLC chromatogram (solvent system B) after iodine staining. The compounds are applied either separately or as the mixture. A = preadsorbent (Celite) layer; MG = monoacylglycerol (15 μg); DG = diacylglycerol (100 μg); FFA = free fatty acid (15 μg); TG = triacylglycerol (15 μg); CE = cholesteryl ester (10 μg); SF = solvent front.

gel boundary is taken as the origin to estimate the R_F value. Importantly it can be seen that the separation characteristics were not altered by the application of the compounds as a mixture.

As an example of an application of the methods described, human endometrial stromal cells maintained in monolayer culture were incubated with [^{14}C]arachidonic acid for 24 h. At the end of the incubation period lipids were extracted from the cells as described. The total lipid content of the cell extract was estimated to be approximately 50 μg, as determined previously by phosphorus assay according to the methods described by Rouser [6]. Thus, it can be calculated that the total amount of lipid material on each lane (standard plus samples) of a phospholipid chromatogram was approximately 650 μg. A radiometric scan (Berthold Instruments LB2760, G.F.R.) of a TLC lane of a chromatogram on which lipids of endometrial stromal cells were separated with solvent system A after incubation with [^{14}C]arachidonic acid for 24 h is presented in Fig. 3. Standards were chromatographed in parallel lanes and visualized after exposure to iodine vapor. Sharp peaks of radioactivity are apparent, indicative of the incorporation of [^{14}C]arachidonic acid into compounds that comigrate with the standards. It should be noted that sphingomyelin, phosphatidylglycerol, lysoglycerophospholipids and cholesterol were not included routinely in our experiments.

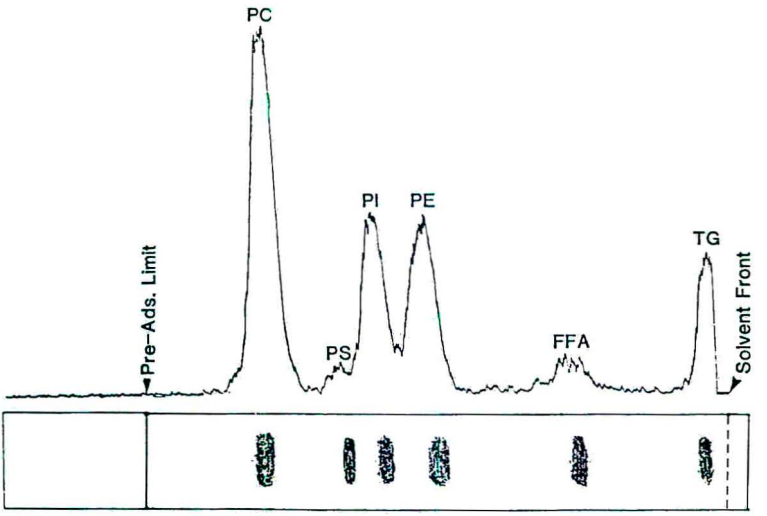

Fig. 3. Radiometric scan of a phospholipid TLC lane with the corresponding standards after iodine staining. Human endometrial stromal cells were maintained in monolayer culture in the presence of [^{14}C]arachidonic acid and thereafter extracted as described in the text. An aliquot (10%) of the cell extract and the standard-mixture was applied to the preadsorbent (Celite) area of the chromatogram. For abbreviations see Figs. 1 and 2.

DISCUSSION

The conditions of isolation of major phospholipids and neutral lipids have been described in detail [1]. However the separation of phospholipids by conventional one-dimensional TLC is limited by the fact that phosphatidylglycerol and phosphatidylethanolamine have very similar R_F values. In addition phosphatidylserine and phosphatidylinositol often do not migrate as sharp bands; rather during chromatography these compounds tend to drag or tail [7, 8]. Recently Touchstone et al. [3] described a method suitable for the separation of six major phospholipids by one-dimensional TLC. These investigators included in the solvent system triethylamine, which has a high selectivity for these critical pairs [9]. The phospholipid TLC solvent system used in the present investigation was devised from the findings of Touchstone and coworkers. However, in many of the methods described previously, including that of Touchstone et al. [3], the quantity of lipid material that can be separated is small, and, therefore, charring of the chromatograms may be necessary for visualization of the compounds. One disadvantage of this process is that the lipids may be altered structurally such that further purification and identification of the original lipid species is precluded. In addition, charring causes severe quenching of β-emitting radioisotopes in liquid scintillation spectrometry [4]. In these experiments we routinely used iodine vapor for visualization of the lipids. The staining process is reversible due to oxidation of the iodine molecules. The silica gel corresponding to standards can be scraped from the chromatogram and the lipids can be eluted quantitatively with organic solvents. If the fatty acid composition of the separated lipids is of interest, the TLC chromatograms can be sprayed with dichlorofluorescein (0.02%) in methanol

and the compounds then can be localized under ultraviolet light. The structure of the fatty acids will be retained and gas chromatography or high-performance liquid chromatography may be employed to identify the fatty acids after hydrolysis and methylation.

The separation of radiolabeled phospholipids, neutral lipids and fatty acids from extracts of cells in culture incubated with radiolabeled precursors is difficult. The quantity of each separated compound must be sufficient to assay β-radiation accurately, but not so great that the TLC plates are overloaded with lipid material. Moreover, according to conventional TLC methods, the sample is applied to the plate at the origin as a single band. This process is extremely time consuming, particularly when the application of large sample volumes is necessary. Therefore, we applied the compounds in a volume of 0.15 ml on the entire preadsorbent (Celite) area (625 mm^2). It was not necessary to respot the origin area and the time required for the application of a sample (2 min) was greatly reduced compared to conventional methods (10 min). A relatively large amount of lipid material can be applied since all the material moves with the solvent front through the inert Celite as a sharp, narrow band to the preadsorbent (Celite)—silica gel junction. The rapidity of sample application together with the achievement of complete separation of a number of lipids are supportive of the proposition that this method is well suited for the separation of relatively large amounts of endogenous or radiolabeled lipids and fatty acids from extracts of biological fluids, tissues, or cells maintained in monolayer culture.

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COMPARISON OF METHODS FOR THE DETERMINATION OF β-ASPARTYLGLYCINE IN FECAL SUPERNATANTS OF LEUKEMIC PATIENTS TREATED WITH ANTIMICROBIAL AGENTS

GJALT W. WELLING

Laboratorium voor Medische Microbiologie, Rijksuniversiteit, Oostersingel 59, 9713 EZ Groningen (The Netherlands)

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SUMMARY

β-Aspartylglycine is an indicator of the absence of bacterial enzymatic activity in the intestinal tract. This study describes and compares four methods — (1) dansylation with thin-layer chromatography, (2) ion-exchange chromatography, (3) thin-layer electrophoresis, (4) high-voltage paper electrophoresis — to determine the concentration of β-aspartylglycine in fecal supernatants of leukemic patients treated with antimicrobial agents.

INTRODUCTION

β-Aspartylglycine was found to be present in cecal contents of germ-free mice and mice treated with certain antibiotics, whereas it was not present in the intestinal tract of control animals [1]. Later on it was shown that this dipeptide was also present in the cecal contents of other germ-free animal species such as rats, lambs and piglets, and in the feces of totally decontaminated leukemic patients [2]. Subsequently, it was found that after association of germ-free mice with an increasing number of different strains of anaerobic bacteria, the concentration of β-aspartylglycine decreased gradually, reaching undetectable levels after association with 50—60 strains of bacteria [3]. The bacterial flora in the gastrointestinal tract is constantly producing enzymes which interact with host enzymes. It was shown that β-aspartylglycine could only be degraded by a bacterial enzyme originating from a number of strains of bacteria [4].

The bacterial flora which is normally present in the gastrointestinal tract protects the host against potentially pathogenic microorganisms from the en-

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vironment [5]. Since colonization and eventually infection occurs much more easily in germ-free animals and animals treated with certain antibiotics [6], it was decided to use β-aspartylglycine as an indicator of the bacterial infection risk during bacterial decontamination of leukemic patients [6, 7]. Results of these investigations will be published elsewhere. The present study describes and compares four methods to determine the concentration or the presence of β-aspartylglycine in feces.

EXPERIMENTAL

Reference solution of β-aspartylglycine

Different amounts (1—20 μl) of 15% dry weight fecal supernatants from germ-free mice were used as reference solutions to determine the relative concentration of β-aspartylglycine in fecal supernatants from patients. A 15% dry weight fecal supernatant from germ-free mice contains 0.8 μmol of β-aspartylglycine per ml.

Commercially available β-aspartylglycine can also be used as a reference solution, but the following has to be taken into account. Commercial β-aspartylglycine (Serva, Heidelberg, G.F.R.; Sigma, St. Louis, MO, U.S.A.) as a powder does not contain any β-aspartylglycine. It consists of the cyclized α, β-form. In solution it slowly converts to the β-form. To prepare a reference solution, the cyclized α,β-aspartylglycine was dissolved in water and incubated for 30 days at 37°C. The resulting mixture of β-aspartylglycine, α,β-aspartylglycine and a small amount of α-aspartylglycine can be used as reference solution. In some cases, however, this β-aspartylglycine-containing solution was further purified by preparative high-voltage paper electrophoresis at pH 3.5 [1]. Different dilutions of a solution of this preparation were subjected to high-voltage paper electrophoresis and compared with the reference solution obtained from 15% dry weight fecal supernatants from germ-free mice. The concentration of the β-aspartylglycine solution was determined by amino acid analysis (Kontron Liquimat III) of an acid-hydrolyzed sample using norleucine as an internal standard.

Dansylation and thin-layer chromatography

To 25 μl of fecal supernatant (25%, w/v) were added 5 μl of 0.1 *M* sodium hydroxide and 30 μl of Dns-Cl (1-dimethylaminonaphthalene-5-sulfonyl chloride) solution (5 mg/ml of acetone). The mixture was incubated for 30 min at 37°C. After centrifugation 50 μl of the supernatant were added to 3 ml of 0.01 *M* acetic acid and subsequently a small amount of Dowex 50 (H^+) equilibrated with 0.01 *M* acetic acid [9]. This suspension was washed three times with 6 ml of 0.01 *M* acetic acid to remove Dns-OH. Finally, the suspension was washed with 2 ml of ammonia—acetone (1:1, v/v) to remove the Dns-amino acids from the resin. The ammonia—acetone wash was freeze-dried and dissolved in 5 μl of ethanol. The Dns-amino acid mixture was applied to both sides of a polyamide layer (5 × 5 cm) (Schleicher and Schüll, Dassel, G.F.R.). To one side also a Dns-β-aspartylglycine reference solution was added. The polyamide sheets were chromatographed in formic acid—water (15:500, v/v) and subsequently perpendicular to the direction of the first solvent in ethyl

acetate—formic acid—methanol (50:2.5:2.5, v/v). The layers were examined under ultraviolet light.

Ion-exchange chromatography

To 50 μl of 25% (w/v) fecal supernatant 500 μl of methanol were added. After mixing and centrifugation 400 μl of the supernatant were removed and dried at 60°C in a stream of nitrogen. The residue was dissolved in 20 μl of the sample buffer for amino acid analysis; 5 μl (the equivalent of 9 μl of fecal supernatant) were applied to the Kontron Liquimat III amino acid analyzer.

Thin-layer electrophoresis

To 40 μl of fecal supernatant (25%, w/v) were added 500 μl of methanol. After mixing and centrifugation the supernatant was dried under a stream of nitrogen at 60°C. The residue was dissolved in 15 μl of the electrophoresis buffer pH 3.5 (see below). Of this solution 2—10 μl were applied as a line 1 cm long at the center of a 20 × 20 cm cellulose MN 300 layer (Polygram cel 300; Macherey-Nagel, Düren, G.F.R.). Electrophoresis was performed at 800 V for 30 min in a home-built apparatus with liquid cooling as described by Whittaker and Moss [10]. After staining with 0.2% ninhydrin in ethanol and drying with a hair-dryer, the layer was heated for 5 min at 110°C, during which the blue to greyish color of β-aspartylglycine may become visible.

High-voltage paper electrophoresis

An 80 μl aliquot of a 25% (w/v) fecal supernatant was applied to Whatman 3 MM chromatography paper (46 × 57 cm) 18 cm from the edge of the paper to be immersed in the anode buffer compartment. A reference solution was applied containing lysine, glycine, glutamic acid, aspartic acid, β-aspartylglycine (see section on reference solution of β-aspartylglycine) and Xylene cyanol FF. The blue-colored dye Xylene cyanol FF (BDH, Poole, Great Britain) migrates slightly ahead of β-aspartylglycine to the anode. High-voltage paper electrophoresis was performed at pH 3.5 (pyridine—acetic acid—water, 1:10:89, v/v) for 1 h at 3000 V in a home-built apparatus similar to the commercially available Savant Model LT48 (Savant, Hicksville, NY, U.S.A.). After elecrtrophoresis the paper was dried at 70—80°C for 10 min and dipped in 0.2% ninhydrin in ethanol. Heating at 70—80°C resulted in purple spots for most of the peptide-like material, except for β-aspartylglycine which shows up greyish. Additional heating at 120—150°C for 10 min gives a clear blue color for β-aspartylglycine. The concentration of β-aspartylglycine in the fecal supernatants was determined by visual comparison with the intensity of the color of various concentrations of β-aspartylglycine reference solutions.

RESULTS

Cyclized α,β-aspartylglycine converted in solution to the more stable β-form for about 70% in 30 days. This solution was used as a reference solution next to a 15% dry weight fecal supernatant from germ-free mice which contained 0.8 μmol of β-aspartylglycine per ml. The concentration of free amino acids varied from 1.4 to 12.8 μmol/l.

Dansylation and thin-layer chromatography

Thin-layer chromatography of a solution containing Dns-α-aspartylglycine, Dns-α,β-aspartylglycine, Dns-β-aspartylglycine and some other Dns-amino acids showed that the different Dns-aspartylglycines were very well separated from the Dns-amino acids (Fig. 1A). To determine the presence of β-aspartylglycine in fecal supernatants, samples have to be pretreated with acetone to remove

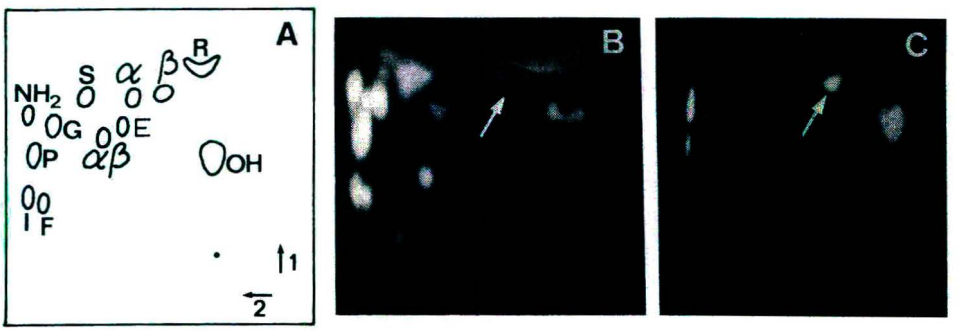

Fig. 1. Thin-layer chromatography on polyamide layers. (A) Dansylated amino acids and peptides: R = arginine, S = serine, E = glutamic acid, G = glycine, P = proline, I = isoleucine, F = phenylalanine, β = β-aspartylglycine, α = α-aspartylglycine; αβ = α,β-aspartylglycine, Dns-NH_2 and Dns-OH are also indicated. (B) Dansylated fecal supernatant containing β-aspartylglycine (arrow). (C) Dansylated β-aspartylglycine (arrow). Solvent in the first dimension was formic acid—water (3:100) and in the second dimension ethyl acetate—formic acid—methanol (20:1:1). The layers were examined under ultraviolet light.

TABLE I

PRESENCE OR CONCENTRATION (μ*M*) OF β-ASPARTYLGLYCINE IN FECAL SUPERNATANTS FROM LEUKEMIC PATIENTS TREATED WITH ANTIBIOTICS

Sample	Dansylation*	Ion-exchange chromatography**	Thin-layer electrophoresis*	High-voltage paper electrophoresis***
1	+	320	++	320
2	+	240	++	300
3	ND†	210	ND	200
4	+	ND	++	180
5	±	210	+	140
6	±	170	+	120
7	–	ND	+	100
8	ND	180	ND	85
9	+	65	–	35
10	–	80	–	18
11	ND	55	ND	10

*Semi-quantitative measurement from – to ++.

**Amount based on peak height; the concentrations were based on the assumption that sample 1 (Fig. 2 bottom) had the same concentration as determined by high-voltage paper electrophoresis.

***Concentration (μ*M*) from semi-quantitative measurements (above 50 μ*M*: ± 10%; below 50 μ*M*: ± 20%).

† ND = not done.

proteins and with Dowex 50 to remove most of the Dns-OH formed during the dansylation reaction. Examples of thin-layer chromatography of a dansylated fecal supernatant and a reference solution are shown in Figs. 1B and C, respectively. The results are shown in Table I.

Ion-exchange chromatography

After removal of protein by methanol precipitation, fecal supernatants were subjected to cation-exchange chromatography on an amino acid analyzer using a short program intended for analysis of acid-hydrolyzed proteins. Part of the analysis of two fecal supernatants is shown in Fig. 2. Pure β-aspartylglycine showed only one peak (see Fig. 2, β). All fecal supernatants showed peaks at almost the same elution position as β-aspartylglycine whether they contained

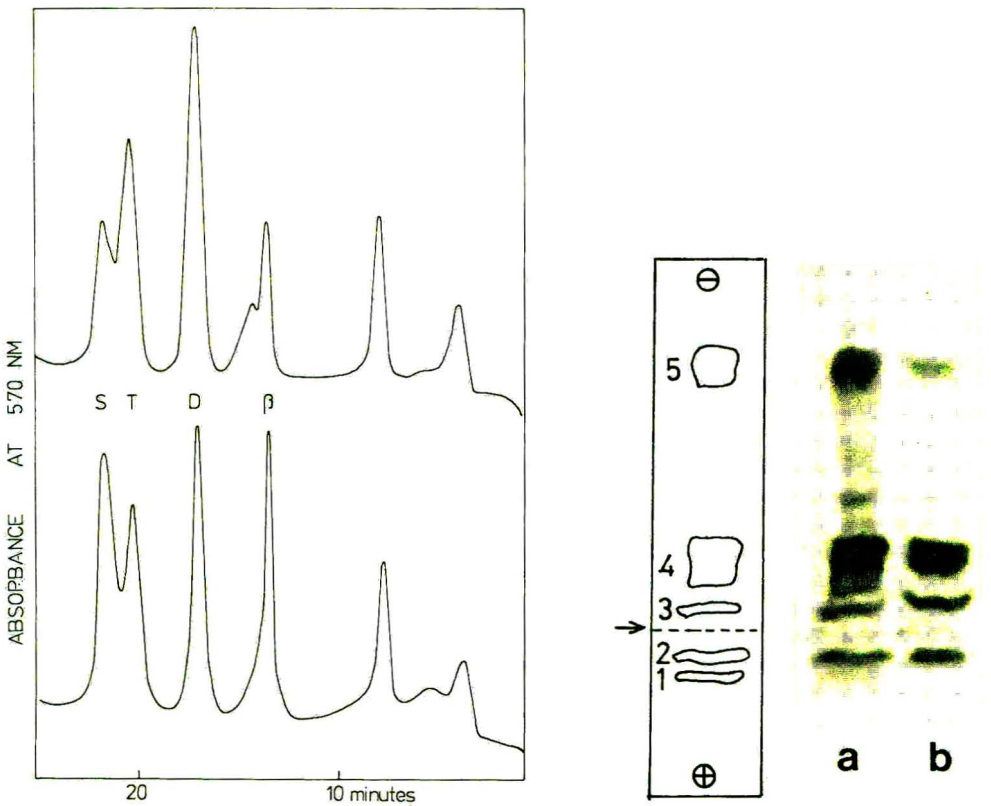

Fig. 2. Ion-exchange chromatography of fecal supernatants from leukemic patients treated with antibiotics. S = Serine, T = threonine, D = aspartic acid, β = β-aspartylglycine. Supernatants obtained after methanol precipitation were applied to an amino acid analyzer. Top: pattern obtained from feces of a patient after 17 days of oral treatment with cephradine (6 g/day). Bottom: pattern from feces of a totally decontaminated patient.

Fig. 3. Thin-layer electrophoresis of fecal supernatants from leukemic patients at 800 V for 30 min. 1 = β-Aspartylglycine, 2 = aspartic acid, 3 = glutamic acid, 4 = neutral amino acids, 5 = basic amino acids. (a) A fecal supernatant containing β-aspartylglycine; (b) a sample containing no β-aspartylglycine. The application line is indicated by an arrow.

β-aspartylglycine or not. These peaks interfered with the accurate determination of β-aspartylglycine. The results based on peak height are shown in Table I.

Thin-layer electrophoresis

Electrophoresis of fecal supernatants without pretreatment often resulted in distorted amino acid and peptide spots and uneven migration. These problems were overcome by removal of the proteins present in the samples by methanol precipitation. An example of thin-layer electrophoresis of fecal supernatants from leukemic patients treated with antibiotics is shown in Fig. 3. The results are shown in Table I.

High-voltage paper electrophoresis

Fecal supernatants were applied to Whatman 3 MM chromatography paper without any pretreatment. Examples of electrophoresis of patient fecal samples containing no β-aspartylglycine, a reference solution of β-aspartylglycine and different amounts of a fecal supernatant from germ-free mice are shown in Fig. 4. The electrophoresis results are summarized in Table I.

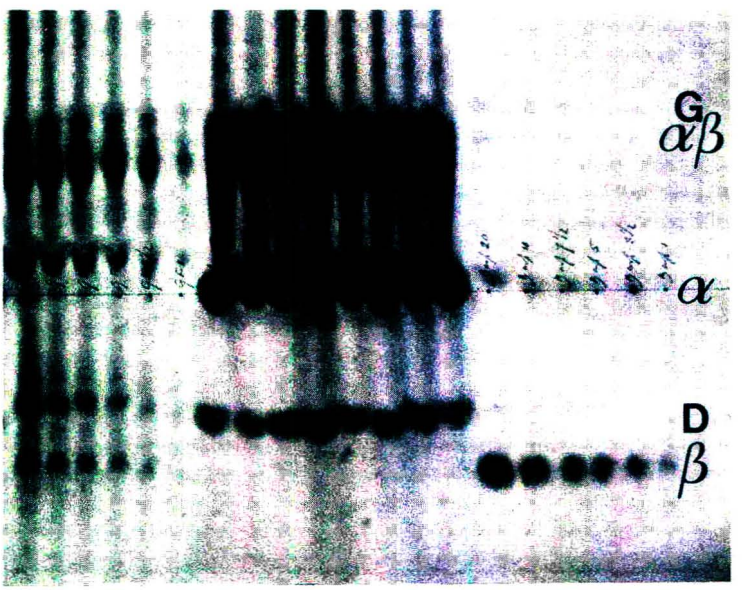

Fig. 4. High-voltage paper electrophoresis at pH 3.5 for 1 h at 3000 V. From left to right: 20, 10, 7.5, 5, 2.5 and 1 μl of a 15% dry weight fecal supernatant from germ-free mice; eight fecal supernatants containing no β-aspartylglycine; a reference solution of β-aspartylglycine (β) containing small amounts of α-aspartylglycine (α), aspartic acid (D), glycine (G) and α,β-aspartylglycine (αβ).

DISCUSSION

In Table I the results obtained with the four methods are compared. Generally there is a good agreement between the methods used. Dansylation is the less sensitive method to determine β-aspartylglycine. Since the other amino acids

are generally present in much higher concentration, the presence of Dns-β-aspartylglycine can only be detected in samples with relatively high concentrations of β-aspartylglycine. Furthermore, the shape and size of the Dns-arginine spot may give problems in distinguishing the presence of β-aspartylglycine.

Thin-layer electrophoresis using the liquid-cooled home-built apparatus described by Whittaker and Moss [10] turned out to be a reliable method to determine the presence of β-aspartylglycine. However, only a limited amount of sample could be applied to the cellulose thin-layer. Furthermore, pretreatment of the supernatants with methanol to remove protein is very often necessary. Although this treatment generally did not have any effect on the recovery of β-aspartylglycine, in a few cases a 25% loss of β-aspartylglycine was observed.

Since a fecal supernatant is rather dirty, analysis on an amino acid analyzer is preferably also preceded by a methanol precipitation step. β-Aspartylglycine can be separated very well from the common amino acids but fecal supernatants appeared to contain substances which eluted at almost the same position as β-aspartylglycine. This makes accurate determination of the concentration impossible, especially at concentrations of β-aspartylglycine of less than 100 μM. Because of this interference, concentrations calculated from the height of the peak at the elution position of β-aspartylglycine are most probably higher than the values obtained with high-voltage paper electrophoresis. Furthermore, other β-aspartylpeptides, i.e. β-Asp-Ala, β-Asp-Ser and β-Asp-Gln, were shown to be present in feces of decontaminated patients [11]. With the short elution program intended for routine analysis of acid-hydrolyzed protein samples, these peptides elute at the same position as β-aspartylglycine, although β-aspartylglycine is most predominantly present. With other gradients however these peptides might be separated [12—14]. The aforementioned factors will result in an over-estimation of the β-aspartylglycine concentration when determined by ion-exchange chromatography (see Table I, column 3). Despite these difficulties, ion-exchange chromatography provides a simple way of determining the presence of β-aspartylglycine in concentrations above 100 μM.

High-voltage paper electrophoresis is the method of choice to determine the concentration of β-aspartylglycine in fecal supernatants. Twenty to twenty-five fecal supernatants can be applied directly, without any pretreatment, to one piece of chromatography paper and subjected to electrophoresis. β-Aspartylglycine is more acidic than aspartic acid at pH 3.5 and because of its blue color after staining with ninhydrin and subsequent heating it can be easily distinguished from the purple spots of other peptide-like substances.

Table I, which summarizes the results, shows that β-aspartylglycine can be detected with all four methods in fecal samples containing more than 0.1 μmol/ml. The most accurate determination of lower concentrations (0.01—0.1 μmol/ml) can only be done with high-voltage paper electrophoresis. The latter method has been applied to monitor disturbance of the bacterial flora of immunocompromised patients who are at high risk of infection. These patients received a preventive treatment with antimicrobial drugs that only removed the aerobic potentially pathogenic microorganisms [7, 8], leaving the major part of the bacterial flora in the gastrointestinal tract intact. β-Aspartylglycine was found in 7% of 793 samples investigated. After additional treatment

with orally administered cephradine, β-aspartylglycine was found in 42% of 81 samples investigated. This indicated that a substantial part of the protective bacterial flora was removed.

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DETERMINATION OF THE ANALGESIC 3-PHENOXY-N-METHYLMORPHINAN IN PLASMA BY GAS CHROMATOGRAPHY USING EITHER POSITIVE CHEMICAL IONIZATION MASS SPECTROMETRIC OR NITROGEN—PHOSPHORUS-SPECIFIC DETECTION ANALYSIS

J.A.F. de SILVA*, J. PAO and M.A. BROOKS

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

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SUMMARY

The compound 3-phenoxy-N-methylmorphinan (I) is under development as an analgesic agent. Studies on the biotransformation of the drug in the rat and in the dog have shown that I is extensively metabolized by N-demethylation to yield the nor-analogue (I-A), by *para*-hydroxylation of the 3-phenoxy ring to yield the phenolic analogue (I-B), cleavage of the ether linkage to yield levorphanol (I-C), and its N-demethylation to yield nor-levorphanol (I-D). The presence of these four metabolites (two of which, I-B and I-C, have analgesic potential) in addition to the parent drug, necessitated the development of sensitive and specific assays for their quantitation in plasma. This was accomplished by the development of (a) a high-performance liquid chromatographic assay using UV detection to obtain a qualitative/semi-quantitative profile of the metabolites present in plasma; (b) a gas chromatographic—nitrogen—phosphorus-specific detection method for the determination of the parent drug (I) for pre-clinical drug evaluation; and (c) a sensitive and specific gas chromatographic—positive chemical ionization mass spectrometric assay for eventual clinical evaluation for the determination of I and a key metabolite levorphanol (I-C). This report presents some preliminary pharmacokinetic data on I and I-C in the dog obtained during pre-clinical development.

INTRODUCTION

The benzomorphinan class of compounds has yielded several clinically effective drugs currently marketed as antitussive (dextromethorphan) and analgesic (levorphanol, levallorphan, dextrorphan) agents [1] (Table I). Clinical interest in this class of compounds continues in the search for a non-narcotic analgesic agent without addiction potential [2]. The analogue 3-phen-

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oxy-N-methylmorphinan [I] is a member of a series of phenoxymorphinan analogues synthesized by Mohacsi [3, 4] and is under development as a non-narcotic analgesic agent. Studies on the biotransformation of I in the rat [5] and in the dog [6,7] have shown that the drug is extensively metabolized not only by N-demethylation to form the nor-analogue (I-A), but also by *para*-hydroxylation of the 3-phenoxy ring to yield I-B and cleavage of the ether linkage to yield levorphanol (I-C) and its subsequent N-demethylation to yield nor levorphanol (I-D) (Fig. 1). The presence of four metabolites in addition to the parent drug necessitated the development of separate assays for their quantitation.

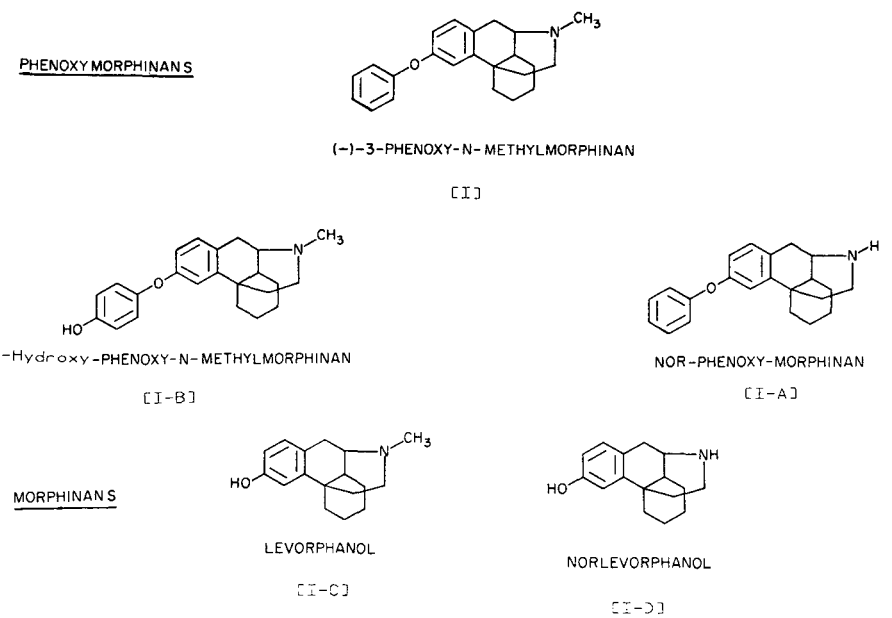

Fig. 1. Chemical structures of phenoxy-N-methylmorphinan and its major metabolites.

The morphinans are amenable to a variety of analytical techniques. Spectrofluorometry was initially used for dextromethorphan (V) [8] and dextrorphan (VI) [9] but was not sufficiently sensitive or specific for pharmacokinetic studies. Gas chromatography (GC) was used for the determination of dextromethorphan [10], 2-hydroxy-N-cyclopropylmethyl morphinan [11] and levorphanol (I-C) [12] by flame ionization detection, dextromethorphan [13] and levorphanol [14] by nitrogen—phosphorus-specific detection (NPD), and dextromethorphan by electron-capture detection [15]. Radioimmunoassay was successfully used for the determination of dextromethorphan [16], 2-hydroxy-N-cyclopropylmethyl morphinan [17], butorphanol [18] and levorphanol [19,20]. Recently, gas chromatographic—mass spectrometric (GC—MS) analysis [21—23], and high-performance liquid chromatographic (HPLC) analysis using either UV [24,25] or electrochemical detection [26] for morphines and morphinans, was also reported, attesting to the intensive analytical endeavor in this class of compounds.

Due to the extensive biotransformation of [I] in the rat [5] and in the dog [6,7], developing a suitable assay in plasma led to the investigation of: (a) an HPLC assay using UV detection to obtain a qualitative/semi-quantitative estimate of [I] and its metabolites detectable in plasma; (b) a GC—NPD method of adequate sensitivity (ca. 10 ng/ml of plasma) for pre-clinical drug evaluation; and (c) a sensitive and specific GC—positive chemical ionization mass spectrometric (GC—PC-MS) assay for I and a key metabolite (I-C) (sensitivity 2.5 ng/ml) for eventual clinical evaluation.

This report presents preliminary pharmacokinetic data on 3-phenoxy-N-methylmorphinan and its metabolite levorphanol in dog plasma using the aforementioned methods obtained during pre-clinical drug development.

EXPERIMENTAL

Reagents

The 1 *M* phosphate buffer, pH 11 was prepared by mixing 530 ml of 1 *M* K_2HPO_4 and 470 ml of saturated Na_3PO_4. Titrate to pH 11.0 with 1 *M* KH_2PO_4 or saturated Na_3PO_4 as needed. The 0.1 *N* hydrochloric acid, and 1.0 *N* sodium hydroxide solutions were made up in distilled water. The *n*-heptane was pesticide grade obtained from Matheson, Coleman and Bell (East Rutherford, NJ, U.S.A.). Methanol (Nanograde) was from Mallinckrodt (St. Louis, MO, U.S.A.) or from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Methylene chloride (Nanograde) from Mallinckrodt (St. Louis, MO, U.S.A.). Pentafluorobenzyl bromide (PFBB) and N,O-bistrimethylsilyltrifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, IL, U.S.A.) and tetrabutylammonium hydrogen sulfate (TBAHS) was from Aldrich (Milwaukee, WI, U.S.A.).

GC—NPD of I in plasma

The sensitivity and specificity of GC—NPD for the determination of morphinans [13,14] prompted this investigation. The primary goal was the quantitation of the parent drug only, since metabolic studies in the dog [6,7] showed low concentrations of the metabolites in plasma which would be impractical to quantitate without derivatization and extensive clean-up.

Analytical standards

Authentic standards of I and II (Table I) of pharmaceutical grade purity (> 99%) were used as the analyte and internal standard, respectively, in the assay. Stock solutions of I containing 10 mg in 10 ml of methanol, and of II containing 1.0 mg in 10 ml of methanol were prepared. Six working standards of solutions containing 5, 10, 25, 50, 75 or 100 ng of I each containing 100 ng of II (internal standard) per 100 μl of solution were prepared using suitable aliquots of serial 1:10 dilutions of the stock solution of I in methanol. Aliquots (5 μl) equivalent to 0.25, 0.5, 1.25, 2.5, 3.75 or 5.0 ng of I and 5.0 ng of II of the working standard solutions were injected into the chromatograph to establish an external calibration curve to determine the linearity and stability of the system.

TABLE I

CHEMICAL NOMENCLATURE FOR PHENOXY-N-METHYLMORPHINAN, ITS MAJOR METABOLITES AND RELATED ANALOGUES

Compound	Chemical name	Molecular weight
Phenoxymorphinans		
I	(—)-3-Phenoxy-N-methylmorphinan	333
I-A	(—)-3-Phenoxymorphinan	319
I-B	(—)-3-(*p*-Hydroxy)phenoxy-N-methylmorphinan	349
II	(—)-3-(*p*-Methyl)phenoxy-N-methylmorphinan	347
III	(—)-3-Pentadeuterophenoxy-N-methylmorphinan	338
Morphinans		
I-C	(—)-3-Hydroxy-N-methylmorphinans; (levorphanol)	257
I-D	(—)-3-Hydroxymorphinan; (norlevorphanol)	243
IV	(—)-3-Hydroxy-N-allylmorphinan; (levallorphan)	283
V	(+)-3-Methoxy-N-methylmorphinan; (dextromethorphan)	271
VI	(+)-3-Hydroxy-N-methylmorphinan; (dextrorphan)	257
Compounds used as internal standards		
II	GC—NPD assay for I.	
III	GC—PCI-MS assay for I.	
IV	GC—PCI-MS assay for I-C.	
V	HPLC assay and carrier substance for GC—PCI-MS assay.	

Instrumental parameters

A Hewlett-Packard Model 5170A gas chromatograph equipped with NPD was used in conjection with a 1.22 m × 2 mm I.D. borosilicate glass column containing 2% OV-17 on 100—120 mesh Gas—Chrom Q. The flow-rates of helium carrier, hydrogen and air, were 1.4 2.5 and 4.0 ml/min, respectively and the operating temperatures for the column oven, injection port and detector were 230°C, 250°C and 300°C, respectively. The silicate bead of the detector was operated at 16 V d.c., the electrometer attenuation was 1 × 8.

Sample preparation

Into a 50-ml PTFE stoppered-centrifuge tube, add 100 μl of stock solution of internal standard (100 ng of II), 1 ml of plasma, 2 ml of phosphate buffer, pH 11 and 2 ml of distilled water and mix well. Extract the sample with 10-ml portions of *n*-heptane twice by shaking at moderate speed for 15 min on a reciprocating shaker (Eberbach). Centrifuge the samples in a refrigerated centrifuge (Damon/IEC, Model PR-J, Rotor No. 253, Needham, MA, U.S.A.), at 5°C for 5 min at approximately 2500 rpm (1500 *g*). Combine each 10-ml extract in another 50-ml PTFE stoppered centrifuge tube. Add 5 ml of 0.1 *N* hydrochloric acid and extract into the acid phase as described above. After centrifugation, remove the heptane phase by aspiration, wash the aqueous phase with another 10-ml portion of heptane and remove the heptane wash by aspiration. Add 0.5 ml of 1 *N* sodium hydroxide, and 2 ml of 1 *M* phosphate buffer, pH 11 (adjust pH to 11.0 ± 0.2). Extract with 10-ml portions of heptane twice as described above and combine these extracts in a tapered 15-ml PTFE-stoppered centrifuge tube. (The first extract is evaporated to near dryness, (ca. 100 μl), before addition of the second). Evaporate the combined

extract to dryness, redissolve in 100 μl of methanol, and inject a 5-μl aliquot for GC—NPD analysis. Typical chromatograms of I and II recovered from plasma, having retention times of 5.9 and 8.2 min, respectively, are shown in Fig. 2A.

Along with the samples, a 1-ml specimen of control plasma and six 1-ml specimens of control plasma containing 100 μl of the working standard solutions equivalent to 5, 10, 25, 50, 75 or 100 ng of I each containing 100 ng of II (internal standard) per ml of plasma, respectively, are run to establish a calibration curve for the quantitation of the unknowns.

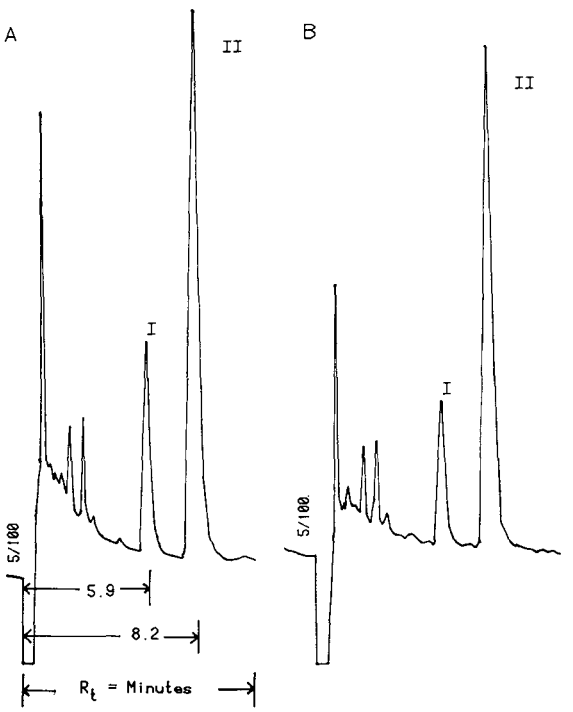

Fig. 2. Chromatograms of GC—NPD analysis of (A) parent drug (I) using the analogue II as the internal standard (equivalent to 75 ng of I and 300 ng of II) recovered from control plasma; (B) dog plasma 1-h post oral dose 10 mg/kg (57 ng of I per ml). R_t = Retention time. Conditions: column, 1.22 m × 2 mm I.D., 2% OV-17 on 100—120 mesh Gas-Chrom Q; temperature, injector 250°C, column 230°C, detector 300°C; flow-rates, helium 1.4 ml/min, hydrogen 2.5 ml/min, air 4.0 ml/min; detector parameters, applied voltage 16 V d.c., attenuation 1 × 8.

Percent recovery and sensitivity limits

The overall recovery of I and II is approximately 65 ± 5% (S.D.) over the concentration range of 10—225 ng/ml and the sensitivity limit is 10.0 ng of I per ml of plasma.

GC—PCI-MS of I in plasma

Metabolic studies in the dog [6,7] showed that the parent drug was the major component in plasma. The presence of levorphanol, (Fig. 1), a metab-

olite with known analgesic activity [27], necessitated the development of a sufficiently sensitive and specific method for the quantitation of the intact drug I, per se in the presence of I-C.

Analysis for intact drug [I] in plasma

Analytical standards. 3-Phenoxy-N-methylmorphinan (I) of pharmaceutical grade purity (> 99%) and 3-pentadeuterophenoxy-N-methylmorphinan (III) (Table I) of purity (> 98.6%) (synthesized by W. Burger and A. Liebman, Radiochemical Synthesis Group Chemical Research Division, Hoffmann-La Roche) were the analytical standards used. Compound IV (levallorphan) (Table I) was used as the carrier substance to minimize adsorption losses in the assay.

Stock solutions of each compound containing 1 mg/ml were prepared in methanol, and serial 1:10 dilutions of each were used to prepare working standard solutions containing 1.0, 2.5, 5.0, 7.5, 10, 25 or 50 ng of I, 25 ng of III (internal standard), and 200 ng of IV (carrier) per 100 μl of methylene chloride—methanol (9:1).

Instrumental parameters. A Finnigan Model 9500 gas chromatograph, equipped with a splitless injector (SGE, Austin, TX, U.S.A.), was coupled via a glass capillary restrictor to a Finnigan Model 3200 mass spectrometer, which was modified in house for chemical ionization in both positive [28] and negative [29] ion modes. Selected ion monitoring (SIM) was performed using a Finnigan Promim® peak monitor with the ion chromatograms recorded by a Rikadenki Model KA-41 four-channel recorder. The GC column was a 1.22 m × 2 mm I.D. pre-siliconized borosilicate glass column packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.), which was conditioned at 300°C with no flow of carrier gas for 4 h followed by overnight conditioning at 280°C with methane carrier gas flow-rate at 10 p.s.i.g. head pressure. The conditioned column was further primed with several (1 μl) injections of BSTFA followed by 10-μl injections of control plasma extract in order to obtain optimal sensitivity and chromatographic peak symmetry for I and III. The operation temperatures for column oven, injector port and interface oven were 265°C, 275°C and 215°C, respectively. Ammonia was introduced through the direct insertion probe inlet as the reagent gas and methane was the carrier gas at a head pressure of 10 p.s.i.g. The methane ion source pressure was 0.5 Torr with a total ion source pressure of 0.6 Torr with ammonia added.

The mass spectrometer was typically operated as follows: ion energy source, repeller and collector at +10 eV, lens at —40 V, electron energy source at —200 V, filament emission at 1.1 mA, the electron-multiplier was set at —2000 V and the continuous dynode electron multiplier at —2500 V. The ion source voltages were optimized daily to give the maximum signal response consistent with Gaussian peak shape and unit mass resolution.

The divert valve was open to vent the first 30 sec of column effluent after which it was closed to permit the effluent to enter the ion source and the filament current turned on. The Promim peak monitor channels were operated at a gain of 10^8 V/A, 100 msec dwell time and a filter setting of 0.5 Hz. The recorder chart speed was 2 cm/min.

The $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 339 (III) (I-D_5) were used for quantitative SIM based on the methane—ammonia-PCI-mass spectra of the respective compounds, (Fig. 3), and were eluted at 1.83 min, (Fig. 4A).

Sample preparation. Into a 50-ml PTFE stoppered centrifuge tube add 25 ng of III (internal standard) and 200 ng of IV (carrier substance) (Table I), in methylene chloride—methanol (9:1) solution, (total volume 100 µl). Add 1 ml of plasma, 2 ml of distilled water and 2 ml of 1 *M* phosphate buffer, pH 11, and mix well at very slow speed on a Vortex mixer.

Add 10 ml of *n*-heptane and extract for 15 min on a Eberbach reciprocating shaker at 80—100 strokes per min. Centrifuge the samples for 10 min at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Damon/IEC-Model PR-J, rotor No. 253) at 5°C. Transfer the heptane layer (ca. 9.5 ml) into a 15-ml conical centrifuge tube, evaporate to dryness, reconstitute the residue in 100 µl of chloroform and inject a 5—10 µl aliquot for GC—MS analysis. Along with the unknowns process seven 1-ml specimens of control plasma, one as the blank specimen and the remaining six samples containing 1.0, 2.5, 5.0, 10, 25, or 50 ng of I, each containing 25 ng of III (internal standard) and 200 ng of IV (carrier), [i.e., 100 µl of each of the working standard solutions], to establish the calibration curve for the quantitation of the unknowns by direct interpolation of the peak height ratio response of I/III vs. concentration of I in ng/ml of plasma.

Statistical validation of the assay for I in plasma. The inter-assay precision and reproducibility of added standards of I over the concentration range of 1.0—50.0 ng/ml of plasma are summarized in Table II. Although 1.0 ng/ml

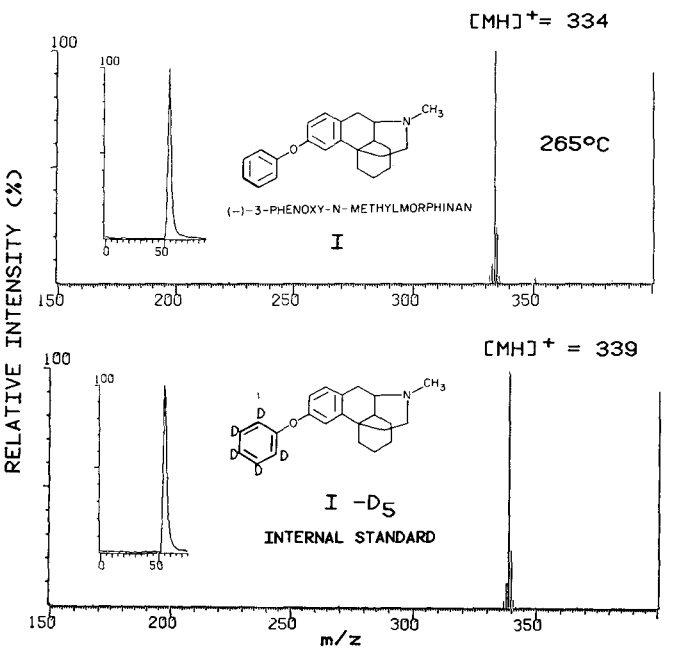

Fig. 3. Methane—ammonia PCI-mass spectra of I and its pentadeuteroanalogue (III) (I-D_5) showing the $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 339 (III), respectively. The inserts represent the total ion chromatogram for each compound.

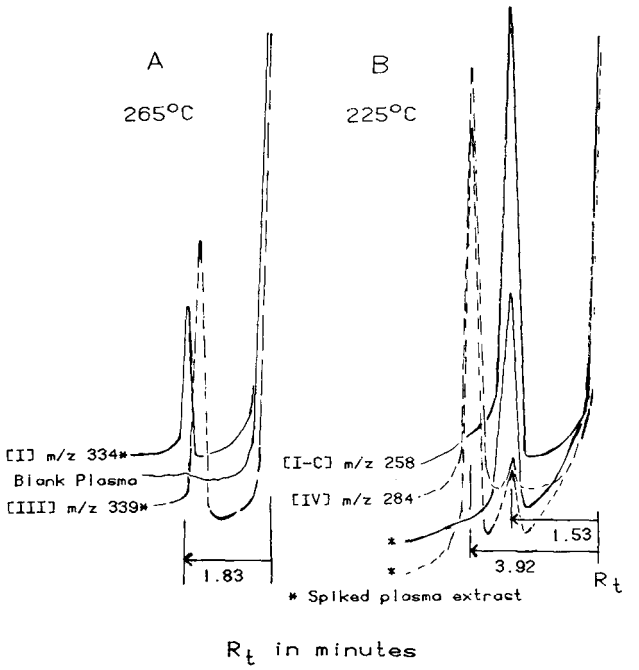

Fig. 4. Ion chromatograms of GC—PCI-MS analysis of: (A) parent drug (I) and its internal standard (III); (B) metabolite, levorphanol (I-C) and its internal standard, levallorphan (IV) determined as underivatized compound. —, $[MH]^+$ of I and I-C; — — —, $[MH]^+$ of internal standards, III and IV.

TABLE II

INTER-ASSAY PRECISION OF RECOVERED CALIBRATION STANDARDS OF I FROM PLASMA DETERMINED BY GC—PCI-MS ANALYSIS

Sample concn. (ng/ml)	n*	Mean concn. found (ng/ml)	Standard deviation	Relative standard deviation (%)
1.0	9	1.34	± 0.55	40.6
2.5	11	2.42	± 0.43	17.8
5.0	9	4.91	± 0.67	13.6
7.5	10	7.45	± 0.55	7.3
10.0	9	9.45	± 0.63	6.6
25.0	8	25.69	± 1.44	5.6
50.0	8	49.79	± 2.63	5.3

*Out of five replicate sets, some standards were injected two to three times during the assay.

of I is measurable its precision is unsatisfactory (40% deviation). Concentrations from 2.5—50.0 ng/ml show good precision especially above 5.0 ng/ml, the mean recovery in this range being 65—70%. The sensitivity limit of the assay was established at 2.5—5.0 ng/ml with a sample/blank response ratio of $>$ 2:1 as the limit of detection.

RESULTS AND DISCUSSION

The extensive biotransformation of 3-Phenoxy-N-methylmorphinian (I) in the rat [5] and in the dog [6,7] resulted in at least four metabolites (Fig. 1), two of which (1-B and I-C) have known analgesic activity. Of these two, I-C (levorphanol) is a marketed analgesic agent (L-Dromoran®), [27], hence the quantitation of this metabolite is relevant.

High-performance liquid chromatographic (HPLC) analysis with UV absorbance at 254 nm was used to obtain a qualitative profile of the plasma concentrations of I as its active metabolites I-B and I-C in animal studies following chronic dosing at 100 mg/kg.

Plasma (0.5—1.0 ml) was extracted with 10 ml of *n*-heptane twice, the combined extracts were evaporated to dryness, the residue was dissolved in 100 μl of methylene chloride—methanol (9:1) and a 50-μl aliquot was injected for HPLC analysis.

Normal-phase chromatography was performed on a Whatman Partisil PXS, 10-μm column 25 cm × 4.6 cm I.D. generating 39,000 plates/m. The mobile phase was a mixture of methylene chloride—methanol—concentrated ammonia (90 : 9.4 : 0.6), the column head pressure was 4.8 MPa (ca. 700 p.s.i.) with a flow-rate of 2 ml/min. The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector, and a Model 440 UV detector with a 254-nm wavelength kit (Waters Assoc., Milford, MA, U.S.A.), operated at a sensitivity of $5 \cdot 10^{-3}$ a.u.f.s., connected to a Hewlett-Packard 5-mV recorder (equivalent to $2.5 \cdot 10^{-3}$ a.u.f.s.) operated at 0.5 in./min. The retention times of I, I-A, I-B and I-C, were 1.6, 13.5, 5.9 and 11.2 min, respectively, using dextromethorphan (V) as the internal standard which had a retention time of 3.2 min. The corresponding capacity factors (k') were 1.0, 8.4, 2.7, 6.0 and 1.6, respectively (Fig. 5). The detector sensitivity yielded full scale peaks for 80 ng of I, 200 ng of I-A, 120 ng of I-B, 200 ng of I-C and 1200 ng of V (internal standard), resulting in sensitivity limits of approximately 10, 30, 20, and 500 ng/ml for I, I-A, I-B and I-C, respectively.

The analysis of plasma specimens from dogs dosed chronically for 90 days at 100 mg/kg showed the presence of measurable amounts of I and I-B (Fig. 5C). Due to the poor UV absorbance of the morphinan analogues I-C and I-D at 254 nm, these two pharmacologically active metabolites could not be determined with adequate sensitivity by HPLC analysis. Even fluorometric detection of these compounds [8,9], (with excitation at 280 nm and fluorescence emission at 315 nm), did not significantly increase the sensitivity limits of their detection. The HPLC method was suitable only for the determination of I and its metabolites I-A and I-B in plasma profiles in the rat and in the dog dosed chronically at high doses (> 100 mg/kg). It was not sufficiently sensitive for single-dose pharmacokinetic studies which necessitated the investigation of the inherently more sensitive GC—NPD analysis [13,14].

Preliminary studies with GC—NPD showed that I, I-A and I-C could be analyzed in a single run using II as the internal standard. The peak for I-C showed tailing indicating the need for derivatization of the phenolic OH group to improve its chromatographic behavior and detector sensitivity, when analyzed at 210°C on a 1.22 m × 2 mm I.D. borosilicate glass column containing 3% OV-17 on 100—120 mesh Gas-Chrom Q.

The retention times for I, I-A, I-C and II (internal standard) were 11.2, 13.3, 3.1 and 15.7 min, respectively, indicating the feasibility of a specific assay. Metabolic studies in a dog administered a single 10 mg/kg oral dose of ^{14}C-labeled I [6] indicated however that I-A, I-B and I-C were minor metabolites in plasma, therefore the assay was modified for the specific determination

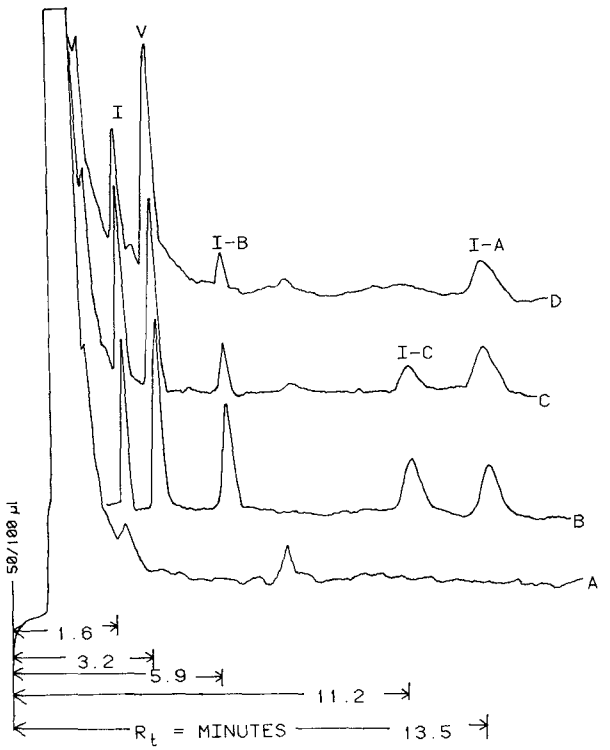

Fig. 5. Chromatograms of the HPLC analysis of (A) control dog plasma extract; (B) authentic standards; (C) authentic standards recovered from plasma equivalent to 25 ng of I, 25 ng of I-A and I-B, 250 ng of I-C and 400 ng of V (internal standard); (D) dog plasma drug—metabolite profile 6-h post chronic oral dosing at 100 mg/kg/day. Conditions: column, Whatman Partisil; mobile phase, chloroform—methanol—concentrated ammonia (90:9.4:0.6); detection, UV at 254 nm, 0.005 a.u.f.s.; recorder, 5 mV; flow-rate 2 ml/min.

of I using II as the internal standard in the assay for preclinical biopharmaceutic—pharmacokinetic studies in the dog (Fig. 2A and B).

The GC—NPD assay was only sufficiently sensitive for monitoring plasma concentrations in the dog at doses of 10 mg/kg or higher. Thus, the need for a more sensitive and specific assay for I capable of also determining I-C (a pharmacologically active metabolite), for eventual clinical pharmacokinetic evaluation of the drug led to the investigation of GC—MS analysis as the method of choice since it has found extensive application in the analysis of narcotics and related analogues [21,22].

Methane—ammonia PCI mass spectra of I using the analogue II as the internal standard (during the early phases of method development), gave intense $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 348 (II), respectively with potential low nanogram sensitivity. This lead was developed into a validated method once the 3-pentadeuterophenoxy-N-methylmorphinan analogue (III) was synthesized, monitoring the $[MH]^+$ ions at *m/z* 334 (I) and *m/z* 339 (III), respectively, for quantitation (Fig. 3).

The methane—ammonia full-scan GC—PCI-mass spectra were determined

using a Finnigan 9500 gas chromatograph coupled to a Finnigan 1015 mass spectrometer [modified in house for PCI analysis [28]] equipped with a Finnigan 6000 computer for spectral analysis. Methane (carrier gas) was introduced at a head pressure of 5 p.s.i.g., and ammonia (reagent gas) was introduced into the ion source at a total source pressure of 0.45 Torr. The operating temperatures ranged as follows: column (225—265°C), injection port (275—300°C) and the interface oven was at 250°C, depending on the individual compound analyzed. The GC effluent was vented for 30—45 sec before the sample was introduced into the ion source of mass spectrometer.

The analysis of the phenolic metabolites I-B and I-C was attempted as the intact compound, and following derivatization either via silylation (O-trimethylsilyl, O-TMS) or extractive alkylation with PFBB using TBAHS in 0.2 *N* sodium hydroxide as the counter ion to yield the corresponding O-PFB derivatives [23], amenable to GC—PCI-MS analysis.

GC—PCI-MS analysis of the phenolic metabolites I-B and I-C

Intact levorphanol (I-C). The analgesic activity of this compound [27] focused attention on its quantitation to demonstrate its presence at pharmacologically significant concentrations in dog plasma. The determination of levorphanol was accomplished using compound IV (levallorphan) as the internal standard (originally added as the carrier substance in the assay for I). The sample remaining after the analysis of I was re-analyzed, at a lower temperature (225°C) and the $[MH]^+$ ions at *m/z* 258 (I-C) (Fig. 6A) and *m/z* 284 (IV) (internal standard) were monitored for quantitation as shown in the

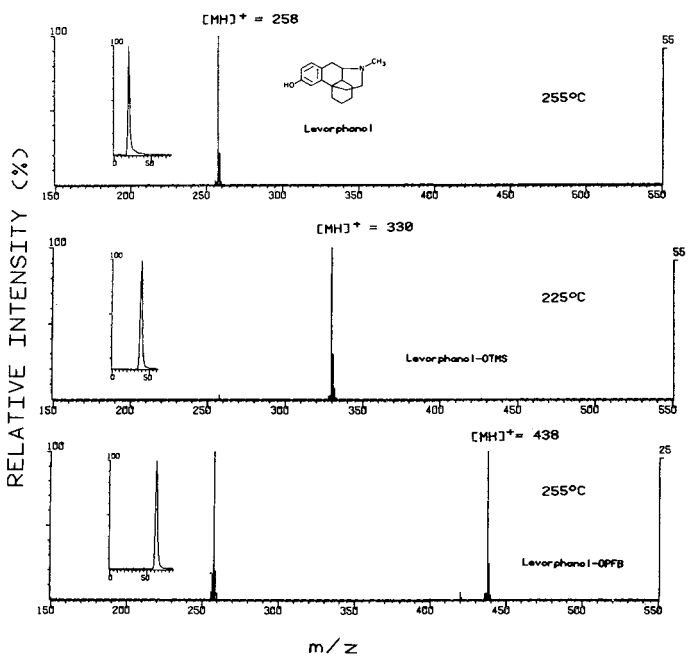

Fig. 6. Methane—ammonia-PCI-mass spectra of levorphanol, levorphanol—O-TMS and levorphanol—O-PFB showing the $[MH]^+$ ions at *m/z* 258, *m/z* 330 and *m/z* 438, respectively. The inserts represent the total ion chromatogram for each compound or derivative.

ion chromatograms (Fig. 4B), at their respective retention times of 1.53 and 3.92 min. The sensitivity limit of quantitation was about 2.5 to 5.0 ng of levorphanol per ml of plasma with an overall recovery of about 50%.

The tailing chromatographic peak for levorphanol indicated the need for derivatization of the phenolic OH group to improve its chromatographic behavior and sensitivity to detection. This was attempted via either silylation or extractive alkylation with PFBB. The former derivative would only improve the chromatographic behavior of the compound by reducing the polarity of the phenolic group and its adsorption on the column. The latter derivative should also increase its sensitivity to GC—PCI-MS due to the introduction of the electron capturing PFB-electrophore.

Trimethylsilyl ethers of I-B and I-C. Varying concentrations of I-B and I-C (2.5—50.0 ng/ml) each containing 50 ng of IV (internal standard) were vacuum dried and treated with 100 μl of BSTFA in glass stoppered 15-ml conical centrifuge tubes, by heating in a water bath at 60—70°C for 1 h. The excess reagent was evaporated to dryness under nitrogen, the residue was reconstituted in 100 μl of chloroform, 10-μl aliquots of which were injected for GC—PCI-MS using the previously described parameters. The $[MH]^+$ ions monitored were: *m/z* 422 (I-B—O-TMS), *m/z* 330 (I-C—O-TMS) and *m/z* 356 (IV—O-TMS) (internal standard), respectively.

The column and injection port temperatures were lowered to 225°C and 250°C respectively, for the analysis of the TMS ethers. Although the formation of I-C—O-TMS in vitro was demonstrated; *m/z* 330 (Fig. 6B), the ion chromatograms of the O-TMS derivatives of I-B, I-C and IV following extraction from plasma were very weak indicating either poor reaction yield and/or chemical instability of the derivatives. However, I-C and IV can be analyzed without derivatization by sequentially monitoring the $[MH]^+$ ions at *m/z* 258 (I-C) and *m/z* 284 (IV) (internal standard) at 225°C, followed by raising the column temperature to 265°C for monitoring *m/z* 334 (I) and *m/z* 348 (II) (internal standard), respectively (Fig. 4B).

Pentafluorobenzyl ethers of I-B, I-C and IV. Direct reaction of I-B, I-C and IV with PFBB using diisopropylethylamine as the catalyst resulted in very low yields of the derivatives. However, extractive alkylation of these compounds dissolved in methylene chloride using PFBB as the alkylating agent and a solution containing 0.1 *M* TBAHS in 0.2 *N* sodium hydroxide as the counter ion for extractive alkylation [23], yielded useable derivatives for levorphanol (I-C) and levallorphan (IV), but not for I-B which apparently did not react.

Varying amounts of I-C (2.5—100 ng/ml) each containing 50 ng of [IV] (as internal standard) were dissolved in 6 ml of methylene chloride to which 10 μl of PFBB reagent was then added and extracted with 2 ml of a solution containing 0.1 *M* TBAHS in 0.2 *N* sodium hydroxide as the counter ion for extractive alkylation. The methylene chloride layer was washed with dilute acid (0.05 *N* sulfuric acid), followed by water to remove the acid, and evaporated to dryness. The residue was dissolved in 100 μl of methylene chloride and a 10-μl aliquot injected for analysis. The $[MH]^+$ ions at *m/z* 438 (I-C—O-PFB) and *m/z* 464 (IV—O-PFB) (internal standard) were monitored for the quantitation of I-C.

Methane—ammonia-PCI-mass spectra of I-C, I-C—O-TMS and levorphanol—O-PFB derivatives showing the $[MH]^+$ ions at *m/z* 258, *m/z* 330 and *m/z* 438, respectively, substantiated the formation of the derivatives (Fig. 6). The second major fragment at *m/z* 258 in the levorphanol—O-PFB mass spectrum is most likely due to rearrangement in the ion source resulting in the loss of the PFB fragment (*m/z* 179) and protonation to levorphanol (*m/z* 258). This competing reaction may reduce the overall potential sensitivity of the derivative.

Typical ion chromatograms (determined on a 1.83 m × 2 mm I.D. column containing 3% OV-1 on 100—120 mesh Gas-Chrom Q at 255°C) of authentic standards of I and II (internal standard) and of I-C and IV (internal standard) recovered from plasma and derivatized by extractive alkylation are shown in Fig. 7 demonstrating the feasibility of the technique for the simultaneous quantitation of phenoxymorphinan and levorphanol.

The overall data for I and I-C—O-PFB were reproducible and linear over the concentration range studied (r=0.9971). The procedure was recently modified

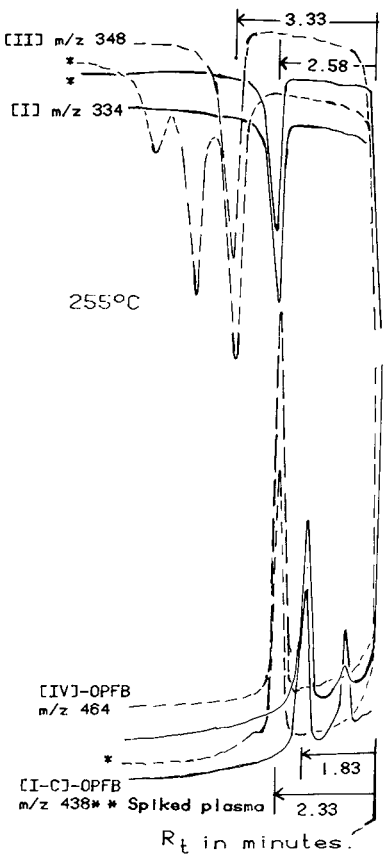

Fig. 7. Ion chromatograms of GC—PCI-MS analysis by selected ion monitoring, of I at *m/z* 334, internal standard (II) at *m/z* 348 and of the metabolite levorphanol (I-C) at *m/z* 438 and its internal standard levallorphan (IV) at *m/z* 464 as their respective pentafluorobenzyl ethers.

to yield a GC—negative chemical ionization (NCI)-MS assay specific for I-C per se as its PFB ester derivative using the trideuteromethyl analogue as the internal standard in the asssay [30].

Application of the methods to biological samples

Plasma samples (0—48 h) from a dog administered a single oral 10 mg/kg dose of I as the tartrate salt (in a hand packed gelatin capsule) were first analyzed by the GC—NPD method, plasma concentrations however, were below the sensitivity limit of the assay ($\leqslant$10ng/ml).

They were reanalyzed by GC—PCI-MS, and the plasma concentration—time profile of I showed an absorption peak of 9.6 ng/ml at 30 min post dose which decline biexponentially to the limit of detectability of the assay (i.e., 2 ng/ml) at 10 h, with an apparent half-life of elimination ($t_{1/2\beta}$) of 4.5 h. In order to evaluate plasma concentrations of the metabolite I-C, plasma samples (0—48 h) from a dog administered a higher oral dose of I·tartrate (three 100-mg tablets) equivalent to 24 mg (free base)/kg were analyzed by GC—PCI-MS for I and I-C using the direct extraction (underivatized) procedure. Plasma concentrations of I were initially seen at 12 min (5.3 ng/ml), reaching a maximum between 30—60 min (47.6 ng/ml) and declined to non-detectable amounts (<2.0 ng/ml) by 12 h (Fig. 8). I-C was detectable at 30 min (3.5 ng/ml), the concentrations gradually increasing to a peak (12.4 ng/ml) at 10 h and declined to 2.4 ng/ml by 73 h (Fig. 8). The concentration of I-C present in this dog was in the same therapeutic concentration range reported for levorphanol in cancer patients treated with this analgesic drug (L-Dromoran) by different routes of administration at doses ranging from 1 mg t.i.d. (i.m.) to 28 mg q.i.d. (oral) determined by radioimmunoassay [19,20], therefore its quantitation would be clinically relevant to the overall analgesic effect of the parent drug.

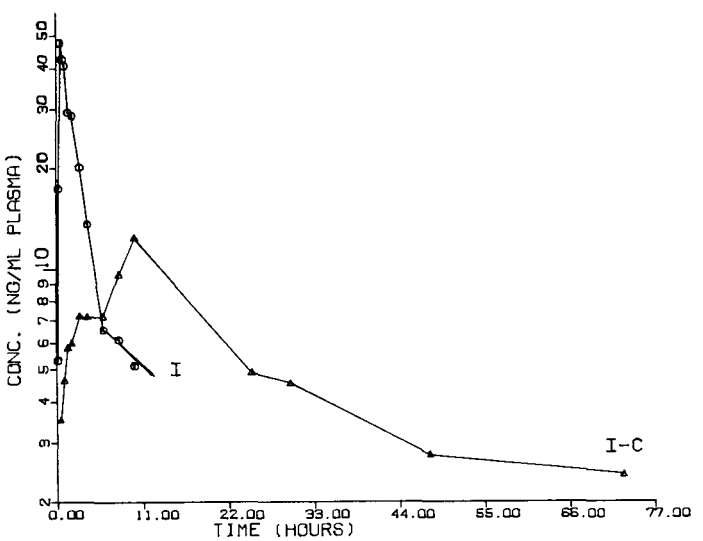

Fig. 8. Plasma concentration of I and metabolite I-C in a dog following a single oral dose of I·tartrate (equivalent to 24.1 mg (free base)/kg) determined by GC—PCI-MS analysis.

These data substantiate the fact that the GC—PCI-MS assay would be the method of choice for use in future clinical pharmacokinetic studies due to its ability to quantitate the parent drug (I) and a pharmacologically active metabolite levorphanol (I-C) with high sensitivity (ca. 2.0 ng/ml) and specificity.

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CHROMBIO. 1343

SEMI-AUTOMATED GAS CHROMATOGRAPHIC METHOD FOR THE ASSAY OF TIARAMIDE (RHC 2592) IN SERUM SAMPLES

HERSCHEL H.C. LI* and WILLIAM P. FEENEY

Drug Disposition Department, Research and Development Division, Revlon Health Care Group, Tuckahoe, NY 10707 (U.S.A.)

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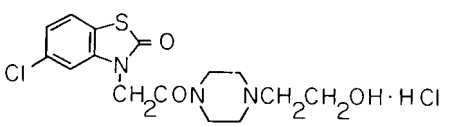
SUMMARY

A rapid and selective semi-automated gas chromatographic method, suitable for the routine assay of tiaramide in serum samples is described. The drug and the internal standard, perphenazine, were extracted with methylene chloride from alkalinized serum samples, and the trimethylsilyl ether derivatives were quantitated by a ^{63}Ni electron-capture detector. Linearity was observed for the range of 0.1—6.0 μg/ml. The average coefficient of variation for all concentration points over a two-week period was 8.5 ± 1.3%. Using an autosampler, the assay rate was 60 to 70 unknown samples in one man-day. The serviceability of the method has been demonstrated in a trial study in which an experimental tablet was given to three dogs. By taking a larger aliquot of the organic extract, a greater sensitivity can be attained if required. This was demonstrated by a trial study with human subjects.

INTRODUCTION

Tiaramide hydrochloride (RHC 2592; Revlon Health Care Group, Tuckahoe, NY, U.S.A.) (I, Fig. 1), 4-[(5-chloro-2-oxo-3(2H)-benzothiazolyl)acetyl]-1-piperazine-ethanol hydrochloride, is marketed in Japan for the treatment of inflammation [1]. Recent studies have shown that RHC 2592 also possesses antianaphylactic and bronchodilatory properties [2, 3]. This agent is now undergoing clinical trial as a new antiasthmatic drug in the U.S.A.



I

Fig. 1. Structure of tiaramide hydrochloride (RHC 2592).

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A gas chromatographic (GC) method for the assay of RHC 2592 in plasma and urine samples has been reported [4]. In this method, RHC 2592 was extracted with ethyl acetate and was then back-extracted with dilute hydrochloric acid. The internal standard was added to the acid extract which was then evaporated to dryness under reduced pressure before derivatization by silylation. The procedure according to this method was lengthy and the evaporation step was time consuming. Therefore, the method was not suitable for the routine assay of a large number of samples.

In order to support formulation development, a rapid and selective semi-automated GC method for the assay of RHC 2592 has been developed in this laboratory such that routine assay of a large number of pre-clinical samples from bioavailability studies in the dog became feasible. The present method permits the assay of 60 to 70 unknown serum samples in one man-day.

EXPERIMENTAL

Gas chromatography

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model Sigma-1 gas chromatograph was equipped with a ^{63}Ni electron-capture detector, and a data station for peak integration and chromatogram display. A 1.8 m × 0.2 cm I.D. glass column packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) was used.

The gas chromatograph was operated in the isothermal mode at 300°C, the injector at 315°C and the detector at 350°C. Nitrogen was used as the carrier gas and the detector purge gas, the respective flow-rates of which were 25 and 35 ml/min.

Reagents and standards

Acetonitrile, methylene chloride and ammonium hydroxide (Fisher Scientific, Fair Lawn, NJ, U.S.A.) were used as provided. Perphenazine (II), 4-[3-(2-chlorophenothiazin-10-yl, propyl]-1-piperazine-ethanol (gift from Schering, Kenilworth, NJ, U.S.A.) was selected as the internal standard, and Tri-Sil/BSA, Formulation P (Pierce, Rockford, IL, U.S.A.) was used as the derivatizing reagent. Control dog serum (Pel-Freez Biologicals, Rogers, AR, U.S.A.) was used for the preparation of standard curves. Standard solutions of RHC 2592 (10.00 mg%) and perphenazine (10.00 mg%) were prepared by dissolving the pure drug in distilled water and were stored at 5°C.

Procedure

A series of standard serum samples with respective concentrations of 6.0, 4.0, 2.0, 1.0, 0.4, 0.2 and 0.1 μg/ml was prepared by adding different aliquots of tiaramide hydrochloride standard solution into a series of PTFE-lined screw-capped tubes containing 1.0 ml of control dog serum. An aliquot of perphenazine standard solution (200 μl) was also added to each tube as an internal standard. These standard serum samples were used to generate a standard curve from which the unknown serum sample concentrations were calculated. Similarly, the same amount of the internal standard was added to serum samples of unknown tiaramide hydrochloride concentration.

To both the standard and unknown serum samples, 1 ml of water and 1 ml of 0.5 *N* ammonium hydroxide solution were added, and the samples were mixed gently. The extraction of the drug and the internal standard was carried out with an aliquot of 5.0 ml methylene chloride by shaking the tubes in a reciprocating shaker for 20 min. After phase separation by centrifugation, an aliquot of 0.5 ml of the organic extract was transferred to an autosampler vial. The extract was then evaporated to dryness at 35—40°C with a stream of nitrogen.

The dried extract was re-dissolved in 525 μl of acetonitrile and 75 μl of the derivatizing reagent. After the vials were sealed with a crimper and the contents were well-mixed, the vials were placed in a block heater at 75°C for 1 h to ensure complete reaction.

Sample injections were made by an auto-sampler (Perkin-Elmer). Sampling parameters specified an injection volume of 2 μl and a sampling time of 12 min. The 12-min time interval between injections allowed for proper detector re-equilibration.

RESULTS AND DISCUSSION

Under the present chromatographic conditions, the silylated derivatives of RHC 2592 and that of the internal standard (perphenazine) were eluted at 2.9 and 4.1 min, respectively. Fig. 2A is a typical chromatogram of a serum standard sample containing 2.0 μg/ml of RHC 2592 and 20.0 μg/ml of perphenazine. Fig. 2B shows the chromatogram of a serum sample from a dog

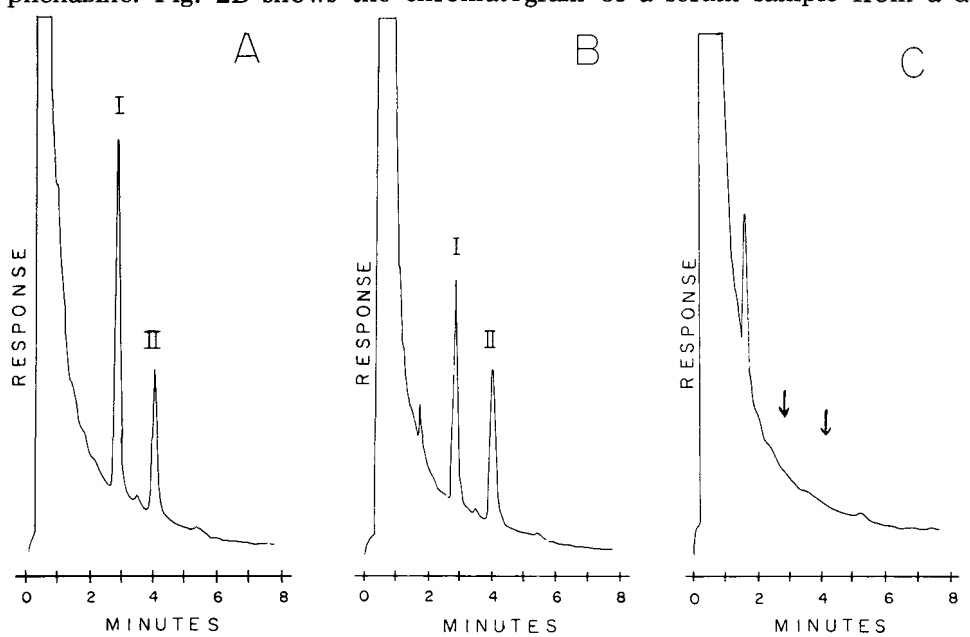

Fig. 2. Chromatograms of serum samples assayed as described. (A) Control dog serum sample which was spiked with 2.0 μg/ml of RHC 2592 (I) and 20.0 μg/ml of perphenazine (II) (internal standard); (B) serum sample from a dog 4.0 h after an oral dose of I, spiked with II; (C) pre-dose dog serum sample showing no interfering peaks at the retention times of I and II (indicated by arrows).

4.0 h post-dose. The peak at 2.9 min represents 1.1 μg/ml of RHC 2592. Fig. 2C is a typical chromatogram of a pre-dose serum sample from a dog. No interfering peaks were observed at the retention time of the derivatives of either RHC 2592 or perphenazine.

Gas chromatography—mass spectrometry

The silylated derivative of RHC 2592 from the extract of a serum standard sample, under the present reaction conditions, was investigated by GC—mass spectrometry (MS) using electron-impact ionization (Finnigan 4000 gas chromatograph—mass spectrometer, Finnigan, Sunnyvale, CA, U.S.A.). The GC—MS data show a molecular ion at *m/z* 427, a base ion at *m/z* 324, and fragmentation ions at *m/z* 412 and 198. The fragmentation ion at *m/z* 412 and the base ion at *m/z* 324 correspond to the loss of a $-CH_3$ and a $-CH_2-O-Si-(CH_3)_2$ group, respectively. The subsequent loss of a $-CO-N\langle\rangle N-CH_2$ group gave rise to the fragmentation ion at *m/z* 198. Based on the above GC—MS analysis, a mono-trimethylsilyl ether derivative of tiaramide was formed under the present reaction conditions.

The pooled serum extract of several dog samples obtained after an oral dose of 200 mg of tiaramide hydrochloride was derivatized and analyzed by GC—MS in a similar manner. The results show that the mass spectrum of tiaramide isolated from dog sera and the mass spectrum of synthetic tiaramide were identical. Based on these studies, the present GC method is considered specific for the assay of tiaramide in dog sera.

Linearity and reproducibility

For each day of sample analysis, a standard curve with seven concentrations was prepared. The regression equation, determined by linear regression analysis was used for the calculation of tiaramide concentrations in the unknown serum samples. Table I lists the regression equations for the standard curves obtained from four different days. The correlation coefficient of the composite standard curve was 0.992 indicating linearity for the range studied (0.1—6.0 μg/ml).

The values for the mean relative area ± standard deviation (S.D.) and the coefficient of variation (C.V.) for each concentration point from four separate runs are shown in Table II. The average coefficient of variation for all con-

TABLE I

PARAMETERS OF REGRESSION EQUATIONS FOR DAILY AND COMPOSITE STANDARD CURVES

Standard curve	Slope	*y*-Intercept	Correlation coefficient
A	0.9974	0.0660	0.998
B	0.8806	0.1384	0.993
C	1.0028	0.0205	0.990
D	0.9931	0.1403	0.993
Composite	0.9601	0.0917	0.992

TABLE II

REPRODUCIBILITY OF EACH CONCENTRATION POINT
Data from standard curves A, B, C and D.

Concentration (μg/ml serum)	*n*	Mean relative area ± S.D.	C.V. (%)
6.0	8	5.8734 ± 0.5806	9.9
4.0	8	3.9607 ± 0.3490	8.8
2.0	8	2.0698 ± 0.1585	7.7
1.0	7	1.1082 ± 0.0729	6.6
0.4	8	0.4631 ± 0.0382	8.2
0.2	8	0.2268 ± 0.0176	7.8
0.1	8	0.1052 ± 0.0108	10.3
		Average C.V. (%)	8.5 ± 1.3

centration points indicating the day-to-day variations for a period of approximately two weeks was 8.5 ± 1.3%.

Application

A trial study to demonstrate the serviceability of the method was conducted in three male beagle dogs weighing 9.5—12.2 kg. An experimental tablet containing 200 mg of the active ingredient was orally administered to the fasting dogs. Fig. 3 shows the composite serum profile of the three dogs.

In another trial study, three human subjects were given an oral solution of 250 mg of RHC 2592. The serum samples were processed by the same procedure with the exception that 2.0 ml of the organic extract was taken in order to increase the assay sensitivity. As shown in the composite human serum profile (Fig. 4), the method was sufficient to assay the 8.0-h samples at 0.06 μg/ml.

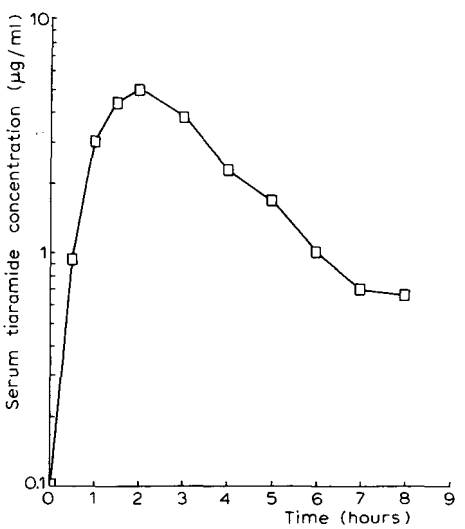

Fig. 3. A composite serum concentration—time profile of three dogs which had received an oral dose of an experimental tablet (200 mg RHC 2592).

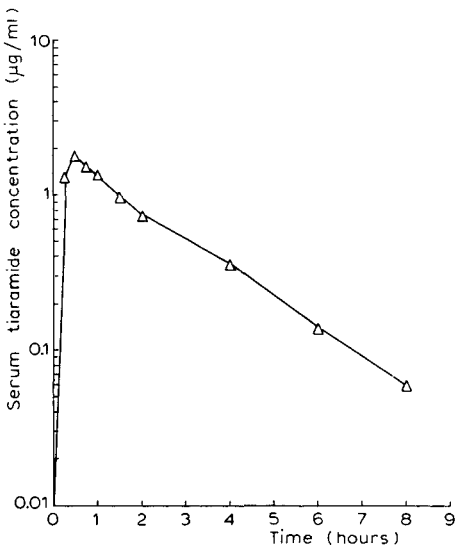

Fig. 4. A composite serum concentration—time profile of three human subjects who had received an oral solution of 250 mg RHC 2592.

CONCLUSION

The present method, as compared to the method reported previously, employed a simpler single extraction. The sensitivity was 0.1 μg/ml when an aliquot of one-tenth of the total extract was used for the assay. With a larger aliquot, as shown in the trial study with human subjects, a greater sensitivity can be attained if required.

Experience in this laboratory in the assay of unknown samples from bioavailability studies demonstrated that the combination of the simplified extraction procedure and the use of an auto-sampler has greatly increased the assay capacity; 60 to 70 unknown samples can be assayed in one man-day.

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CHROMBIO. 1364

SENSITIVE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CINNARIZINE AND FLUNARIZINE IN BIOLOGICAL SAMPLES

R. WOESTENBORGHS*, L. MICHIELSEN, W. LORREYNE and J. HEYKANTS

Department of Drug Metabolism and Pharmacokinetics, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse (Belgium)

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SUMMARY

A sensitive method has been developed for the determination of the vasoactive compounds cinnarizine and flunarizine in plasma, urine and milk samples from man and animals. The procedure involves the extraction of the drugs and their internal standard from the biological samples at alkaline pH, back-extraction into sulphuric acid and re-extraction into the organic phase (heptane—isoamyl alcohol).

The analyses were carried out by gas chromatography using a nitrogen-selective thermionic specific detector. The detection limit was 0.5 ng/ml of biological fluid and extraction recoveries were sufficiently high (87—94%).

The method was applied to plasma samples from bioavailability studies of both cinnarizine and flunarizine in healthy volunteers, and to plasma, urine and milk samples from flunarizine-treated dogs.

INTRODUCTION

Cinnarizine, (*E*)-1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)piperazine (I, Fig. 1), and its difluoro analogue flunarizine, (*E*)-1-[bis(4-fluorophenyl)-methyl]-4-(3-phenyl-2-propenyl)piperazine (II, Fig. 1) are Ca^{2+}-entry blockers, both widely used in the treatment of cerebral and peripheral vascular insufficiency [1].

Gas chromatographic (GC) methods [2—4] and a high-performance liquid chromatographic (HPLC) procedure [5] have been described for determining cinnarizine in biological samples with detection limits ranging from 2 to 10 ng/ml. No determination method for flunarizine has been described thus far.

The present paper describes a more sensitive GC method for both cinnarizine and flunarizine in biological samples. The method was used to obtain more

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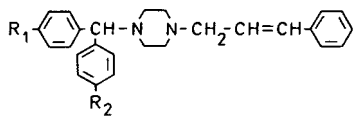

Compound	R_1	R_2
I	H	H
II	F	F
III	F	Cl

Fig. 1. Chemical structure of cinnarizine (I), flunarizine (II) and the internal standard (III).

detailed information about the pharmacokinetics of the drugs in man and animals.

EXPERIMENTAL

Reagents

Cinnarizine (R 516 base), flunarizine hydrochloride (R 14 950) and the internal standard (R 13 415), (*E*)-1-[(4-chlorophenyl)(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine dihydrochloride (III, Fig. 1), were obtained from the Life Sciences Products Division of Janssen Pharmaceutica (Beerse, Belgium) and were of analytical grade.

Spectrophotometric grade *n*-heptane and methanol were used (Uvasol; E. Merck, Darmstadt, G.F.R.). All the other reagents were of analytical grade. The inorganic reagents were prepared in double-distilled water. A borate buffer (pH 8.5) was prepared by adding 15.2 ml of 0.1 *M* hydrochloric acid to 50 ml of 0.025 *M* sodium borate decahydrate (borax). A final volume of 100 ml was prepared.

Standard solutions

Stock solutions, corresponding to 1 mg of the free base per ml of methanol, were prepared for compounds I, II, and III. Standard solutions were obtained by diluting the stock solution of I and II to concentrations ranging from 0.01 to 20 μg/ml of methanol.

To spike the samples with the internal standard, the stock solution of III was further diluted to 1 μg/ml.

Extraction procedure

Plasma. Two millilitres of plasma (unknown samples, drug-free plasma, or plasma standards containing known amounts of the drugs) were transferred to 15-ml glass centrifuge tubes, spiked with 0.1 μg of the internal standard and buffered with 2 ml of the borate buffer solution (pH 8.5). After addition of 4 ml of a heptane—isoamyl alcohol mixture (98.5:1.5, v/v), the tubes were carefully rotated for 10 min (10 rpm, Cenco rotary mixer) and then centrifuged (5 min, 1000 *g*). The upper organic layer was transferred to a second

centrifuge tube by means of a disposable pasteur pipet and the plasma was extracted again with 4 ml of the heptane—isoamyl alcohol mixture. The combined organic layers were back-extracted with 3 ml of 0.05 *M* sulphuric acid and removed after centrifugation. The remaining acidic phase was made alkaline with 0.15 ml of concentrated ammonia and extracted twice with 2-ml aliquots of the heptane—isoamyl alcohol mixture. The combined organic layers were finally evaporated to dryness under a gentle stream of nitrogen in a water bath at 55°C.

Urine and milk samples. Volumes of 1 ml were processed in the same way as the plasma samples.

Calibration procedure

Using the cinnarizine and flunarizine standard solutions, separate series of blank control plasma samples (2 ml) were spiked with either I or II at concentrations ranging from 0.0005 to 2 μg/ml, and with the internal standard at a fixed concentration of 0.05 μg/ml. These calibration plasma samples were then taken through the extraction procedure described above.

In the same way, standard curves for both cinnarizine and flunarizine were prepared in blank control milk and urine samples.

Apparatus

The instrument used was a Varian Model 3700 gas chromatograph equipped with the thermionic specific detector containing an electrically heated ceramic-alkali bead. The glass column (100 × 0.2 cm) was packed with 3% OV-17, coated on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The column, injector and detector temperatures were maintained at 275°C, 300°C and 340°C, respectively. Nitrogen was used as a carrier gas at a flow-rate of 25 ml/min. The detector was operated at a bias voltage of −4 V and the bead heating current was adjusted to 3 A, corresponding to a bead temperature of about 800°C. To ensure optimal detectability of the investigated compounds, the detector bead was in the path of a gas stream comprising hydrogen and air at flow-rates of 4.5 and 175 ml/min, respectively. A Spectra-Physics Model 4000 data system was used for the integrations, calculations and plotting of the chromatograms.

Gas chromatography

The various extraction residues were redissolved in 50 μl of methanol by vigorous vortexing. Sample volumes of 0.5—2 μl were then directly injected into the gas chromatograph.

Calculations

Ultimate sample concentrations were calculated by determining the peak area ratios of cinnarizine or flunarizine, related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of the calibration samples.

TABLE I

EXTRACTION RECOVERIES FOR CINNARIZINE AND FLUNARIZINE FROM BIOLOGICAL SAMPLES

Compound	Percentage recovery* (mean ± S.D., n = 5)		
	Plasma	Milk	Urine
Cinnarizine (I)	93 ± 3	91 ± 4	94 ± 3
Flunarizine (II)	89 ± 2	91 ± 4	87 ± 5

*Percentage recovery = recovery of the extraction procedure, obtained after analysis of 100 ng of the appropriate compound added to 2 ml of control plasma.

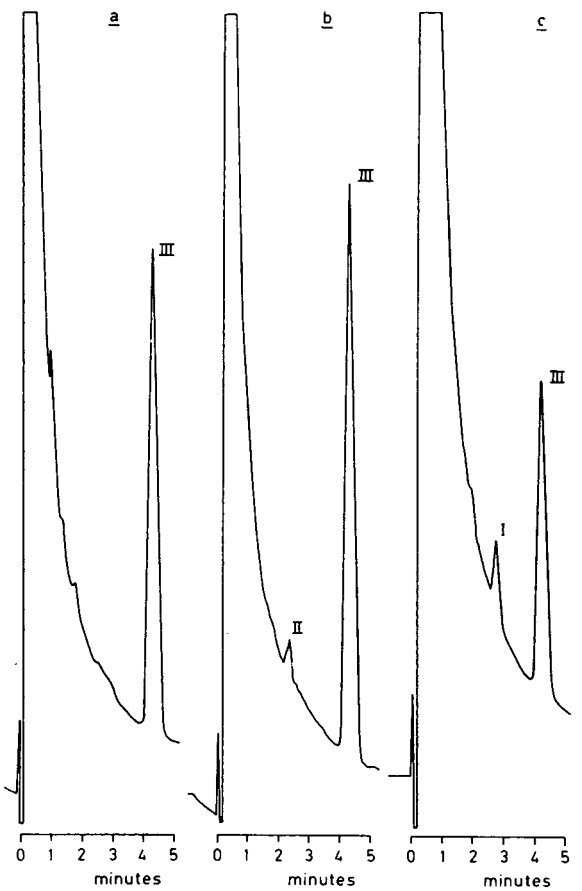

Fig. 2. Chromatograms of extracts from (a) blank control plasma, (b) plasma from a volunteer, seven days after oral intake of 30 mg of flunarizine, and (c) plasma from a volunteer, 30 min after oral intake of 20 mg of cinnarizine. I = cinnarizine (1.67 ng/ml); II = flunarizine (1.02 ng/ml); III = internal standard (50 ng/ml for chromatograms a and b; 40 ng/ml for chromatogram c). Chromatographic conditions were as indicated in the text.

RESULTS

The recoveries of the extraction procedure for cinnarizine and flunarizine (100 ng) from 2-ml control plasma, urine and milk samples are summarized in Table I.

Fig. 2 shows that no interfering peaks occurred at the retention times of cinnarizine, flunarizine or their internal standard under the described chromatographic conditions. All compounds were eluted as completely separated symmetrical peaks. The retention times for compounds I, II, and III were 2.8, 2.3, and 4.3 min, respectively. A linear relationship (r = 0.9999) was found when the ratios of the peak area of cinnarizine and flunarizine to the peak area of their internal standard (50 ng/ml) were plotted on the y-axis against various concentrations of either cinnarizine or flunarizine (in ng/ml) on the x-axis. Equations by the least-squares method were $y = 0.037\,x + 0.007$ for cinnarizine and $y = 0.035\,x - 0.020$ for the flunarizine calibration samples.

The accuracy and precision of the procedure was ascertained by adding different amounts of I and II to drug-free plasma and analyzing four samples of each concentration with the method described. The results are summarized in Tables II and III.

The detection limit was 0.5 ng/ml for both investigated compounds.

The method described has been used in a bioequivalence study of two flunarizine formulations (a 5-mg capsule and a 10-mg tablet) in healthy volunteers. Mean plasma levels for six subjects receiving oral doses of 30 mg of flunarizine in either dosage form are shown in Fig. 3. The method allowed the quantification of the plasma levels up to 28 days in all but two subjects and thus enabled the accurate measurement of the plasma elimination half-life ($t_{1/2\beta}$) which appeared to be 18.3 ± 8.47 and 17.3 ± 5.75 days for the capsule and tablet formulation, respectively. The method has also proved to be valuable for the determination of flunarizine in plasma, milk and urine of dogs orally treated with a single 20 mg/kg doze of flunarizine [6, 7].

TABLE II

ACCURACY AND PRECISION OF THE GC METHOD FOR THE DETERMINATION OF CINNARIZINE IN PLASMA SAMPLES

Theoretical cinnarizine plasma concentration (ng/ml)	Observed cinnarizine plasma concentration (ng/ml, mean ± S.D., n = 4)	C.V. (%)*	Accuracy
0.5	0.59 ± 0.05	9.0	118.0
1	0.89 ± 0.07	7.4	89.0
2.5	2.71 ± 0.15	5.4	108.4
5	4.85 ± 0.30	6.1	97.0
10	9.86 ± 0.38	3.9	98.6
25	24.5 ± 1.0	4.0	98.0
50	51.3 ± 1.1	2.2	102.6
100	100.6 ± 2.8	2.8	100.6
250	247.0 ± 4.7	1.9	98.8

*C.V. = coefficient of variation.

TABLE III

ACCURACY AND PRECISION OF THE GC METHOD FOR THE DETERMINATION OF FLUNARIZINE IN PLASMA SAMPLES

Theoretical flunarizine plasma concentration (ng/ml)	Observed flunarizine plasma concentration (ng/ml, mean ± S.D., n = 4)	C.V. (%)	Accuracy
0.5	0.56 ± 0.08	14.7	111.3
1	1.16 ± 0.15	12.6	116.0
2.5	2.64 ± 0.34	12.9	105.5
5	4.77 ± 0.40	8.4	95.4
10	10.7 ± 0.8	7.7	106.5
25	23.5 ± 2.2	9.3	93.9
50	48.5 ± 3.4	7.0	97.0
100	97.4 ± 6.3	6.5	97.4
250	229.4 ± 14.7	6.4	91.8
500	517.7 ± 31.6	6.1	103.5
1000	992.9 ± 4.0	0.4	99.3

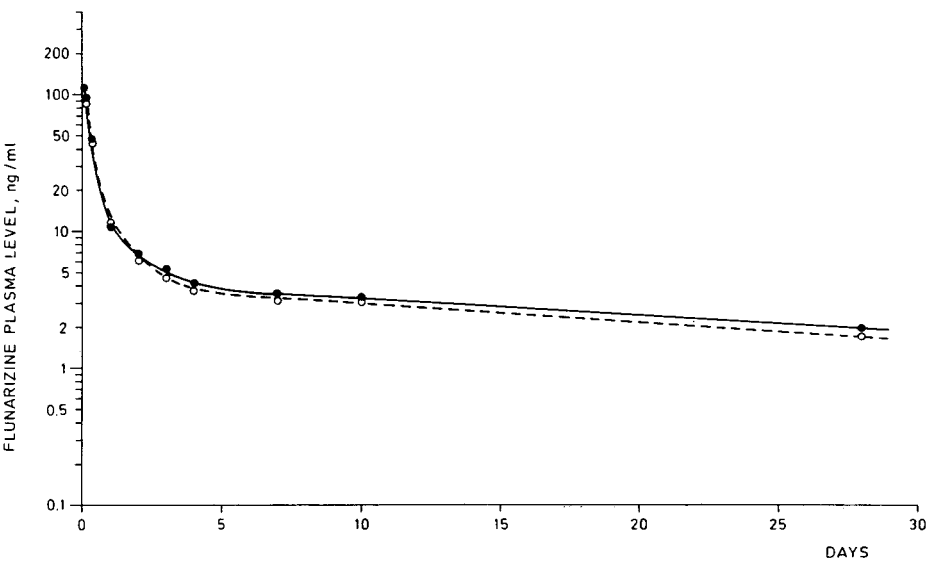

Fig. 3. Mean plasma levels of flunarizine up to 28 days after 30-mg doses either as 5-mg capsules (●) or as 10-mg tablets (○).

Cinnarizine plasma levels were measured in five healthy subjects up to 8 h after oral intake of 20 mg of the drug. The results are presented in Table IV.

DISCUSSION

The solvent system heptane—isoamyl alcohol (98.5:1.5, v/v) was selected for the extraction of cinnarizine and flunarizine since it is efficient, and also since the amount of co-extractants and water which interfere in the chromato-

TABLE IV

CINNARIZINE PLASMA LEVELS IN FIVE HEALTHY VOLUNTEERS AFTER ORAL ADMINISTRATION OF A 20-mg CINNARIZINE TABLET

Time (h)	Cinnarizine (ng/ml, mean ± S.D.)
0.25	1.7 ± 0.9
0.50	2.4 ± 1.2
1	10.4 ± 6.0
2	31.6 ± 14.3
4	36.6 ± 15.7
6	27.1 ± 9.5
8	17.8 ± 6.9

graphic step are minimal. Recovery experiments with different alkaline buffer systems indicated a maximum extraction efficiency at pH 8.5. The application of the selected buffer system enabled the use of standard extraction conditions for plasma, milk and urine samples. A recovery above 85% was reached for all samples. Standardization of the method was also obtained by the use of the same internal standard and the application of the more universal thermionic specific detector.

Although one can expect some benefit from the use of the electron capture detector for the detection of flunarizine, the absolute lower limit of detection proved to be about 1 ng, being about 50 times the absolute lower limit of detection of the thermionic specific detector which was less than 20 pg.

The application of the method to the assay of a few hundred plasma, urine and milk samples demonstrated its suitability; interferences were not observed and the GC column proved to be stable under the conditions used for at least 2—4 weeks.

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ANALYSIS OF HYDROXYLATED AND DEMETHYLATED METABOLITES OF MEPHENYTOIN IN MAN AND LABORATORY ANIMALS USING GAS—LIQUID CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A. KÜPFER, R. JAMES, K. CARR and R. BRANCH*

Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232 (U.S.A.)

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SUMMARY

Separation of urinary mephenytoin metabolites was evaluated under various gas—liquid chromatographic (GLC) and high-performance liquid chromatographic (HPLC) conditions. A simple and rapid alkylation procedure is described for GLC using a nitrogen sensitive thermionic detector. The in situ formation of sodium methinylsulfinylmethide is used as base for the perpropylation of hydantoins and their metabolites. Normal-and reversed-phase HPLC of the underivatized compounds was performed using four different types of stationary phases. None of the GLC systems separated all the six hydantoin compounds tested, whereas, normal- and reversed-phase HPLC were able to obtain a complete separation of these compounds.

The major metabolites of mephenytoin were 5-phenyl-5-ethylhydantoin, 3-methyl-5-(4-hydroxyphenyl)-5-ethylhydantoin and 5-(4-hydroxyphenyl)-5-ethylhydantoin in man, rat, mouse, rabbit, and guinea pig. 3-Methyl-5-phenyl-5-(2-hydroxyethyl)-hydantoin and 3-methyl-5-(3-hydroxyphenyl)-5-ethylhydantoin are major metabolites in the dog.

INTRODUCTION

The antiepileptic drug, mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin), is methylated at position 3 of the hydantoin ring. The 5-phenyl-5-ethyl substitution makes it a close congener of diphenylhydantoin (DPH). Oxidative demethylation of mephenytoin yields the pharmacologically active metabolite, 5-phenyl-5-ethylhydantoin (PEH, nirvanol) [1, 2]. In man, this metabolite accumulates in the body and contributes to the pharmacological response [3, 4]. In addition, mephenytoin can be hydroxylated to a variety of products [3, 5—8] the predominant one being 3-methyl-5-(4-hydroxyphenyl)-5-ethyl-

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hydantoin (4-OH-M). There is stereoselective formation of this metabolite in man [9] with preferential formation of S-4-OH-M. The hydroxylated metabolites are rapidly excreted largely as glucuronides. Rapid renal excretion of hydroxylated products prevents their accumulation in plasma under normal circumstances [6, 9].

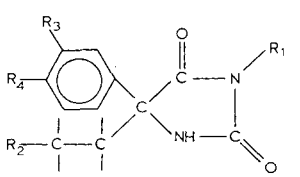
Quantitative assays for the determination of concentrations of mephenytoin, PEH and 4-OH-M by gas—liquid chromatography (GLC) [10—16] and high-performance liquid chromatography (HPLC) [6] have been published in several papers. However, a detailed description of the separation characteristics of major metabolites of mephenytoin using a variety of GLC and HPLC systems has not previously been presented. The objective of this paper is to present the results of our experience obtained while developing analytical methods for this group of compounds.

The thermionic nitrogen-sensitive detector provides a high sensitivity towards N-alkylated drugs and their metabolites [15]. As a consequence, we concentrated on alkylation of compounds to obtain suitable derivatives for GLC separation. In contrast, HPLC did not require derivatization and normal-phase chromatography was compared with reversed-phase chromatography. The assay methods developed have been used to investigate species differences in mephenytoin metabolism. The information presented in this paper should facilitate the selection of the appropriate GLC or HPLC procedure in the future investigation of mephenytoin dispostion in laboratory animals and man.

EXPERIMENTAL

Materials

The syntheses for mephenytoin [9], 4-OH-M [17], PEH, 5-phenyl-5-propylhydantoin (PPH) [17, 18], 5-(4-hydroxyphenyl)-5-ethylhydantoin (4-OH-PEH) [8] and 5-(4-bromphenyl)-5-ethylhydantoin (4-Br-PEH) [9] were carried out according to the references given in square brackets. The 3-OH- and 2-OH-isomers of 4-OH-M were kindly donated by Professor U.P. Schlunegger from the Department of Organic Chemistry at the University of Berne (Berne, Switzerland). 3-Methyl-5-(2-hydroxyethyl)-5-phenylhydantoin (OH-ethyl-M) was obtained from Professor T.M. Harris of the Department of Organic Chemistry at Vanderbilt University (Nashville, TN, U.S.A.) (Fig. 1). Details of synthesis nuclear magnetic resonance (NMR) and gas chromatographic—mass spectrometric (GC—MS) data of OH-ethyl-M will be reported in detail elsewhere. Trimethylanilinium hydroxide 0.1 *N* in methanol (TMA) was prepared as earlier reported [19].



R_1	R_2	R_3	R_4	
CH_3	H	H	H	Mephenytoin
CH_3	H	H	OH	4-OH-M
CH_3	H	OH	H	3-OH-M
CH_3	OH	H	H	OH-ethyl-M
H	H	H	OH	4-OH-PEH
H	H	H	H	PEH, Nirvanol

Fig. 1. Mephenytoin and its major metabolites in various laboratory animals and in man.

1-Iodoethane and 1-iodopropane were obtained from Eastman-Kodak (Rochester, NY, U.S.A.) for ethylation and propylation, respectively. The alkyl halides were redistilled to remove traces of the rapidly alkylating methyl iodide from the derivatization reaction. Dimethylsulfoxide (DMSO, methylsulfoxide) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) and dried over powdered calcium hydride (Fisher Scientific). Sodium hydride (NaH), obtained from Aldrich (Milwaukee, WI, U.S.A.) in a 50% suspension in mineral oil was washed with heptane prior to its use.

Methods

Racemic mephenytoin was administered to rats (60 mg/kg), mice (80 mg/kg), dogs (40 mg/kg), rabbits 80 mg/kg, guinea pigs (80 mg/kg) and man (5 mg/kg) and urine was collected for 24 h with the exception of the dog studies (8 h).

To one ml of urine either 2 μmol of PPH for GLC assay or 2 μmol of PPH, DPH, or 4-Br-PEH for HPLC were added as internal standards. Acid hydrolysis to liberate the aglycone was performed with hydrochloric acid (6 *N* final concentration) at 100°C for 2 h [20]. The hydrolyzed urines were extracted with 5 ml of ethyl acetate and the ethyl acetate was transferred to a dry test tube. The organic solvent was evaporated at 60°C under a stream of nitrogen. The residue was either dissolved in 200 μl of methanol and injected for HPLC or derivatized for GLC.

GLC separation

Compounds were injected in the GLC system underivatized or derivatized by peralkylation by methylation, ethylation, and propylation. On-column methylation, using trimethylanilinium hydroxide (0.1 *N*) in methanol (TMA), was performed as previously reported [19]. Ethylation and propylation were performed at room temperature using a modified Hakamori procedure [21—23], where the DMSO anion is formed in situ by direct addition of NaH to the final reaction mixture containing approximately 50 μg of the dry standard drug, 200 μl of dry DMSO, 50 μl of the iodoalkane and 50 mg of washed sodium hydride suspended in 1 ml of heptane. After 30 min of constant vortexing, the reaction was stopped by addition of 4 ml of water and the derivatives were extracted into 6 ml of diethyl ether. The diethyl ether was evaporated to dryness and after addition of 200 μl of methanol, an aliquot of 4 μl was injected into a perkin-Elmer Model 3920 B gas chromatograph equipped with temperature programmer and a thermionic nitrogen—phosphorous-sensitive detector (NPD, Perkin-Elmer). Stationary phases and temperature conditons are given in Table I. Carrier gas flow-rates and detector gas supply (air and hydrogen) were adjusted according to the manufacturers recommendations. Peak detection and retention times were obtained by a Varian CDS 211 C electronic integrator as well as by a standard pen recorder.

HPLC separation

A Waters Model 6000A solvent delivery system (Waters Assoc. Milford, MA, U.S.A.) equipped with a Waters Model 450 UV/VIS spectrophotometric variable-wavelength detector was used at a flow-rate of 1 ml/min under iso-

TABLE I

RETENTION TIMES (min) OF MEPHENYTOIN AND ITS METABOLITES UNDER VARIOUS DERIVATIZING AND GLC CONDITIONS

Column length and materials used: 1.8 m OV-17 5% on Chromosorb W HP 80—100 mesh (Applied Science Lab., State College, PA, U.S.A.); 1.8 m SP-2100 3% on Supelcoport 100—120 mesh (Supelco, Bellefonte, PA, U.S.A.).

Column	Derivatization	Temperature	MEPH	PEH	PPH	2-OH-M	3-OH-M	OH-ethyl-M	4-OH-M	4-OH-PEH
OV-17	None	220°C isothermal	3.4	6.5	—	—	—	9.2	11.0	—
OV-17	Methylation	200°C isothermal	5.6	5.6	6.6	11.3/13.0	12.9	11.0	16.0	16.0
OV-17	Propylation	230°C isothermal	2.2	3.5	4.1	—	6.6	6.0	8.8	12.3
SP-2100	Ethylation	8 min 180°C, 8°C/min to 220°C	3.6	4.0	—	8.4	9.6	11.6	10.8	11.6
SP-2100	Propylation	8 min 180°C, 8°C/min to 220°C	4.0	6.5	8.0	10.6	11.7	11.2	13.3	15.1

TABLE II

RETENTION TIMES OF UNDERIVATIZED MEPHENYTOIN AND ITS METABOLITES IN NORMAL- AND REVERSED-PHASE HPLC USING VARYING SOLVENT COMPOSITIONS

Column	Mobile phase			Retention time (min)										
				MEPH	PEH	2-OH-M	3-OH-M	OH-Ethyl-M	4-OH-M	4-OH-PEH	PPH	Phenobarbital	DPH	4-BrPEH
Normal-phase HPLC														
μPorasil	3% 2-propanol in heptane			7.5	12.0	9.6	15.3	85.2	28.2	39.9	8.7	8.1	9.3	11.0
Zorbax CN	11% 2-propanol in heptane			6.3	6.5	7.8	10.8	12.6	15.3	15.9	6.0	6.9	6.9	4.8
Reversed-phase HPLC														
	Water	Methanol	Acetonitrile											
Zorbax CN	400	—	100	18.3	10.5	10.5	10.3	7.5	9.0	6.6	—	—	—	—
Zorbax C_8	500	—	100	41.0	16.3	16.2	13.1	10.1	11.2	6.0	41.0	22.0	—	72.0
μBondapak C_{18}	140	100	—	12.0	8.3	—	7.5	7.0	6.5	4.7	12.8	—	—	21.0
μBondapak C_{18}	280	100	—	46.0	23.0	—	19.0	17.0	15.0	10.0	—	—	—	—
μBondapak C_{18}	400	100	—	—	—	—	34.0	30.0	26.4	13.2	—	—	—	—

cratic conditions. The columns used were (a) Waters μPorasil 10 μm particle size 30 cm × 3.9 mm and a Waters μBondapak C_{18} column (30 cm × 3.9 mm). The nitrilo column CN (cyanopropyl—silica) and the C_8 column were both of the Zorbax series (6 μm particle size, 25 cm × 4.6 mm) as commercially supplied by DuPont (Wilmington, DE, U.S.A.). Solvents were glass-distilled as obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Underivatized samples were analyzed after acid hydrolysis. Peak detection was performed at 211 nm according to a previously established UV scan of mephenytoin and PEH in methanol—water (1:1, v/v). Retention times were reported uncorrected according to the peak time obtained by measuring peak location on the strip chart recorder.

RESULTS AND DISCUSSION

Table I summarizes the retention times for mephenytoin, its various metabolites and the internal standard 5-phenyl-5-propylhydantoin (PPH) under various GLC conditions. In their underivatized form, the compounds separated well on the OV-17 column and we used this approach successfully for the structural analysis of urinary metabolites in conjunction with MS [6, 17]. This method had the advantages inherent with the injection of smaller, underivatized molecules with respect to GC—MS identification. However, there was relatively low sensitivity in comparison to alkylated derivatives. Furthermore, a pronounced tailing phenomenon made this procedure inadequate for quantitative metabolite determinations.

On-column methylation (flash-heater methylation) is a simple derivatizing procedure recommended for flame ionization detectors only. However, the TMA reagent is of limited use in connection with the thermionic nitrogen sensitive detector because the excess of TMA present (and its breakdown products) result in a high and prolonged background signal. The utility of this approach is also limited because the permethylated products of mephenytoin and PEH, as well as 4-OH-M and 4-OH-PEH are the same. A separate analysis of these compounds, however, can be achieved by the use of deuterated TMA in combination with MS, where the native and the added methyl groups are distinguishable by their isotopic composition [24].

An additional problem was encountered with the flash-heater methylation of 2-OH-M. This *ortho*-substituted phenolic compound consistently produced two peaks (Table I) of similar peak height ratios. This problem has not been pursued but it seems probable that steric hindrance of the *ortho*-OH-group prevents quantitative methylation, at least under the conditions of flash-heater methylation.

Ethylation derivatization was considered to be unsatisfactory in the separation of compounds as the separation between mephenytoin and PEH was small, and OH-Ethyl-M had the same retention time as 4-OH-M (Table I). In contrast, propylation provided baseline separation for mephenytoin, PEH, 3-OH-M, 4-OH-M, 4-OH-PEH, and internal standard on both OV-17 and SP-2100 columns; however, OH-ethyl-M separation from 3-OH-M was incomplete. Attempts to achieve the total separation of OH-ethyl-M from 3-OH-M was not

undertaken because these two metabolites could only be identified as major metabolites in the dog (see Table III). Thus, derivatization by propylation followed by column separation on either OV-17 and SP-2100 columns provided adequate methods for the development of quantitative assays for studies in other species.

Table II contains the retention times of the underivatized hydantoins in normal- and reversed-phase HPLC with varying qualitative and quantitative solvent compositions. Table II also contains the retention times for phenobarbital and DPH as potential internal standards since PPH was not an appropriate internal standard under all the various conditions presented. In addition, we evaluated 4-Br-PEH because it was used in the synthesis of 3-methyl-5-[^{3}H-phenyl]-5-ethylhydantoin by catalytic tritiation in some of our experiments [9].

For practical purposes, mephenytoin can be omitted in the urinary analysis of samples since less than 2% of the administered dose is excreted in urine within 24 h and 2-OH-M was not identified within the limits of GLC or HPLC detection in any of the species studied (Table III).

TABLE III

MAJOR PRODUCTS OF MEPHENYTOIN METABOLISM IN VARIOUS LABORATORY ANIMALS AND MAN

Species	Oxidative demethylation of mephenytoin	Aromatic hydroxylation to *meta*- or *para*-phenols	Aliphatic hydroxylation of mephenytoin	2-Hydroxy-mephenytoin (2-OH-M)
Man	+ mainly during chronic mephenytoin therapy	*para*	—	—
Dog	+	*meta* and *para*	+	—
Rat	+	*para*	—	—
Mouse	+	*para*	—	—
Guinea pig	+	*para*	—	—
White New Zealand rabbit	+	*para*	—	—

Complete separation of all samples of interest was obtained with normal-phase HPLC using a μPorasil column under isocratic conditions (Table II). However, long collection times were required for 4-OH-PEH and OH-Ethyl-M. The cyanopropyl column (Zorbax CN) in normal-phase chromatography did not fully separate 4-OH-M and 4-OH-PEH but had good resolution for the remaining compounds.

For reversed-phase chromatography, optimal resolution between compounds was obtained with the Zorbax C_8 column (Table II). Mephenytoin, PEH, and metabolites separated within a 17-min retention time. The reversed-phase cyanopropyl (CN) column provided baseline separation of all metabolites except for 3-OH-M and PEH. It was important to use acetonitrile in both C_8 and CN systems, as substitution with methanol yielded substantially lower resolution. Reversed-phase chromatography with the commonly used C_{18} column did not give baseline separation for 3-OH-M, 4-OH-M and OH-ethyl-M.

Resolution was not improved either by increasing the polarity of the solvents because corresponding peak width increased at at higher retention times, or by using acetonitrile instead of methanol. Thus, both normal-phase chromatography with a μPorasil column or reversed-phase chromatography with a C_8 column were adequate for quantitative assay development for all species. The latter system had the advantages of an easy solvent system do use, and a relatively short retention time for all compounds of interest except mephenytoin itself.

A qualitative assessment of urinary metabolites found in a number of species is presented in Table III. Two aspects deserve special attention. Firstly, the dog has a distinctive pattern of aromatic as well as of aliphatic hydroxylation of mephenytoin. In addition to 4-OH-M, the isomeric phenolic metabolite, 3-OH-M, as well as OH-ethyl-M represent major metabolic end products in this species. One of these routes of metabolism is analogous to DPH, where it has been shown, that the *meta*-isomer of the phenolic DPH metabolite is a major urinary constituent in addition to *para*-substituted phenol [25-27]. Secondly, in man, the demethylated metabolite PEH is a minor urinary metabolite in single mephenytoin does studies, but accounts for approximately 30% of the total metabolites present during chronic drug administration [4].

Thus, the requirements of the separation procedure are dependent on the range of metabolic products to be expected and this in turn is dependent on the species under investigation. If the species to be studied is known, then the information provided in this paper can be used to select an appropriate analytical separation procedure to develop a quantitative assay to study the fate of mephenytoin under various experimental conditions.

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DETERMINATION OF MORPHINE IN CEREBROSPINAL FLUID AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION*

RICHARD D. TODD*, SHEILA M. MULDOON and ROBERT L. WATSON

Department of Anesthesiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20014 (U.S.A.)

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SUMMARY

Two methods for the extraction of morphine from cerebrospinal fluid or plasma with quantitation by high-performance liquid chromatography with electrochemical detection were compared for accuracy, precision and ease of preparation. One procedure was a standard extraction procedure and the other utilized a commercially available liquid—liquid extraction column. Both methods produced linear calibration curves over the concentration range 1—200 ng/ml with coefficients of correlation of 0.999. Since the electrochemical detector is capable of detecting 20 pg of morphine, biological samples as small as 0.1 to 0.4 ml can be quantified with an average relative precision of 4.1 ± 3.9% over the concentration range 1—200 ng/ml. The potential clinical importance of the assay is demonstrated using a time course distribution study of morphine in the cerbrospinal fluid and plasma of a Rhesus monkey.

INTRODUCTION

In recent years, several sensitive methods for the determination of low levels of morphine in biological fluids have been reported. Radioimmunoassays (RIA) are capable of detecting picogram amounts of morphine, but they lack specificity and therefore cannot distinguish between morphine and compounds which are structurally related to morphine [1, 2]. Spectrofluorometric and radiolabeled morphine assays also have low specificity unless morphine is purified by chromatography prior to quantitation [3—5]. Assays using gas

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chromatography (GC) with electron-capture detection (ECD) require extraction and formation of a volatile morphine derivative prior to measurement [6—8]. The sensitivity of GC—ECD methods is comparable with that of radioimmunoassay [7]. Recently, Wallace et al. [9] reported the use of high-performance liquid chromatography (HPLC) with electrochemical detection (ElCD) to precisely determine morphine concentrations as low as 1 ng/ml in 1—2 ml of plasma with sensitivity exceeding that of GC—ECD methods [9].

In order to determine the pharmacokinetics of morphine in cerebrospinal fluid (CSF) and plasma after epidural administration of morphine sulfate, repeated sampling of the CSF and blood is required. GC—ECD and HPLC—ElCD methods require a minimum of 1 ml of CSF for each sample. The repeated sampling of such volumes from a small animal model cannot be performed without seriously impairing the physiological state of the animal.

We report on an HPLC—ElCD system which can accurately and precisely determine morphine levels as low as 1 ng/ml in 0.1—0.4 ml of CSF or plasma after extraction by a modification of Wallace et al.'s procedure [9]. An alternative extraction method is also presented which has the advantage of reduced sample preparation time. The application of the assay to the analysis of CSF and blood samples drawn following epidural administration of morphine sulfate is discussed.

EXPERIMENTAL

Reagents and materials

Methanol, acetonitrile, chloroform, isobutanol, and isopropanol were HPLC grade and used as received. Potassium monobasic phosphate (primary standard grade), boric acid and sodium borate (ACS grade) required no further purification. Clin-Elute CE-1001 extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). Morphine sulfate pentahydrate and nalorphine hydrochloride were purchased from Merck (Darmstadt, G.F.R.).

Chromatographic apparatus and conditions

The HPLC—ElCD system was composed of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery pump, Rheodyne injector equipped with a 200-μl sample loop, and a Waters μBondapak C_{18} column, 300 mm × 4 mm I.D., was used in conjunction with an amperometric detector system (LC-4A, Bioanalytical Systems, Lafayette, IN, U.S.A.). A guard column packed with Corasil-C_{18} was placed before the reversed-phase column. The electrochemical cell contained a glassy carbon working electrode modified as described by Moyer and Jiang [10], a stainless-steel auxiliary electrode, and a Ag/AgCl reference electrode. The working electrode was maintained at an applied potential of +0.79 V.

The chromatography was performed at ambient temperature using an isocratic mobile phase composed of 0.07 *M* KH_2PO_4 containing 0.5 m*M* EDTA and modified with 5% acetonitrile and 8% methanol. The flow-rate was 1.0 ml/min.

Determination of cell potential

The applied cell potential was determined by constructing a hydrodynamic voltammogram for the oxidation of morphine sulphate in the mobile phase.

Sample collection and preparation

All blood samples were drawn from an indwelling catheter with disposable syringes and transfered to Veneject® collection tubes containing Na_2EDTA. The tubes were centrifuged at 1600 *g* for 20 min and the plasma transfered to a polypropylene test tube, which was capped and stored at −80°C. CSF samples were collected in chilled polypropylene test tubes and stored at −80°C.

Extraction method

This procedure is a modification of the procedure of Wallace et al. [9]. CSF (0.1—0.4 ml) or plasma (0.4 ml) was added to a 14-ml polypropylene centrifuge tube containing borate buffer (0.1 *M*, pH 8.9, 0.5 ml) sodium chloride (0.25 g) and nalorphine (25 ng) as an internal standard. The morphine was extracted by adding chloroform—isobutanol (95:5, 9 ml) and the mixture was shaken for 30 min on a mechanical shaker. Organic and aqueous layers were separated by centrifugation (5 min, 900 *g*) and the aqueous layer removed by aspiration. Phosphate buffer (2 *M*, pH 10, 0.5 ml) was then added to the organic extract. The mixture was shaken for 10 min, centrifuged and the aqueous layer was removed by aspiration. This washing procedure was repeated a second time. Morphine was extracted from the organic phase by adding 0.5 *N* hydrochloric acid (3 ml) and shaking the mixture for 15 min. Following centrifugation, the aqueous layer was transfered to a polypropylene centrifuge tube (12 ml) and the pH adjusted to 8.9 ± 0.2 with solid potassium carbonate. Morphine was extracted from the aqueous phase with chloroform—isopropanol (95:5, 9 ml) by shaking the mixture for 30 min. The samples were centrifuged and the aqueous layer removed by aspiration. The organic extract was transfered to a 12 × 75 mm glass test tube and the solvent evaporated to dryness at 55°C under a stream of filtered air. The residue was reconstituted in the HPLC mobile phase (0.20 ml) and 10—100 µl injected into the HPLC system or the sample was stored at −80°C.

Extraction method II

Borate buffer (pH 8.9, 0.4 ml), nalorphine hydrochloride (25 ng) and plasma or CSF (0.1—0.4 ml) were applied to a Clin-Elute CE-1001 extraction column. After waiting 3 min for the sample to adsorb onto the column packing, chloroform—isopropanol (95:5, 5 ml) was added to the column and the eluate collected in a glass test tube. The extract was evaporated to dryness in a water bath maintained at 55°C under a gentle stream of filtered air. The residue was reconstituted in 0.2 ml of the HPLC mobile phase and 10—100 µl injected or the sample was stored at −80°C.

Determination of morphine in unknown samples

Morphine sulfate standards (1—200 ng/ml) were prepared with pooled human plasma. The plasma standards were then extracted and the peak height

ratios of morphine/nalorphine (M/N) obtained from chromatogams were plotted against the original morphine concentrations. Standard curves were determined by linear regression. Unknown samples were spiked with the same amount of internal standard and assayed. The morphine concentration of the unknown sample was determined from the calibration curve using the calculated peak height ratio (M/N).

Animal study

Rhesus monkeys were anesthetized with ketamine and equipped with indwelling catheters in the femoral vein and in the upper lumbar region of the spinal column. Morphine sulphate pentahydrate was dissolved in 0.9% saline and injected epidurally into the caudal space of the spinal column. Blood (3—5 ml per sample) was drawn for 6 h and CSF was continuously collected for 24 h. The samples were processed as described above.

RESULTS AND DISCUSSION

Determination of the electrochemical cell potential

The hydrodynamic voltammogram was established with two concentrations of morphine sulfate (Fig. 1). A voltage range was determined from a cyclic voltammogram of morphine sulfate dissolved in the mobile phase. The cyclic voltammogram contained an oxidation wave between +0.6 and +0.8 V using a pair of platinum electrodes. Allowing for different oxidation responses between platinum and glassy carbon electrodes, the potential range was expanded from +0.5 to +0.9 V for construction of the hydrodynamic voltammogram. The optimum applied cell potential was found to be +0.79 V.

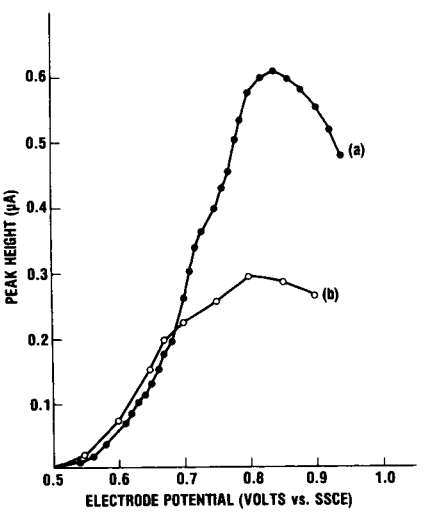

Fig. 1. Hydrodynamic voltammograms for morphine sulfate dissolved in the mobile phase at concentrations of (a) 1.32 μM and (b) 0.65 μM. Chromatographic conditions: column, μBondapak C_{18}; mobile phase, NaH_2PO_4 (0.07 M) with Na_2EDTA (0.5 mM)—methanol—acetonitrile (87:8:5, v/v), pH 4.5; flow-rate 1.0 ml/min; electrode potential, +0.79 V vs. Ag/AgCl reference electrode (SSCE); temperature, ambient.

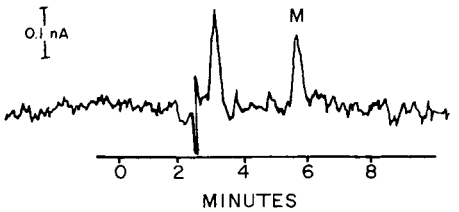

Fig. 2. Chromatogram of morphine sulfate (20 pg) injected into the HPLC system. Signal-to-noise ratio equals 4. Chromatographic conditions as in Fig. 1.

Sensitivity

The minimum amount of morphine sulfate detected by the electrochemical cell was 20 pg (signal-to-noise ratio 4, Fig. 2). When extracted from plasma (0.4 ml) the minimum quantifiable concentration of morphine was 1 ng/ml.

Electrochemical response

The glassy carbon electrode produced a linear response using a range of 20 pg to 10 ng of morphine sulfate when operated at the applied potential of +0.79 V. The electrode has remained stable for over five months without a significant decrease in sensitivity (<5%). However, an anomalous response to morphine sulfate was obtained with the glassy carbon electrode. A maximum cell response was reached at an applied potential of +0.84 V which decreased as the applied potential increased (Fig. 1). A similar electrochemical response is reported for the pesticide Aminocarb [11]. This hysteresis is reported to be the result of pacification of the electrode by polymerization of oxidation products onto its surface at the more positive potentials. At a lower concentration of morphine the hysteresis is still present but is not as pronounced. This observed phenomena may be due to less materials at the electrode surface.

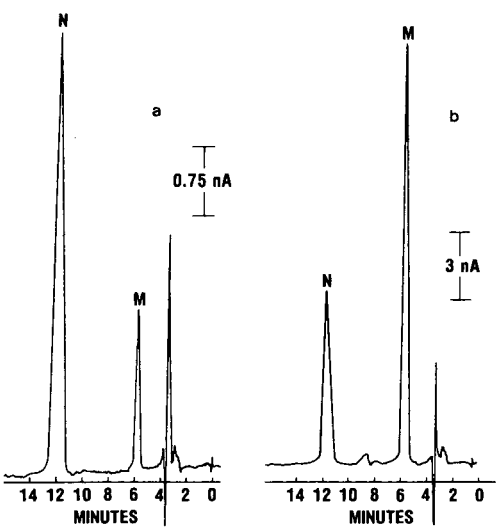

Fig. 3. Chromatograms of plasma (0.4 ml) extracted by method I: (a) plasma spiked with morphine (M, 10 ng/ml) and nalorphine (N, 25 ng) and (b) CSF drawn from a monkey 16 h after epidural administration of morphine sulfate (morphine concentration calculated to be 57.2 ng/ml). Chromatographic conditions as in Fig. 1.

Chromatography

Chromatogram obtained from samples of plasma and CSF after extraction (method I or II) show that both methods provide samples which are relatively free from interfering peaks (Figs. 3 and 4). Under the chromatographic conditions specified morphine and nalorphine eluted at 5.6 and 11.7 min, respectively; therefore a sample can be injected every 15 min.

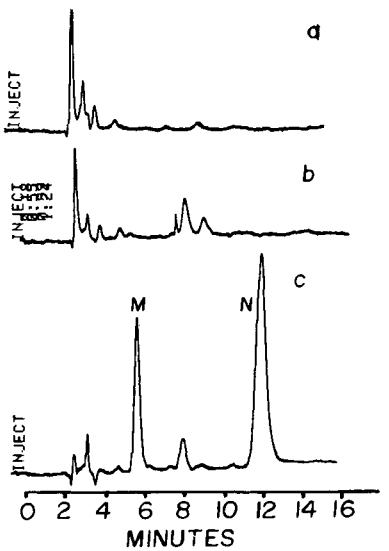

Fig. 4. Chromatograms of samples (0.4 ml) extracted by method II: (a) drug-free CSF, (b) drug-free plasma and (c) plasma containing morphine (M, 47 ng/ml) and nalorphine (N, 25 ng). Chromatographic conditions as in Fig. 1.

Extraction efficiency

Recoveries of morphine and nalorphine for each method were determined by extracting a plasma sample containing 100 ng/ml of morphine sulfate and 50 ng/ml of nalorphine hydrochloride. Six aliquots were assayed by each method and the peak heights compared to those obtained from a standard. The calculated mean recoveries for morphine and nalorphine were respectively 78.0 ± 4.7% and 79.0 ± 3.4% for method I and 84.8 ± 4.0% and 78.4 ± 2.2% for method II. The time required to prepare samples by each method for injection was compared. Using extraction method I 6 h were required to prepare 40 samples but when using extraction method II only 1.5 h were required.

Linearity

Calibration curves derived for morphine-spiked plasma prepared by extraction methods I and II were linear over the range 1—200 ng/ml (Table I and II). Correlation coefficients were typically 0.999. Accuracy decreased for samples containing less than 10 ng/ml of morphine when the above curves were used to quantify morphine. Therefore, to obtain optimal accuracy, the range of the calibration curve was decreased to 1—20 ng/ml.

TABLE I

PEAK HEIGHT RATIOS OF MORPHINE SULFATE (M) TO INTERNAL STANDARD, NALORPHINE HYDROCHLORIDE (N) OBTAINED WITH PLASMA SAMPLES EXTRACTED BY METHOD I

Values are expressed as the mean ± standard deviation.

Morphine concentration (ng/ml)	Peak height ratio (M/N, n = 4)	
1.25	0.04 ± 0.002	
2.50	0.10 ± 0.003	
10.00	0.38 ± 0.035	
20.00	0.71 ± 0.019	
50.00	1.75 ± 0.056	
100.00	3.41 ± 0.218	
200.00	6.84 ± 0.293	
	Range of curve (ng/ml)	
Linear regression analysis	1.25—20	1.25—200
Coefficient of correlation	0.999	0.999
Slope	0.035	0.034
y-Intercept	0.008	0.021

TABLE II

PEAK HEIGHT RATIOS OF MORPHINE SULFATE (M) TO INTERNAL STANDARD, NALORPHINE HYDROCHLORIDE (N) OBTAINED WITH PLASMA SAMPLES EXTRACTED BY METHOD II

Values are expressed as the mean ± the standard deviation.

Morphine concentration (ng/ml)	Peak height ratio (M/N, n = 4)	
1.07	0.02 ± 0.003	
5.60	0.10 ± 0.014	
9.90	0.18 ± 0.037	
22.80	0.36 ± 0.026	
47.60	0.71 ± 0.054	
104.00	1.51 ± 0.042	
200.00	3.00 ± 0.045	
	Range of curve (ng/ml)	
Linear regression analysis	1.07—22.80	1.07—200.00
Coefficient of correlation	0.999	0.999
Slope	0.016	0.015
y-Intercept	0.014	0.014

Precision and accuracy

The precision obtained with method I was determined in a blind study using a set of standards prepared by an independent laboratory. The precision, ex-

TABLE III

DETERMINATION OF THE WITH-IN RUN PRECISION OF EXTRACTION METHOD I WITH PLASMA SPIKED WITH MORPHINE SULFATE

Values are expressed as the mean and coefficient of variation.

Morphine concentration (ng/ml)		Coefficient of variation (%)
Theoretical	Calculated	
2.48	2.43	9.9
18.60	18.15	1.2
92.90	91.20	2.5
174.00	174.60	2.7

pressed as the average of the relative standard deviations obtained over the concentration range 2.5—200 ng/ml was 4.1% (Table III). Using standards prepared in our laboratory, the average precision obtained with method II over the concentration range 1.07—200.00 ng/ml was 9.2% with relative standard deviations of 7.1% at 22.8 ng/ml and 17.1% at 1.1 ng/ml. Analysis of a pooled plasma sample containing a morphine concentration of 7.29 ng/ml gave a value of 7.23 ± 0.34 ng/ml by method I (n = 5) and 7.34 ± 0.30 ng/ml by method II (n = 5).

Specificity

Plasma containing morphine and internal standard was spiked with codeine, acetominophen, or acetylsalicylic acid and assayed by methods I and II to determine whether these commonly used drugs interfere with the assay. At concentrations of 1 μg/ml no interference was found in the chromatography or quantitation of morphine. Retention times for the drugs tested were 8.7 and 8.8 min, respectively for acetominophen and codeine and no peak was detected within 20 min for acetylsalicylic acid.

Animal study

To illustrate the utility of the assay, unchanged morphine was quantitated in the plasma and CSF of Rhesus monkeys following an epidural administration of morphine sulfate. Blood and CSF samples were prepared for injection by extraction method I. The results obtained for one monkey are listed in Table IV.

Morphine entered the blood and CSF within 5—30 min. The maximum concentration of morphine in plasma occurred 10—15 min after epidural administration and declined rapidly ($t_{½}$, 0.5—1 h). Morphine fell below detectable limits after 4 h. In contrast, morphine reached maximal concentration in CSF within 30—35 min and disappeared more slowly ($t_{½}$ > 2 h). Morphine was still detectable in CSF 23 h after administration of the drug. This seems to correlate with the clinical observation that morphine administered epidurally in man provides pain relief for 16—24 h while the same dosage given intramuscularly provides pain relief for only 4—6 h [1, 12—14].

TABLE IV

PLASMA AND CSF MORPHINE LEVELS IN ONE RHESUS MONKEY FOLLOWING AN EPIDURAL ADMINISTRATION OF MORPHINE SULFATE (2 mg/10 kg OF BODY WEIGHT)

Plasma (0.4 ml) and CSF (0.15 ml) samples were prepared with extraction method I.

Time (h)	Morphine concentration (ng/ml)	
	Plasma	CSF
0.00	n.d.*	n.d.
0.25	35.6	n.d.
0.50	27.4	3714.0
1.00	18.6	3173.0
2.00	14.4	1261.0
4.00	9.4	553.2
8.00	n.d.	125.1
16.00	n.d.	22.0
23.00	n.s.**	10.0
24.00	n.d.	n.d.

*n.d. = None detected (< 1 ng/ml).
**n.s. = No sample.

CONCLUSION

In the development of pharmacokinetic profiles for drugs many samples must be drawn from the test animal; therefore it is necessary to utilize as small a sample as possible in order to not disrupt the physiological state of the animal. The precision and accuracy obtained with extraction method I makes it a most suitable method for analyzing small volumes of CSF and plasma. Method II has similar accuracy and precision but has the advantage of a faster analysis time. Extraction method II would be very useful to forensic laboratories requiring trace morphine analyses.

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CHROMBIO. 1352

DETERMINATION OF 7-AMINO-FLUNITRAZEPAM (Ro 20-1815) AND 7-AMINO-DESMETHYLFLUNITRAZEPAM (Ro 5-4650) IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

Y.C. SUMIRTAPURA, C. AUBERT, Ph. COASSOLO and J.P. CANO*

I.N.S.E.R.M. SC No. 16, Laboratoire de Pharmacocinétique et de Toxicocinétique, 27 Boulevard Jean Moulin, F-13385 Marseille (France)

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of 7-amino-flunitrazepam (Ro 20-1815) and 7-amino-desmethylflunitrazepam (Ro 5-4650) in plasma is described. After extraction with an organic solvent, the compounds and their internal standard (7-amino-methylclonazepam or Ro 5-3384) are derivatized with fluorescamine and chromatographed on a reversed-phase μBondapak C_{18} column using pH 8 buffer solution—acetonitrile (3:1) as mobile phase. The detection is performed by a fluorometer at excitation and emission wavelengths of 390 and 470 nm, respectively. The sensitivity limit is about 1 ng/ml of plasma for both 7-amino-flunitrazepam and 7-amino-desmethyl-flunitrazepam. The method has been applied to the determination of plasma levels of these substances during pharmacokinetic studies of flunitrazepam, desmethylflunitrazepam and 7-amino-flunitrazepam.

INTRODUCTION

Flunitrazepam is a benzodiazepine derivative which possesses all the activities typical of the class of compounds, namely anxiolytic, anti-convulsant, muscle-relaxant and central sedative effects which cause the onset of sleep, prolong the duration of sleep and tone down reactions to stress factors [1]. Its major metabolites found in the human plasma are 7-amino-flunitrazepam (Ro 20-1815, 7-AF) and desmethylflunitrazepam (Ro 5-4435), which are further metabolized to 7-amino-desmethylflunitrazepam (Ro 5-4650, 7-ADF) [2] (Fig. 1).

The determination of plasma levels of 7-AF and 7-ADF after administration of flunitrazepam, 7-amino-flunitrazepam or desmethylflunitrazepam provides important information/data for the psychopharmacological and

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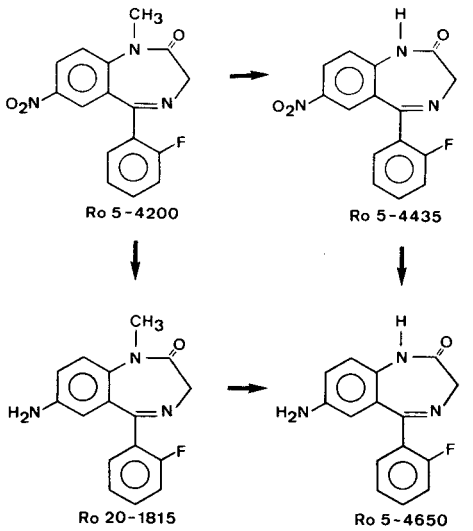

Fig. 1. Chemical structures of flunitrazepam and its major metabolites.

pharmacokinetic studies of these substances.

Several gas—liquid chromatographic methods are available for the determination of unchanged flunitrazepam and desmethylflunitrazepam [3—7], but 7-AF and 7-ADF cannot be measured by this technique at the concentrations that occur in human blood following therapeutic doses.

However, a thin-layer chromatographic assay for the determination of 7-AF and 7-ADF, involving derivatization with fluorescamine, has been developed by Haefelfinger [8]. Subsequently, the derivatized substances were quantified fluorometrically by direct scanning. The sensitivity limits reported were 1 ng/ml and 2 ng/ml of plasma for 7-AF and 7-ADF, respectively.

This paper describes a highly selective and sensitive high-performance liquid chromatographic method for the determination of 7-AF and 7-ADF involving fluorescamine derivatization and fluorescence detection. 7-Amino-methylclonazepam (Ro 5-3384, 7-AMC) was used as internal standard.

MATERIALS AND METHODS

Reagents and standards

All aqueous solutions were prepared with double-distilled water. Dipotassium hydrogen phosphate, sodium hydroxide, and pH 8 and pH 10 buffer solutions were obtained from Merck (Darmstadt, G.F.R.). Sulfuric acid "Ultrex" was provided by J.T. Baker Co. (Phillipsburg, NJ, U.S.A.). Acetonitrile, RS grade, and isoamyl alcohol, RPE grade, were purchased from Carlo Erba (Milan, Italy). Acetone, diethyl ether and hexane (Pestipur grade) were obtained from Solvant-Documentation-Synthese Co. (Peypin, France). Methanol was from Prolabo (Paris, France).

Fluorescamine solution (0.5 %, w/v) was prepared by dissolving a quantity of the solid substance (Fluram®, Hoffmann-La Roche, Basel, Switzerland) in acetone.

Standard substances of 7-amino-flunitrazepam, 7-amino-desmethylflunitrazepam and 7-amino-methylclonazepam were provided by Hoffmann-La Roche. Individual standard stock solutions (1 mg/ml) were prepared in acetone—methanol (1:1). Working solutions (varying from 0.01 to 10 ng/μl) were obtained by dilution with acetone—hexane (1:4).

Extraction and derivatization

Into a 20-ml glass-stoppered centrifuge tube, 20—500 ng of internal standard (7-AMC), 0.5—4 ml of plasma, 2 ml of pH 10 buffer solution and 10 ml of diethyl ether containing 1% of isoamyl alcohol were added. (The quantity of the internal standard added was about two times higher than that of 7-AF or 7-ADF to be analyzed; plasma volumes of 0.5, 1, 2, and 4 ml were used for plasma drug concentrations above 50 ng/ml, between 5 and 50 ng/ml, between 1 and 5 ng/ml and lower than 1 ng/ml, respectively.) The mixture was shaken on a three-dimensional shaker (type EM 4; Desaga, Heidelberg, G.F.R.) for 15 min and centrifuged for 5 min at 3750 *g*. The organic layer was transferred into another centrifuge tube containing 2 ml of 0.5 *M* sulfuric acid. The tube was shaken for 10 min and centrifuged for 5 min. The organic layer was discarded without removing any of the acid phase which was then adjusted to pH 9—10 with 1 *M* sodium hydroxide solution containing 1 *M* dipotassium hydrogen phosphate. The mixture was extracted with 10 ml of diethyl ether by shaking for 10 min and centrifuging for 5 min. The organic phase was transferred into another tube and evaporated to dryness in a 45°C water-bath under vacuum and a stream of nitrogen. To the residue, 100 μl of the mobile phase and 20 μl of 0.5% fluorescamine solution were added and the tube was mixed on a Vortex mixer for 60 sec. An appropriate volume of the aliquot was injected onto the column. (The volume to be injected was determined according to the detector sensitivity range used and the estimated drug concentration contained in the sample, to obtain an optimum detector response; with the detector we used, this could vary from 10 to 100 μl.)

Chromatographic conditions

A Waters Model M 45 pump and a Waters Model 6000A pump were used with a Waters Model 660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). One pump (A) contained pH 8 buffer solution (obtained by diluting 20 ml of concentrated pH 8 buffer solution to 1 l with double distilled water) and the other pump (B) contained acetonitrile. The solvent programmer was set to 74—75% of A and 26—25% of B for an isocratic mode. (The percentage of acetonitrile varied from 25 to 26%. It was adjusted to optimize the separation and the retention times of the compounds analyzed.) The mobile phase flow-rate was 2.5 ml/min.

The chromatography was performed on a 30 cm × 4 mm μBondapak C_{18} reversed-phase column (10 μm, Waters) at ambient temperature.

The detection was carried out with a Schoeffel Model GM 970 fluorometer (Schoeffel, Westwood, NJ, U.S.A.) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm.

Calibration curves

Standard calibration curves (ratios of 7-AF-fluorescamine or 7-ADF-fluorescamine to 7-AMC-fluorescamine peak areas versus concentrations of 7-AF or 7-ADF) were obtained by analyses of blank plasma samples to which known quantities of 7-AF and 7-ADF were added together with a constant amount of internal standard. The areas of the chromatographic peaks were calculated by electronic digital integration (Hewlett-Packard 3352 B Data System).

The quantities of 7-AF and of 7-ADF used for the establishment of the calibration curves were of the same magnitude as the concentrations of the compounds contained in the samples. For both compounds there was good assay linearity over the concentrations range tested (2.5—500 ng/ml of plasma) with correlation coefficients of > 0.999. Moreover, the regression curves practically passed through the origin.

RESULTS AND DISCUSSION

Derivatization

The quantity of reagent required for complete derivatization of 7-AF, 7-ADF and 7-AMC at concentrations below 500 ng/ml of plasma has been investigated. For this purpose, a set of mixtures, containing 500 ng of 7-AF, 500 ng of 7-ADF, 500 ng of 7-AMC and the extract of 4 ml of plasma blank were derivatized with 20 μl of fluorescamine solutions of various concentrations (0.25, 0.5, 1, 2.5, 5 and 10 μg/μl) using 100 μl of mobile phase as reaction medium. For each mixture, an identical volume of aliquot was injected onto the column and fluorescence was continuously recorded.

As can be seen in Fig. 2, the peak heights of fluorescamine derivatives of 7-AF, 7-ADF and 7-AMC are constant in the presence of fluorescamine at concentrations of 2.5 μg/μl and above. Consequently, we used routinely 20 μl of 0.5% (w/v) reagent solution for the quantitative determination of 7-AF and 7-ADF in our pharmacokinetic studies.

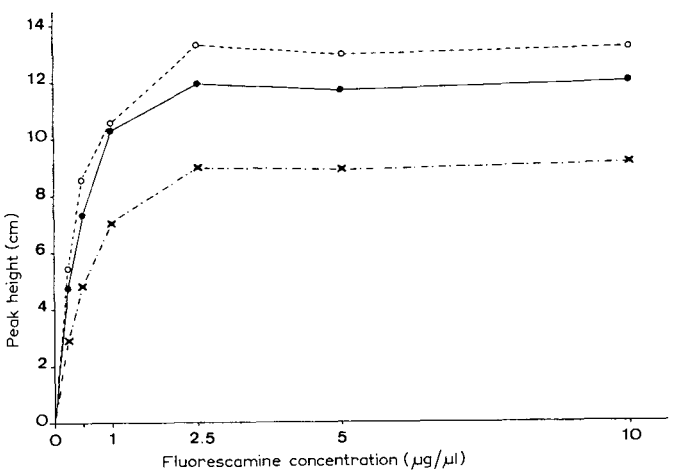

Fig. 2. Evolution of signal intensity observed for 7-AF-fluorescamine (•), 7-ADF-fluorescamine (○) and 7-AMC-fluorescamine (×) as a function of fluorescamine concentration.

Overall recoveries

Extraction characteristics of 7-AF and 7-ADF have been studied. Both compounds were quantitatively extracted with diethyl ether from an aqueous phase of pH 10 and they were quantitatively back-extracted into 0.5 *M* sulfuric acid from this solvent. The overall recoveries of 7-AF and of 7-ADF from plasma samples after complete extraction by this method were about 70% and 50%, respectively. These recoveries could be improved by performing twice extractions at the first step of extraction described above. In this case, the first portion of the ether phases must be concentrated.

Sensitivity

Using fluorescence detection and derivatization with fluorescamine, both 7-AF and 7-ADF can be detected at picogram levels. By this method, a relatively great volume of plasma sample can be utilized. Using 4 ml of the sample the sensitivity limit is about 0.5—1 ng/ml for both 7-AF and 7-ADF.

Selectivity

In this study, fluorescamine was chosen as derivatizing agent for 7-AF, 7-ADF and 7-AMC (internal standard) for several reasons: high reactivity, sensitivity and particularly for its high selectivity. Only primary amine compounds can react and produce an intensely fluorescent derivative with fluorescamine, while fluorescamine by itself is not fluorescent [9, 10]. Moreover, by performing back-extraction into an acidic medium, the interfering substances were limited to the basic compounds.

Chromatograms obtained from human and monkey plasma blanks show that no endogenous substances interfered with the compounds of interest.

By the present method, 7-AF and 7-ADF can be quantitatively analyzed in the presence of 7-amino derivatives of clonazepam and nitrazepam. The retention times of the five derivatized substances were about as follows: 7-ADF-fluorescamine (7-ADF-Fl), 4.5 min; 7-amino-clonazepam-Fl and 7-amino-nitrazepam-Fl, 6 min; 7-AF-Fl, 9.2 min; and 7-AMC-Fl, 13.6 min (Fig. 3). 7-Acetamido-flunitrazepam (Ro 20-9800), another possible metabolite of 7-AF, is not detected by the proposed method; we did not notice the acid hydrolysis of Ro 20-9800 to 7-AF during the extraction described above, especially during the back-extraction into 0.5 *M* sulfuric acid.

Furthermore, the selectivity of this assay has been tested with plasma of intensive care unit patients receiving several other drugs frequently administered to such patients [7]. Fig. 4 illustrates a representative chromatogram obtained from a patient in an intensive care unit.

Reproducibility

The reproducibility of the assay was determined at concentrations ranging from 1 to 500 ng/ml for 7-AF and from 1.25 to 50 ng/ml for 7-ADF, by assaying six plasma samples at each concentration on the same day. The results obtained (expressed as variation coefficient) are presented in Table I. The variation coefficients within the range tested varied from 3 to 9.8%.

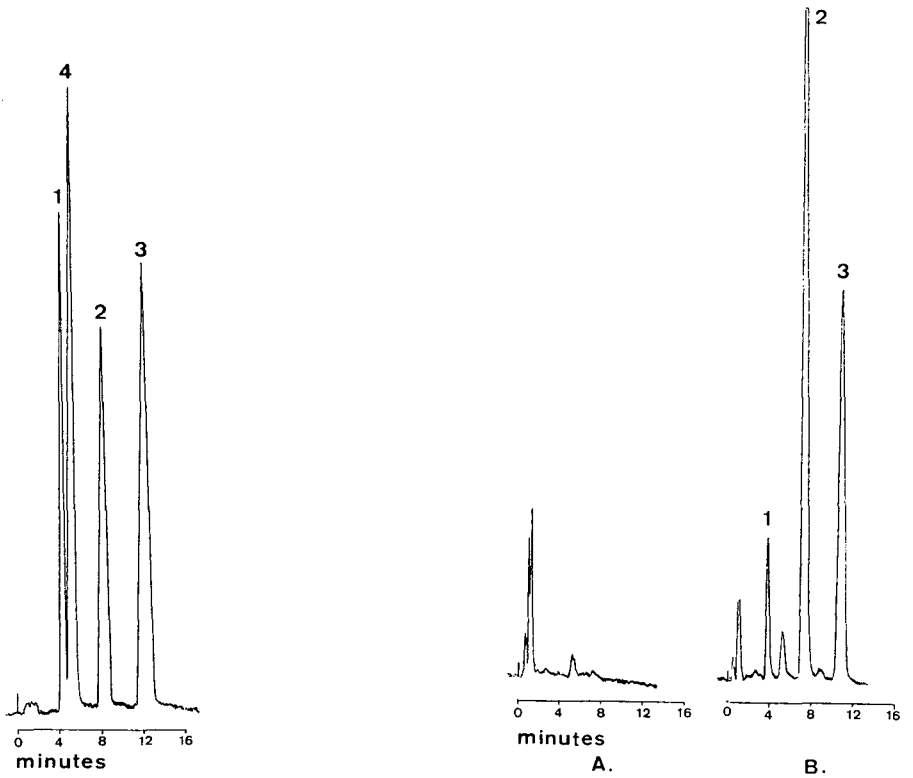

Fig. 3. HPLC separation of fluorescamine derivatives of 7-ADF (1), 7-amino-clonazepam (4), 7-amino-nitrazepam (4), 7-AF (2) and 7-AMC (3).

Fig. 4. Chromatograms of (A) plasma of a patient receiving diazepam, droleptan, dopamine and other drugs, 5 min prior to flunitrazepam administration, and (B) plasma of the same patient 8 h following the ninth intravenous injection of 2 mg of flunitrazepam (injections at time 0, 0.33, 2, 6, 10, 14, 22, 30 and 38 h) with addition of 15 ng/ml of 7-AMC. (Amount of plasma used = 2 ml; volume of the last aliquot injected = 50 μl; detector sensitivity range = 0.2 μA.) 1 = 7-ADF-Fl; 2 = 7-AF-Fl; 3 = 7-AMC-Fl.

TABLE I

REPRODUCIBILITY OF THE ASSAY

Compound	Concentration (ng/ml)	Amount of plasma used (ml)	Volume of the last aliquot injected (μl)	Coefficient of variation (%)
Ro 20-1815	1	4	100	4.7
	5	2	50	6.2
	50	1	25	5.0
	500	0.5	10	3.0
Ro 5-4650	1.25	4	100	9.8
	5	2	50	6.6
	50	1	25	7.9

Application to biological samples

The present method has been successfully applied to the assay of plasma levels of 7-AF and 7-ADF in man and monkey during pharmacokinetic studies of flunitrazepam, desmethylflunitrazepam and 7-amino-flunitrazepam.

The plasma level—time course of 7-AF and of 7-ADF following a single intramuscular dose of 1.2 mg/kg 7-AF in one monkey is presented in Fig. 5. In this study we observed significant differences between the pharmacokinetic behaviour of 7-amino-flunitrazepam in man from that observed in rhesus monkey. The drug clearance in this monkey is found to be approximately 0.94 l/h per kg of body weight. This value is about 20 times higher than that obtained in the human subject (0.05 l/h per kg). The elimination half-lives of

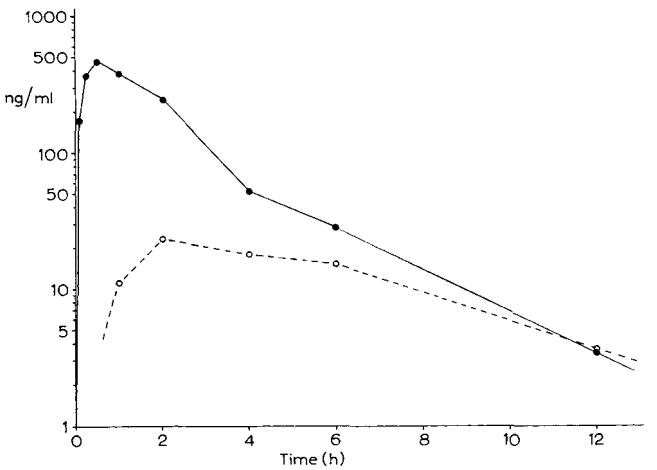

Fig. 5. Plasma level—time course of 7-AF (•) and 7-ADF (○) in a monkey after a single intramuscular dose of 1.2 mg/kg 7-AF.

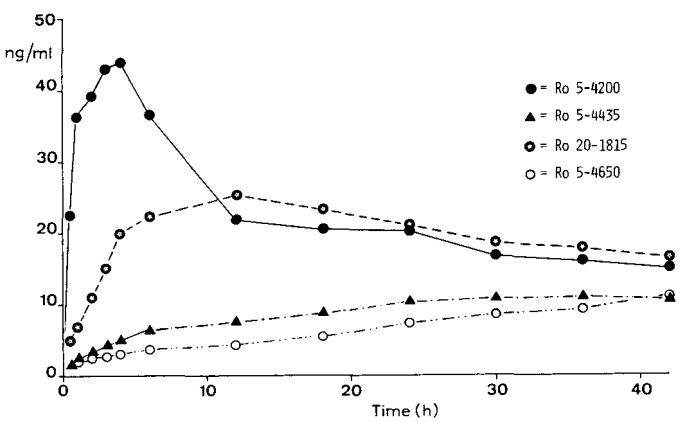

Fig. 6. Evolution of plasma concentrations of flunitrazepam and its major metabolites as a function of time in a patient in an intensive care unit during multiple intravenous administration of 2 mg of flunitrazepam (injections at time 0, 0.5, 1, 2, 3, 4, 6 h and then every 6 h).

the drug are about 2 h in the monkey and 13 h in man. Meanwhile, the plasma levels of the desmethyl metabolite (7-ADF) compared with those of the unchanged compound are negligible.

Fig. 6 shows the accumulation of 7-AF and 7-ADF plasma concentrations during a multiple intravenous administration of 2 mg of flunitrazepam to one patient in an intensive care unit (concentrations of unchanged flunitrazepam and of desmethylflunitrazepam presented in this figure were determined by a gas—liquid chromatographic method developed in our laboratory). As can be seen in this figure, during chronic administration of flunitrazepam, concentrations of 7-AF can slightly exceed those of the unaltered drug, while 7-ADF levels remain inferior.

Another matter of interest of this specific and sensitive method is that it allowed us to calculate the pharmacokinetic parameters of 7-AF after intravenous administration of 7-AF itself, to correlate plasma levels of the derivative to pharmacological responses and to estimate the extent of flunitrazepam biotransformation to this metabolite [11].

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SIMULTANEOUS DETERMINATION OF TOLMETIN AND ITS METABOLITE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RAVI K. DESIRAJU*, DONALD C. SEDBERRY, Jr. and KUNG-TAT NG

Department of Drug Metabolism, McNeil Pharmaceutical, Spring House, PA 19477 (U.S.A.)

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SUMMARY

A highly sensitive and selective high-performance liquid chromatographic assay has been developed for the separation and quantitation of tolmetin and its major metabolite in human biological fluids, viz. plasma, urine and synovial fluid. Analysis of plasma and synovial fluid required only 0.5 ml of the sample. The sample was washed with diethyl ether and extracted with diethyl ether—chloroform (2:1). The extracted compounds were injected onto a reversed-phase column (RP-2) and absorbance was measured at 313 nm. The standard curves in plasma were found to be linear for both tolmetin and the metabolite at concentrations from 0.04 to 10.0 μg/ml. Urine samples (0.5 ml) were diluted (1:1) with methanol containing the internal standard and were directly injected onto the reversed-phase (RP-2) column. Standard curves of tolmetin and metabolite in urine were linear in the range 5—300 μg/ml. Serum and synovial fluid concentrations of tolmetin and its metabolite in patients receiving multiple doses of tolmetin sodium were determined using the assay procedure.

INTRODUCTION

Tolmetin (1-methyl-5-(4-methylbenzoyl)-1H-pyrrole-2-acetic acid) is a non-steroidal anti-inflammatory agent [1, 2] effective in the treatment of rheumatoid arthritis and osteoarthritis. Tolmetin is rapidly adsorbed after oral administration, both in healthy subjects and in arthritic patients [3, 4]. It is eliminated by metabolism to 1-methyl-5-(4-carboxybenzoyl)-1H-pyrrole-2-acetic acid (MCPA) [5], and both tolmetin and MCPA are excreted via kidneys.

Several methods involving spectrophotometric [6], gas chromatographic (GC) [7, 8] and high-performance liquid chromatographic (HPLC) procedures [9] have been developed for the determination of tolmetin in plasma and urine. GC procedures involved derivatization prior to analysis, while the spectrophotometric assays required large volumes of plasma for analysis. Ayres

and co-workers [9, 10] developed an HPLC assay procedure for the analysis of tolmetin and its metabolite in plasma and urine but the lower limit of quantitation was 0.250 μg/ml. Samples of plasma or urine containing less than 0.250 μg/ml of drug were assayed by utilizing 2.5 ml of plasma or urine.

Since the above procedures either did not quantitate the major metabolite or were too time consuming and laborious, an HPLC assay that could accurately and rapidly determine the levels of tolmetin and its metabolite in plasma, urine and synovial fluid was developed.

The distinct advantages of this method over previous methods are: (a) a lower limit of quantitation (0.04 μg/ml) resulting in greater sensitivity, thus allowing smaller volumes of plasma to be used; (b) accurate determination of the major metabolite, MCPA; and (c) applicability of the assay for both tolmetin and MCPA in synovial fluid.

EXPERIMENTAL

Chromatographic conditions

The HPLC system was equipped with the following components: Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph, Model ALC/GPC 204, equipped with a U6K injector, a M-6000A solvent delivery system and a UV detector (Model 440) with 313-nm wavelength filter. A 25 cm × 4.6 mm I.D. column (E. Merck, Darmstadt, G.F.R.) packed with LiChrosorb® RP-2 (10 μm) was used.

The mobile phase was methanol—water (1:1) with 0.005 *M* tetrabutylammonium hydrogen sulfate (TBA) and 0.0016 *M* dibasic sodium phosphate. The individual components of the mobile phase were filtered through a 0.45-μm Millipore® filter and the mobile phase was prepared fresh daily. The LiChrosorb RP-2 column was conditioned with at least 30 ml of the mobile phase prior to use at a flow-rate of 2.0 ml/min which was then maintained constant for sample analysis. The retention times for MCPA, the internal standard and tolmetin were 3.3, 4.6 and 6.5 min, respectively.

Chemicals and reagents

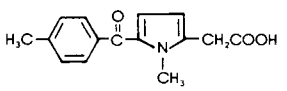
Sulfuric acid (analytical grade), anhydrous diethyl ether (analytical grade), methanol (nanograde), and chloroform (reagent grade) were all purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Tetrabutylammonium hydrogen sulfate was purchased from Aldrich (Milwaukee, WI, U.S.A.). Both monobasic potassium phosphate (KH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4) were certified ACS grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Citrated human plasma was purchased from Interstate Blood Bank (Philadelphia, PA, U.S.A.).

Fig. 1 illustrates the structural formulae of tolmetin, MCPA and the internal standard, McN-2512 (McNeil Pharmaceutical, Spring House, PA, U.S.A.) used in the assay.

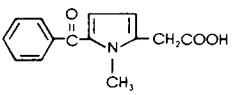
Glassware treatment

Prior to use, all glassware was soaked in detergent (Micro, International Products, Trenton, NJ, U.S.A.) for 2 h, rinsed thoroughly with distilled water

A. Tolmetin: 1-methyl-5-(4-methylbenzoyl)-1H-pyrrole-2-acetic acid



C. McN-2512: 1-methyl-5-benzoyl-1H-pyrrole-2-acetic acid



B. MCPA: 1-methyl-5-(4-carboxybenzoyl)-1H-pyrrole-2-acetic acid

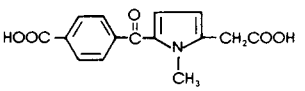


Fig. 1. Chemical structures of tolmetin, MCPA and the internal standard, McN-2512.

and heat treated for 3 h at 270°C. Polyethylene-lined screw-caps were soaked in *n*-heptane for at least 2 h and dried in an oven until all the solvent had evaporated.

Standard curves in plasma

The solutions used in the construction of the standard curve (0.02—5.0 μg/ml) were prepared by serial dilution of a stock solution containing 1 mg/ml each of tolmetin and MCPA with distilled water and were prepared daily. The stock solution was prepared by dissolving sodium salts of tolmetin and MCPA in distilled water.

Stock solution of the internal standard was prepared in methanol (1 mg/ml). A 1-ml volume of this solution was diluted to 500 ml with distilled water (2 μg/ml) and utilized for analysis.

Extraction procedure

Citrated human plasma (0.5 ml) and 1.0 ml of solutions containing 0.02, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 3.0 and 5.0 μg/ml of tolmetin and MCPA were added to 15-ml disposable screw-cap bottles each containing 1.0 ml of phosphate buffer, pH 7.4, 0.5 ml of internal standard solution (2 μg/ml) and 10.0 ml of diethyl ether. Duplicate sets were prepared at each concentration. The samples were shaken on a table-top shaker for 15 min and centrifuged for 10 min at 800 *g*. The diethyl ether (upper) layer was aspirated and discarded. A 0.5-ml volume of 6 *N* sulfuric acid and 9.0 ml of the diethyl ether—chloroform (2:1) mixture were added to the samples which were then shaken for 15 min and centrifuged for 10 min. An 8-ml volume of the organic (upper) layer was transferred to a 12-ml centrifuge tube and evaporated to dryness under nitrogen. The sample extract was reconstituted with 30 μl of methanol, vortexed and 20 μl of this solution was injected into the liquid chromatograph using the stated chromatographic conditions at attenuations between 0.05 and 0.2 a.u.f.s. depending on the concentration of tolmetin and MCPA in the sample.

Extended standard curve

In order to analyze plasma samples containing more than 10 μg/ml of tol-

metin and/or MCPA, four plasma pools containing tolmetin and MCPA, each in concentrations of 20, 30, 40 or 50 μg/ml, were prepared. These pools were analyzed in exactly the same manner as described before, except that only 0.1 ml of plasma sample was taken for analysis and the volume was made up to 0.5 ml with 0.4 ml of blank plasma. Following analysis, the peak height ratios were multiplied by five in order to account for the dilution of the two compounds in plasma.

Synovial fluid assay

Concentrations of both tolmetin and MCPA in synovial fluid were quantitated using the assay procedure described for plasma. Plasma standards were used for the daily calibration curve. Synovial fluid samples were assayed starting at step one of the extraction procedure for plasma and there were no changes in the assay procedure.

Standard curves in urine

The solutions used in the construction of the standard curve were prepared by diluting a stock solution containing 1 mg/ml each of tolmetin and MCPA in distilled water with urine and were prepared daily. Solutions of the following concentrations were prepared in urine: 5, 10, 25, 50, 100, 200 and 300 μg/ml. Internal standard solution (0.2 mg/ml) was prepared in methanol.

Procedure

A 0.5-ml aliquot of each of the above solutions was added to 12-ml tapered centrifuge tubes. Internal standard solution (0.5 ml, 0.2 mg/ml) was added to each tube, the samples were vortexed for 30 sec and 20-μl aliquots were injected into the liquid chromatograph.

Analysis of patient samples

Five patients with rheumatoid arthritis were placed on a regimen of 400 mg of tolmetin every 6 h for seven days. On day 8, following the administration of a single dose of 400 mg of tolmetin, blood, synovial fluid and urine samples were obtained from these patients at selected time intervals. The blood samples were processed for serum. The serum, synovial fluid and urine samples were analyzed for tolmetin and MCPA by the procedure described here.

RESULTS AND DISCUSSION

Typical chromatograms of blank plasma and plasma spiked with tolmetin, MCPA and the internal standard are shown in Fig. 2A and B, respectively. Good correlation was obtained between the peak height ratios and tolmetin plasma concentrations. The standard curve data were obtained by analyzing duplicate samples at each of the concentrations, each day for three days (n=6, at each concentration). Linear regression analysis using 1/variance weighting of the data gave a correlation coefficient (r) of 0.9998, Student's t of 433.0, a slope of 0.363 ± 0.00084 and a y-intercept of −0.0036 ± 0.00043. The standard curve was reproducible from day-to-day. Coefficients of variation (C.V.) in the peak height ratios of tolmetin plasma concentrations be-

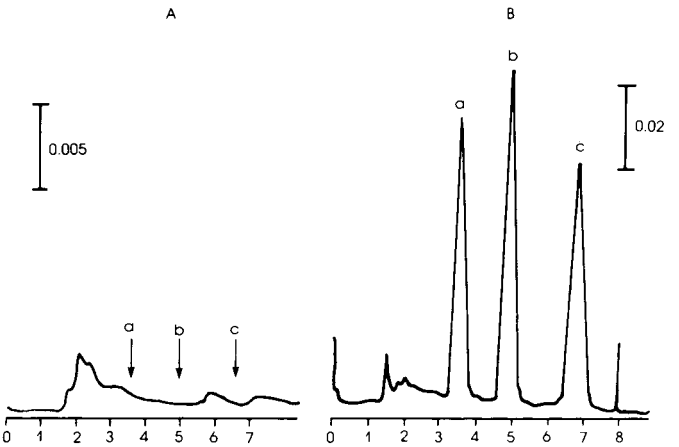


Fig. 2. HPLC assay of tolmetin and MCPA in human plasma. Chromatograms of: A, blank plasma (a.u.f.s. = 0.05); B, plasma spiked with 2 μg/ml each of MCPA (a) internal standard, McN-2512 (b) and tolmetin (c); a.u.f.s. = 0.2.

tween 0.2 and 10 μg/ml were less than 2% and less than 11% for 0.1 and 0.04 μg/ml.

Good correlation was also obtained between the peak height ratios and MCPA plasma concentrations. Linear regression analysis using 1/variance weighting of the data gave a slope of 0.435 ± 0.0012, y-intercept of 0.0016 ± 0.0005, r=0.9998, t=376.0. The standard curve was reproducible from day-to-day. C.V. values for MCPA plasma concentrations between 0.1 and 10.0 μg/ml were less than 3% and 20% at 0.04 μg/ml.

Regression analysis of the plasma standard curve data using 1/variance gave the best statistical evaluation, i.e., Student's t value, r, and accuracy of the back calculated concentrations. The back calculated tolmetin and MCPA concentration values were within 2% of the theoretical concentrations in the range 0.1—10.0 μg/ml. At 0.04 μg/ml, the percent deviation was within 12.5% for both compounds. A summary of both tolmetin and MCPA standard curve data in plasma is presented in Table I.

The standard curves for both tolmetin and MCPA were found to be non-linear above 10.0 μg/ml using 0.5 ml of plasma. Regression analysis of the data, obtained by analyzing 0.1-ml aliquots of plasma samples containing tolmetin and MCPA in concentrations greater than 10.0 μg/ml (i.e. 20, 30, 40 and 50 μg/ml), using 1/variance weighting factor again gave excellent back calculated concentration values within ±4% of the theoretical values. The percent deviation of the slopes of the two regression lines, i.e. 0.04—10.0 μg/ml versus 20—50 μg/ml, was 3.7% from the mean for tolmetin and 0.7% for MCPA. Thus, samples containing tolmetin and MCPA in excess of 10 μg/ml can be analyzed by using a 0.1-ml aliquot of the samples, calculating the concentrations from the regular standard curve, i.e. 0.04—10.0 μg/ml, and then multiplying the concentrations by five to give the actual sample concentrations.

The extraction efficiencies for both tolmetin and MCPA were determined

TABLE I

SUMMARY STATISTICS FOR TOLMETIN AND MCPA STANDARD CURVES IN PLASMA

Concentration in plasma (μg/ml)	Tolmetin (n = 6)				MCPA (n = 6)			
	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration
0.04	0.012	0.0013	10.6	10.0	0.017	0.0033	19.7	12.5
0.1	0.034	0.0028	8.2	0	0.045	0.0013	2.9	0
0.2	0.069	0.0012	1.7	0	0.089	0.0023	2.6	0
0.4	0.140	0.0014	1.0	1.0	0.176	0.0036	2.1	0
0.8	0.284	0.0055	1.9	1.3	0.351	0.0056	1.6	0
1.2	0.433	0.0036	0.8	0	0.524	0.0038	0.7	0
1.6	0.578	0.0065	1.1	0	0.699	0.0210	2.9	0
2.0	0.721	0.0100	1.4	0	0.866	0.0190	2.2	0.5
6.0	2.205	0.0176	0.8	1.7	2.608	0.067	2.6	0
10.0	3.610	0.0350	1.0	0.5	4.292	0.093	2.2	1.3
Slope	0.363 ± 0.0008				0.435 ± 0.001			
Intercept	−0.0036 ± 0.00043				0.0016 ± 0.0005			
Correlation coefficient	0.9998				0.9998			
Student's t	433.0				376.0			
Weighting procedure	1/variance				1/variance			

at 0.8 and 3.0 μg injected and that for McN-2512, at 1.0 μg injected. Results are shown in Table II and show a reduction in extraction efficiency at lower concentrations although the differences were marginal.

TABLE II

EXTRACTION EFFICIENCIES OF TOLMETIN, MCPA AND INTERNAL STANDARD FROM PLASMA

Compound	Amount injected (μg)	Extraction efficiency from plasma* (%)
Tolmetin	3.0	75.3
	0.8	66.9
MCPA	3.0	75.2
	0.8	70.6
Internal standard	1.0	72.8

*(Mean peak height of six extracted samples)/(mean peak height of six directly injected samples).

The synovial fluid samples for analysis were obtained from patients who were already on a multiple dosing regimen of tolmetin. Hence, pre-dose samples also contained tolmetin and MCPA. Therefore, it is not known whether any endogenous substances in synovial fluid would interfere with tolmetin and MCPA peaks. However, from the characteristics of the peaks in the chromatograms (i.e. peak sharpness, lack of front or back tailing), it would seem to indicate that no endogenous material in synovial fluid was eluting from the column at the same time as tolmetin and/or MCPA.

Tolmetin and MCPA were identified in synovial fluid samples by spiking a sample with 1.0 μg each of tolmetin and MCPA and comparing the peak shape and retention times of compounds in the spiked sample to those in the unspiked sample. The retention times of tolmetin and MCPA in the spiked sample were the same as for the peaks seen in the unspiked sample. Fig. 3A and B show the chromatograms of a synovial fluid sample from a rheumatoid arthritic patient receiving tolmetin sodium and the same sample spiked with 1.0 μg each of tolmetin and MCPA, respectively. A 1-μg sample of McN-2512 was used as the internal standard in both samples.

Typical chromatograms of blank urine and urine spiked with tolmetin, MCPA and the internal standard are shown in Fig. 4A and B, respectively. Good correlation was obtained between the peak height ratios and tolmetin urine concentrations. Linear regression analysis using 1/variance weighting of the data gave a correlation coefficient of 0.995, a slope of 0.0074 ± 0.00011 and a y-intercept of −0.0053 ± 0.0014. The back calculated tolmetin concentrations were within 5% of the theoretical concentrations (5—300 μg/ml). C.V. values for tolmetin peak height ratios were less than 11% at all concentrations.

Good correlation was also obtained between the peak height ratios and

TABLE III

SUMMARY STATISTICS FOR TOLMETIN AND MCPA STANDARD CURVES IN URINE

Concentration in urine (μg/ml)	Tolmetin (n = 6)				MCPA (n = 6)			
	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration	Mean peak height ratio	Standard deviation	Coefficient of variation (%)	Percent deviation from theoretical concentration
5.0	0.031	0.003	10.4	2.0	0.040	0.005	12.4	4.0
10.0	0.073	0.008	11.0	5.0	0.090	0.009	10.4	4.0
25.0	0.187	0.018	9.6	3.2	0.230	0.019	8.3	3.2
50.0	0.372	0.034	9.1	1.4	0.461	0.025	5.5	0
100.0	0.728	0.076	10.4	1.4	0.907	0.078	8.6	2.2
200.0	1.470	0.142	9.7	0.9	1.800	0.163	8.8	0.6
300.0	2.190	0.130	6.0	1.6	2.700	0.151	5.4	0.5
Slope	0.0074 ± 0.00011				0.0093 ± 0.00012			
Intercept	−0.00533 ± 0.00143				−0.0054 ± 0.002			
Correlation coefficient	0.9949				0.9965			
Student's t	62.943				75.646			
Weighting procedure	1/variance				1/variance			

MCPA urine concentrations. Linear regression analysis using 1/variance weighting of the data gave a correlation coefficient of 0.997, a slope of 0.0093 ± 0.00012 and a y-intercept of −0.0054 ± 0.002. The back calculated MCPA concentrations were within 4% of the theoretical concentrations (5—300 μg/

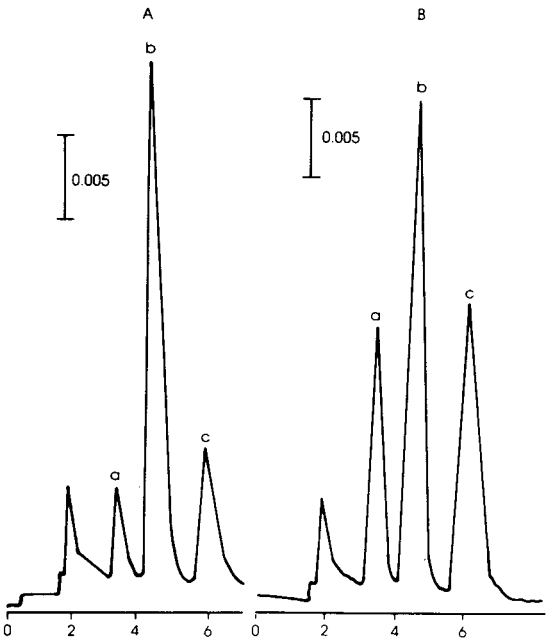


Fig. 3. HPLC assay of tolmetin and MCPA in human synovial fluid. Patient No. 1, A, 8-h synovial fluid sample with 1 μg internal standard (a.u.f.s. = 0.05); B, 8-h synovial fluid sample spiked with 1 μg each of tolmetin (c), MCPA (a) and internal standard (b) (a.u.f.s. = 0.05).

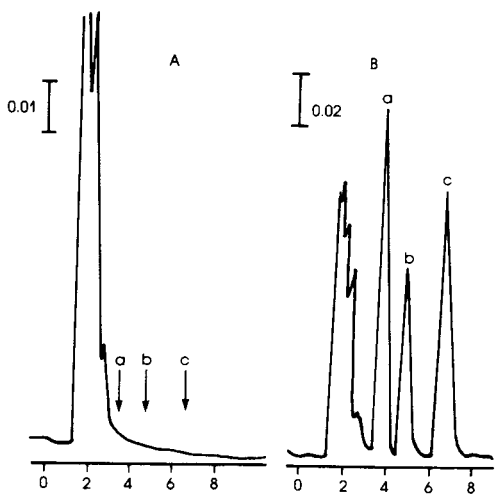


Fig. 4. HPLC assay of tolmetin and MCPA in human urine. Chromatograms of: A, blank urine sample (a.u.f.s. = 0.1); B, MCPA (a), internal standard (b), and tolmetin (c) each in concentrations of 200 μg/ml in urine (a.u.f.s. = 0.2).

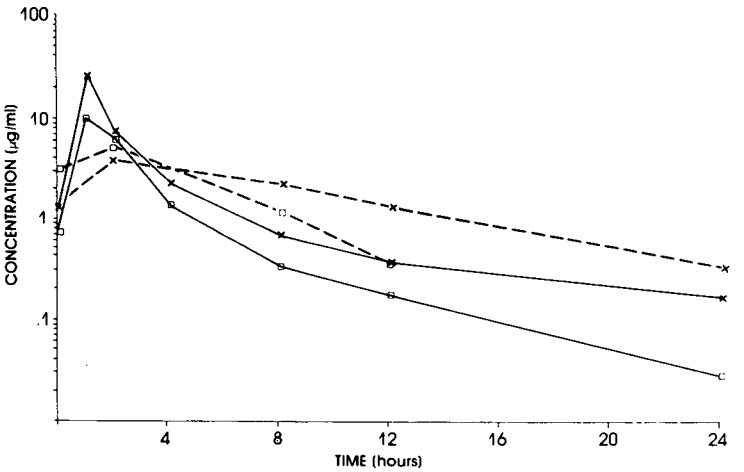


Fig. 5. Serum (——) and synovial fluid (- - - -) concentration profiles of tolmetin (×) and MCPA (□) in Patient No. 1 following a single oral dose of 400 mg of Tolectin®, tolmetin sodium on day 8 (on day 1 through 7, the patient was receiving 400 mg of Tolectin®, tolmetin sodium four times a day).

ml). C.V. values for MCPA peak height ratios were 12.4% or less at all concentrations. A summary of tolmetin and MCPA standard curve data in urine are presented in Table III. No interference peaks due to endogenous material in urine were observed during the analysis of samples from normal volunteers and from patients with rheumatoid arthritis.

To show the applicability of the technique, the results of analysis of serum and synovial fluid samples obtained from one patient are presented here. Fig. 5 illustrates the semi-logarithmic plot of serum and synovial fluid concentration—time profiles of tolmetin and MCPA in a rheumatoid arthritic patient. The serum samples obtained at 1 and 2 h were analyzed using 0.1 and 0.5 ml aliquots since historical data suggested tolmetin concentrations above 10 μg/ml at these time points. The synovial fluid concentration of MCPA in this patient at 24 h was below the quantitation limit of the assay.

This technique has been successfully utilized in the analysis of samples from arthritic patients receiving tolmetin sodium. The pharmacokinetic results will be presented in a subsequent publication.

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A SIMPLE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PENTA- AND TETRACHLOROPHENOLS IN URINE OF EXPOSED WORKERS

KAIJA PEKARI*,* and ANTERO AITIO**

Laboratory of Biochemistry, Department of Toxicology and Industrial Hygiene, Institute of Occupational Health, Arinatie 3, 00370 Helsinki 37 (Finland)

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SUMMARY

A liquid chromatographic method was developed for the simultaneous determination of penta- and tetrachlorophenols in urine. The method is more rapid than gas chromatographic methods and does not involve the use of such potentially dangerous compounds as benzene, diazomethane or pyridine, which have been used in several methods described previously.

INTRODUCTION

Chlorinated phenols and their sodium salts are used extensively as wood preservatives and pesticides. About 200,000 tonnes of chlorophenols are manufactured annually [1]. Technical chlorophenol products manufactured by chlorination of phenol vary in composition; those studied in this investigation contained 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol as their main components. Since chlorophenols are absorbed through the skin, occupational exposures have been monitored by measuring urinary excretion or blood levels rather than by measuring air concentrations [2—8]. Several sensitive gas chromatographic methods exist for the analysis of chlorophenols in biological fluids [3—20]; however, these involve extensive, time-consuming purification and derivatization steps. Some of these methods also require use of chemicals such as benzene, diazomethane and pyridine, which pose a health risk to the analyst. We describe a rapid and convenient liquid

*Present address: Laboratoire de Toxicologie, Faculté des Pharmaceutiques et Biologiques, Université de Paris-Sud, Rue Jean-Baptiste Clément, 92290 Chatenay-Malabry, France.
**Present address: International Agency for Research on Cancer, 150, cours Albert-Thomas, 69372 Lyon Cédex 08, France.

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chromatographic (LC) method for the analysis of chlorophenols in the urine of exposed workers.

EXPERIMENTAL

Principle

After acid hydrolysis of conjugates, tetra- and pentachlorophenols are extracted in a mixture of *n*-hexane and isopropanol. The chlorophenols in the organic phase are quantitated by reversed-phase LC.

Equipment

The following liquid chromatographs were used: Spectra-Physics 3500B with "mixed-wavelength" (254 and 280 nm) absorbance detector, Model 230 and Rheodyne Injector No. 7120, or a Hewlett-Packard 1084B with 79875A variable-wavelength detector, 200—540 nm, with automatic sampling system 79842A. The columns used were: Spherisorb ODS, RP-18, 10 μm, 30 cm × 5 mm I.D. (Spectra-Physics, Santa Clara, CA, U.S.A.), Radial Pak A, RP-18, 10 μm, 10 cm × 8 mm I.D. (Waters Assoc., Milford, MA, U.S.A.), or Hewlett-Packard (Avondale, PA, U.S.A.) 79918B, RP-8, 10 μm.

Chemicals

The chlorophenols, 2,3,4,5-tetrachlorophenol (2,3,4,5-TCP) purum, 2,3,4,6-tetrachlorophenol (2,3,4,6-TCP) techn (with approx. 20% pentachlorophenol as contaminant), 2,3,5,6-tetrachlorophenol (2,3,5,6-TCP) purum, and pentachlorophenol (PCP) puriss, were purchased from Fluka (Buchs, Switzerland); hydrochloric acid pa., *n*-hexane rein, ammonium carbonate pa., and isopropanol pa., were purchased from Merck (Darmstadt, G.F.R.). The methanol was of high-pressure liquid chromatography grade and was purchased from Orion, Finland. Water was purified by double-distillation after deionization.

Procedure

Four millilitres of 6 mol/l hydrochloric acid were added to 8 ml of urine in a 25-ml test tube with a screw cap. After 60 min in a boiling-water bath, the tubes were cooled, and penta- and tetrachlorophenols were extracted in 8 ml of hexane—isopropanol (5:1, v/v) by shaking for 15 min in a mechanical shaker. After centrifugation, 5 ml of the organic phase were evaporated at 80°C to dryness in a covered water bath (there were holes in the cover for the tubes); the residue was dissolved in 0.5 ml of methanol—water (1:1, v/v) by vigorous shaking in a Rotamix test-tube shaker. Of this solution, 20—30 μl were injected into the liquid chromatograph. In the isocratic analysis, the mobile phase consisted of methanol and 0.05% ammonium carbonate in water; the optimal ratio of the two varied somewhat depending on the column, but was generally about 50% of methanol. The flow-rate was 2.0—3.0 ml/min. In separating different isomers of tetrachlorophenols, a linear gradient of 36 to 48% methanol within 12 min was used, after an isocratic elution at 36% for 10 min. The chlorophenols were detected with an ultraviolet detector at 254 nm. The standards, which consisted of urine from non-exposed persons and contained 0—15 μmol/l chlorophenols, were treated in the same way as the samples.

Quantitation was achieved by measurement of peak heights by the Spectra-Physics LC system, or by area integration with the Hewlett-Packard LC system.

RESULTS AND DISCUSSION

Hydrolysis of chlorophenol conjugates

Penta- and tetrachlorophenols occur in the urine of exposed workers partly as conjugates with glucuronic acid [20, 21], and such conjugates must be hydrolyzed before analysis. 2,3,4,6-Tetrachlorophenol conjugates are totally hydrolysed by about 2 mol/l hydrochloric acid at 100°C after 15 min; however, in confirmation of the findings of Edgerton and Moseman [9], 1 h was required to complete the hydrolysis of pentachlorophenol conjugates (Fig. 1).

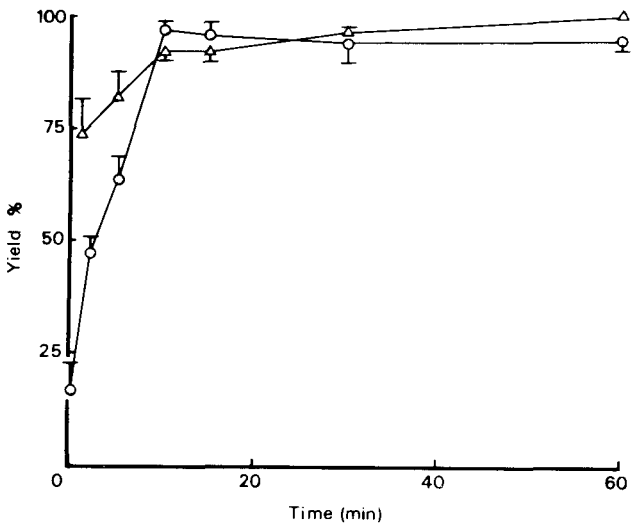


Fig. 1. Effect of hydrolysis time on the yield of 2,3,4,6-tetrachlorophenol (○) and pentachlorophenol (△) in urine. The points represent mean ± S.E.M. of three separate samples from workers exposed to chlorophenols.

The hydrolysis did not decrease the yield of added penta- and tetrachlorophenols (data not shown), indicating that the released aglycones are not destroyed by this procedure. It was shown earlier [3] that treatment with sulphuric acid, even at low temperatures, is enough to break conjugates of pentachlorophenol. We prefer to use hydrochloric acid, however, since sulphuric acid constitutes a health hazard in the laboratory, and since the addition of sulphuric acid to urine can easily cause overflow due to foaming of the mixture.

Extraction procedure

Chlorophenols are weak acids and can be extracted by organic solvents under acidic conditions. Various combinations of organic solvents have been used, including benzene [7, 9—13], hexane [14—16], diethyl ether [12, 18—20], isopropyl ether [8] and isopropanol [14—16]. In the present study a

mixture of hexane and isopropanol was used, which has the advantage that even when acidic urine is extracted no emulsion is formed.

Extraction efficiency for both 2,3,4,6-tetra- and pentachlorophenol is 85–87%. As chlorophenols have some volatility, there is, however, additional loss at 80°C in the evaporation. The final yield for 2,3,4,6-tetrachlorophenol after evaporation is 54.6 ± 2.0% (mean ± S.D. from six different concentrations between 1.7 and 17.3 mol/l) and for pentachlorophenol 83.3 ± 3.7% (mean ± S.D. from six different concentrations between 1.5 and 15.3 mol/l). Thus, although the yield was rather low, the reproducibility was good. A nitrogen evaporator and an aluminium heater block did not give as reproducible results as the covered water bath.

Chromatographic separation

Using gradient elution with 36 to 48% methanol in ammonium carbonate, after 10 min of isocratic elution at 36%, the three isomers of tetrachlorophenol could be separated from each other and from pentachlorophenol (Fig. 2). With this technique the analysis time was nearly 25 min. However, since no more than trace amounts of 2,3,4,5- and 2,3,5,6-tetrachlorophenols were

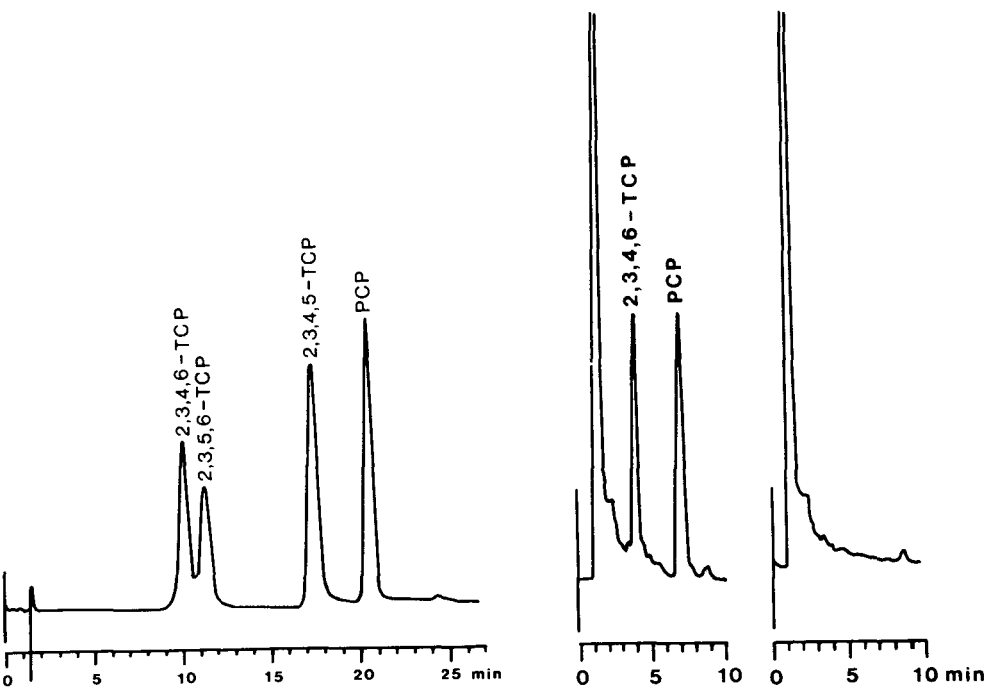


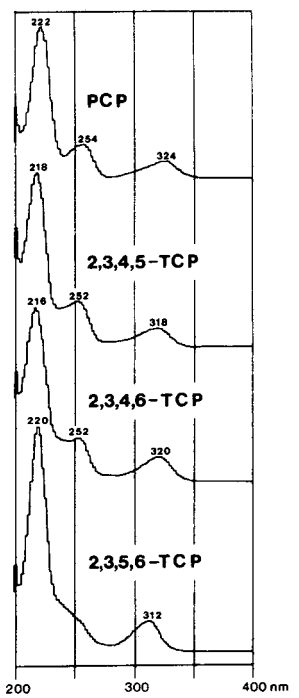
Fig. 2. Separation of tetrachlorophenol (TCP) isomers and pentachlorophenol (PCP) by the gradient elution technique; column; Radial Pak A (RP-18). Linear gradient of 36 to 48% methanol, after 10 min of isocratic elution at 36%. Ultraviolet detection at 254 nm.

Fig. 3. Left: separation of pentachlorophenol (PCP) (15 μmol/l) and 2,3,4,6-tetrachlorophenol (2,3,4,6-TCP) (17 μmol/l) added to urine of non-exposed persons. Right: tracing after no addition. Column, Radial Pak A (RP-18); elution with 53% methanol in 0.05% ammonium carbonate. Ultraviolet detection at 254 nm.

present in the urine of exposed workers, a simple isocratic analysis at about 50% methanol in ammonium carbonate was found to be generally sufficient (Fig. 3). In this way, an excellent separation of penta- and 2,3,4,6-tetrachlorophenol from each other and from interfering chemicals present in urine was achieved in less than 10 min.

Detection

Penta- and tetrachlorophenols have three distinct absorption maxima in the ultraviolet region (Fig. 4). The highest values were encountered at about 220 nm; however, that short wavelength is inconvenient, since many chemicals tend to interfere there. Efficient detection with low background was, however, achieved at 254 nm. It should be pointed out that the molar absorptivities of the tetrachlorophenol isomers differ and their quantitation may thus not depend on a common standard. At 254 nm, calibration graphs (prepared by adding known amounts of penta- and tetrachlorophenols to urine from non-exposed persons) were linear up to a concentration of at least 15 μmol/l. The regressions y (peak height) = 12.075x (concentration — 0.210 for pentachloro-



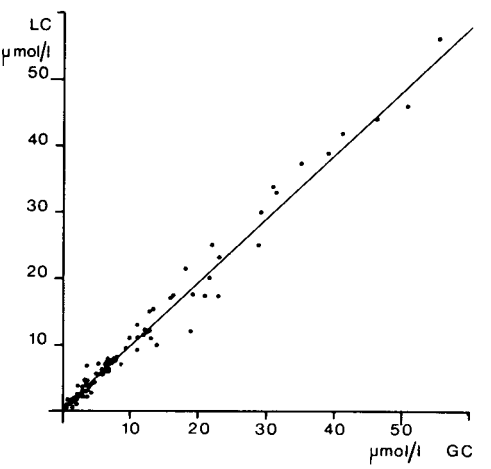


Fig. 4. Ultraviolet spectra of penta- (PCP) and tetrachlorophenol (2,3,4,5-TCP; 2,3,4,6-TCP; 2,3,5,6-TCP) standards, measured with a Hewlett-Packard 1084B liquid chromatograph equipped with a 79875A variable-wavelength detector, column RP-8 Hewlett-Packard 79918B, 10 μm; elution with 50% methanol in 0.05% ammonium carbonate.

Fig. 5. Comparison of the liquid (LC) and gas—liquid chromatographic (GC) analysis of 2,3,4,6-tetrachlorophenol. Calculations were based on 115 samples of 2,3,4,6-TCP; each point represents one or more equal results. The regression line y = 0.976 x + 0.034 (r = 0.988) is shown.

phenol and $y = 9.625x + 0.157$ for 2,3,4,6-tetrachlorophenol, had correlation coefficients in excess of 0.998.

Comparison with a gas—liquid chromatographic method

To further validate this LC procedure for the analysis of chlorophenols in urine, it was compared with a gas—liquid chromatographic method [15], based on Rudling's method [14], which makes use of hexane—isopropanol extraction, sodium tetraborate purification, acetylation and detection with electron capture. Fig. 5 shows that the two methods give similar results. The regression line had an equation of y (LC method) = $0.976\,x + 0.034$, and the correlation coefficient was 0.988.

Sensitivity and precision

The concentration of chlorophenol that gave rise to an absorption peak twice that of the background was 0.1 μmol/l for both penta- and 2,3,4,6-tetrachlorophenol when 8 ml of urine were used in the LC analysis. This sensitivity is lower than those of gas chromatographic methods: that of the gas chromato-

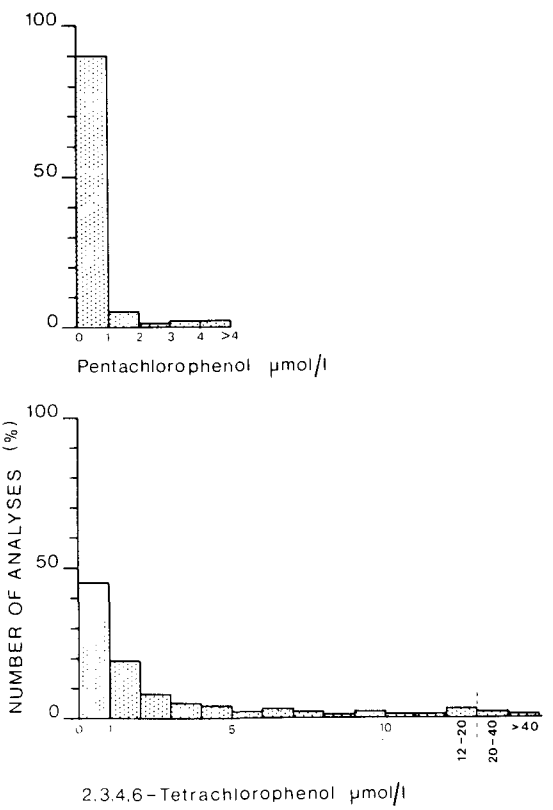


Fig. 6. The frequency distribution of the results of the analyses of penta- and 2,3,4,6-tetrachlorophenols in the urine of exposed workers in wood preservation and chlorophenol manufacture. During the years 1975—1976 the analysis was done gas chromatographically [15] (n=338), thereafter with the LC method (n=1038).

graphic method used for comparison in this study was 0.05 μmol/l with only 2 ml of urine used as the starting material. However, for biological monitoring of workers exposed to chlorophenols the sensitivity of the LC method presented is sufficient.

The coefficient of variation (between series, determined from ten separate preparations made during one day), was 2.6 and 2.5% for pentachlorophenol and 2,3,4,6-tetrachlorophenol, respectively, at concentrations of 12—13 μmol/l.

Application

The method described for the analysis of penta- and tetrachlorophenols was used for three years in Finland in the biological monitoring of workers exposed to chlorophenols during preservation of wood and manufacture of chlorophenols. The frequency distribution of the results is depictedin Fig. 6, showing that 90% of the workers excreted less than 1 μmol/l penta- and less than 9 μmol/l 2,3,4,6-tetrachlorophenol in the urine. In nearly all cases 2,3,4,6-tetrachlorophenol was the prevailing chlorophenol in the urine. The exposure of these workers was thus, in general, considerably less than, for example, that of those employed in wood preservation in Hawaii [2].

ADDENDUM

While this manuscript was in preparation, two related methods were published. A reversed-phase LC method was described for confirmation of the presence of different chlorinated phenols in the urine, using electrochemical detection [22]. Di-, tri-, tetra- and pentachlorophenols could be separated with an LC run of approximately 1.5 h. In the other method, chlorophenols in tissue specimens were analyzed with high-performance LC on silica [23].

ACKNOWLEDGEMENTS

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CHROMBIO. 1357

SIMULTANEOUS DETERMINATION OF BUNITROLOL AND ITS METABOLITE IN BIOLOGICAL FLUIDS, PLASMA AND URINE

AKIHITO NAGAKURA and HIROSHI KOHEI*

Department of Biochemistry, Nippon C.H. Boehringer Sohn Co., Ltd., Kawanishi Pharma Research Institute, Yato, Kawanishi, Hyogo 666-01 (Japan)

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of bunitrolol (Koe 1366) and its metabolite, *p*-hydroxybunitrolol (Koe 1801) has been developed. Using the method, the sensitive and selective determination of Koe 1366 and Koe 1801 can be performed with a simple extraction with diethyl ether and spectrofluorometric detection. The detection limits of Koe 1366 and Koe 1801 in plasma are both less than 2 ng using 1-ml samples.

This method was applied to human and rabbit plasma samples collected after oral administration of bunitrolol tablets (20 mg). The results show the species difference in the metabolism of bunitrolol.

INTRODUCTION

O-[3-(*tert.*-Butylamino)-2-hydroxypropoxy]-benzonitrile hydrochloride (Koe 1366 Cl), a β-adrenergic blocking agent, is highly metabolized in rats [1] and man [2]. Propranolol, one of the β-blocking agents, is also metabolized in the liver in rats, dogs and man [3, 4]. The main metabolites of Koe 1366 are Koe 1801 and its glucuronide.

It is necessary to determine the plasma levels of Koe 1366 and its metabolites for pharmacokinetic study of Koe 1366 in man. Previously the plasma level of Koe 1366 was determined by gas chromatography—mass spectrometry (GC—MS) [5]. However, this technique can measure only Koe 1366 and the analytical procedure is complicated because of the necessity for derivatization.

Recently we developed a simple, sensitive method for the determination of both Koe 1366 and Koe 1801 by high-performance liquid chromatography (HPLC). We could determine the plasma levels of Koe 1366, Koe 1801 and conjugated Koe 1801 in man and rabbits.

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MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade purity. Diethyl ether used for extraction was distilled. DL-Propranolol HCl was purchased from Sigma (St. Louis, MO, U.S.A.). β-Glucuronidase (100 U/ml) was purchased from Boehringer (Mannheim, G.F.R.). Methanol, hydrochloric acid, Na_2HPO_4, and *d*-camphor sulfonic acid were purchased from Wako Pure Chemicals, Osaka, Japan.

HPLC conditions

The HPLC system consisted of a Shimadzu LC-3A constant flow pump with an injector (Shimadzu, SIL-1A) and a column oven (Shimadzu, CTO-2A), and two fluorescence detectors (Shimadzu RF-510 LC fluorescence spectromonitor). The two detectors were connected in series directly after the column. The column was Zorbax ODS (5 μm) prepacked column (150 mm × 4.6 mm I.D., Du Pont). The temperature of the column was set at 40°C. The mobile phase for HPLC was the mixture of 350 ml of water, 650 ml of methanol and 1 g of *d*-camphor sulfonic acid. The flow-rate was 1 ml/min. The first fluorescence detector (D_1) was set for detection of Koe 1366 and propranolol (excitation wavelength, 295 nm; emission wavelength, 330 nm). The second detector (D_2) was set for detection of Koe 1801 (excitation, 323 nm; emission, 371 nm). The signals from the two detectors were led to a two-pen recorder and the chromatogram was drawn. Two digital integrators (Chromatopac E1A, Shimadzu) were also connected to the respective detectors (Fig. 1).

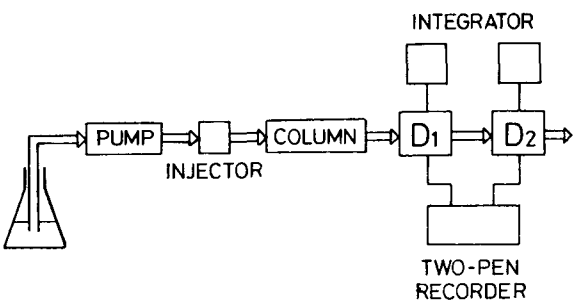

Fig. 1. Flow diagram. D_1 = fluorescence detector for Koe 1366; D_2 = fluorescence detector for Koe 1801.

Extraction procedure

A 0.1-ml volume of propranolol HCl aqueous solution (200 ng/ml for plasma, 10 μg/ml for urine) was added to an aliquot of plasma or urine in a 10-ml glass-stoppered centrifuge tube. To the sample was added 0.5 ml/ml sample of aqueous 1 *M* Na_2HPO_4 solution. Then extraction with 5 ml of diethyl ether was done twice. The ether layers were combined and transferred to another centrifuge tube. A 0.25-ml (1.0 ml in the case of urine) volume of 0.01 *M* HCl was added to the ether. After vigorous shaking and centrifugation, the tube was immersed in a water bath at 40°C to evaporate the ether. An aliquot of the residue (10—100 μl) was injected into the chromatograph.

Preparation of samples

Koe 1366 and free Koe 1801 in plasma (urine). We used 1–2 ml of plasma (1 ml of urine). Koe 1366 and Koe 1801 were extracted by the method described above.

Koe 1801 glucuronide in plasma (urine). A 0.1-ml volume of β-glucuronidase was added to 1 ml of plasma (urine). The mixture was incubated at 37°C for 2 h. The subsequent procedure was the same as that for free Koe 1801.

Calibration curves

Standard curves were constructed with 1 ml of blank plasma containing 0, 2.5, 5, 10, 20, 40 and 80 ng of both Koe 1366 Cl and Koe 1801 Cl; 20 ng of propranolol HCl were added to the sample as internal standard. These samples were extracted by the method described above. Standard curves for urine samples were constructed with 1 ml of water containing 0.25, 0.5, 1.0, 2.0 and 4.0 μg of both Koe 1366 Cl and Koe 1801 Cl. One microgram of propranolol HCl was added to the sample and then extracted. Peak heights of Koe 1366, Koe 1801 and propranolol were measured. These standard curves were obtained by linear regression.

Plasma levels and renal excretion of Koe 1366 and Koe 1801 in man

Two healthy volunteers took 20 mg of Koe 1366 Cl tablets. Then 7 ml of blood were collected at 0.5, 1, 1.5, 2, 4, 6 and 8 h after administration. A 20-μl volume of heparin solution (1000 U/ml) was added to the blood and the blood was centrifuged immediately to separate the plasma. The plasma samples were stored at −20°C until analyzed.

Urine was collected over the periods 0–3, and 3–6 h after administration. Urine samples were stored at −20°C until analyzed.

Plasma levels of Koe 1366 and Koe 1801 in rabbits

Two Himalayan rabbits (male, 2 kg body weight) were used; 20 mg of Koe 1366 Cl (5 ml) were administered by a stomach tube after 16 h fasting. Blood was collected at 0.5, 1, 1.5, 2, 3 and 4 h after administration. After the addition of heparin, the blood was immediately centrifuged to separate the plasma. The plasma samples were stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Chromatography

We can determine the plasma concentration of both Koe 1366 and Koe 1801 simultaneously using fluorescence detectors. Fig. 2 shows the chromatograms obtained from extracts of standard plasma (B, C) and a human plasma sample (D). Chromatogram A was obtained from control plasma. Peak heights of the substances were calculated by digital integrators. A fluorometric method has been used for analysis of propranolol. Maximum fluorescence of propranolol which is dissolved in the mobile phase is observed at 295 nm (excitation wavelength) and 340 nm (emission wavelength); 84% of maximum fluorescence is obtained in this analytical system for Koe 1366 when propranolol is injected into the system. The relative fluorescence intensity of propranolol is 1.9

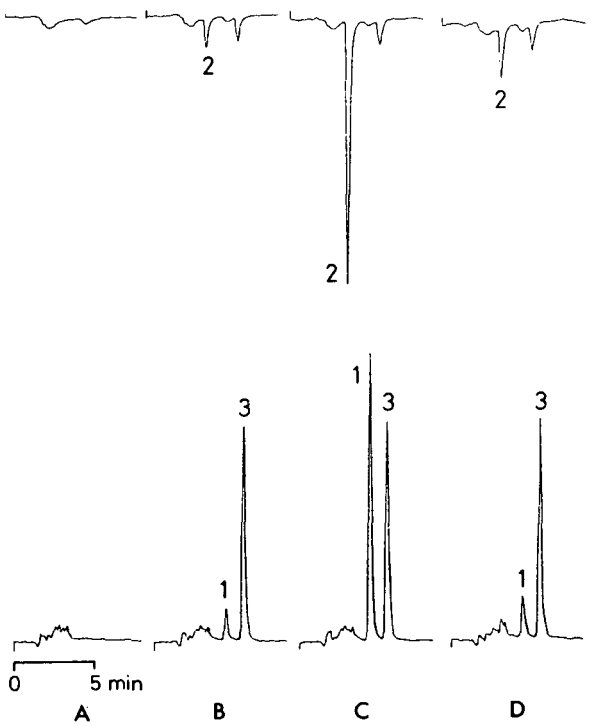

Fig. 2. HPLC chromatograms of control plasma (A), standard plasma (B, C) and a human plasma sample (D). Standard plasma B contains 5 ng of both Koe 1366 and Koe 1801, and C contains 40 ng of both. Propranolol, 20 ng, was added to the plasma as internal standard. 1 = Koe 1366; 2 = Koe 1801; 3 = propranolol.

(Koe 1366 = 1.0) in this condition. We used the peak height of propranolol from the chromatogram of detector D_1 to calculate the Koe 1366 and Koe 1801 peak height ratio.

TABLE I

PRECISION AND ACCURACY

	Concentration (ng/ml)	*n*	Peak height ratio (mean ± S.D.)	Accuracy (ng/ml) (mean ± S.D.)	C.V. (%)
Koe 1366	2.5	4	0.0870 ± 0.0027	2.6 ± 0.10	3.1
	5	4	0.1683 ± 0.0016	5.2 ± 0.05	1.0
	10	4	0.3145 ± 0.0074	9.8 ± 0.24	2.3
	20	4	0.6321 ± 0.0116	19.8 ± 0.36	1.8
	40	4	1.2693 ± 0.0158	40.0 ± 0.51	1.2
	80	4	2.5397 ± 0.0608	80.1 ± 1.89	2.3
Koe 1801	2.5	4	0.0622 ± 0.0045	2.5 ± 0.26	7.2
	5	4	0.1219 ± 0.0053	5.1 ± 0.25	4.3
	10	4	0.2410 ± 0.0012	10.1 ± 0.08	0.5
	20	4	0.4749 ± 0.0073	20.0 ± 0.33	1.5
	40	4	0.9432 ± 0.0069	39.8 ± 0.29	0.7
	80	4	1.8963 ± 0.0242	80.1 ± 1.02	1.3

Calibration curves, precision and accuracy

In the range of 2.5 80 ng/ml, the calibration curves for Koe 1366 and Koe 1801 in plasma showed excellent linearity (r = 0.999). Calibration curves for Koe 1366 and Koe 1801 in urine were also linear in the range 0.25—4 μg/ml.

Table I shows the results of precision and accuracy tests. High levels of precision and accuracy were obtained by this method. The recoveries of Koe 1366 and Koe 1801 by this extraction method were 95.5 ± 3.7% (S.D., n = 24) and 63.0 ± 2.3% (S.D., n = 24), respectively.

Plasma levels and renal excretion in man and rabbits

Plasma levels and renal excretion of Koe 1366, free Koe 1801 and total Koe 1801 after administration of 20 mg of Koe 1366 Cl were studied in two healthy volunteers. Figs. 3 and 4 show plasma levels of Koe 1366 and its major

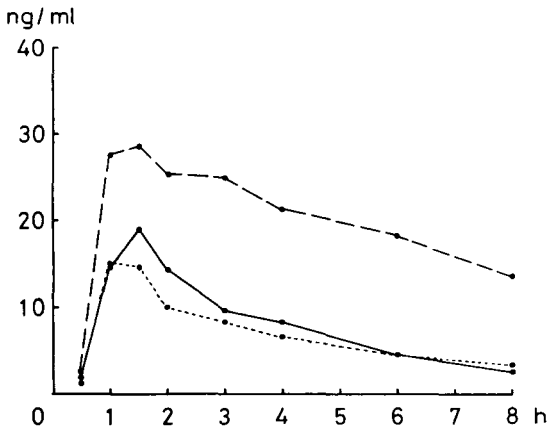

Fig. 3. Plasma levels of Koe 1366 (——), free Koe 1801 (- - - - -) and total Koe 1801 (— — — —) after administration of bunitrolol tablets (2 × 10 mg) in man. Total Koe 1801 means the concentration after enzymatic hydrolysis. Subject: H.K.

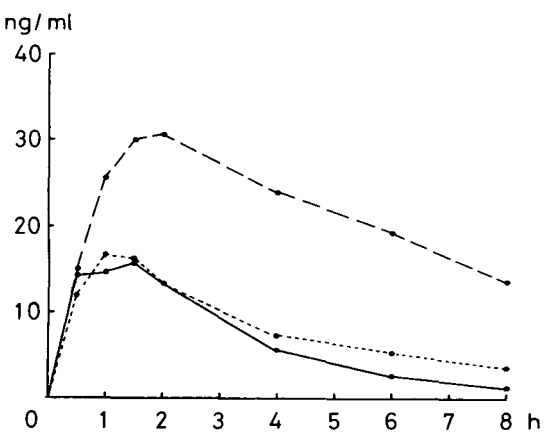

Fig. 4. Plasma levels of Koe 1366 (——), free Koe 1801 (- - - - -) and total Koe 1801 (— — — —) after administration of bunitrolol tablets (2 × 10 ng) in man. Subject: M.I.

TABLE II

URINARY EXCRETION

Subject	Dose (mg)		Amount excreted (μg)	
			0—3 h	3—6 h
H.K.	20	Koe 1366	145	64
		Free Koe 1801	1255	615
		Koe 1801 glucuronide	<10	103
M.I.	20	Koe 1366	378	166
		Free Koe 1801	1222	1104
		Koe 1801 glucuronide	<10	52

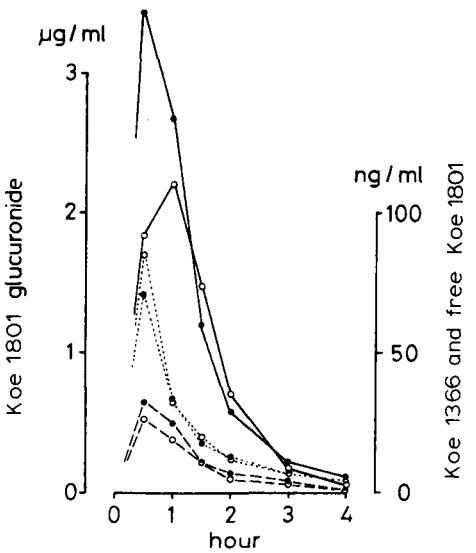

Fig. 5. Plasma levels of Koe 1366 (- - - -), free Koe 1801 (— — — —) and Koe 1801 glucuronide (——) after administration of 20 mg of bunitrolol in rabbits (n = 2).

metabolite, Koe 1801. Plasma concentration of Koe 1366 reached a maximum at 1.5 h after administration. The concentration of free Koe 1801 was about the same as that of Koe 1366.

Table II shows the urinary excretion of Koe 1366 and its major metabolites. It is interesting that only a small amount of Koe 1801 glucuronide was found in urine. However, it is not clear at this stage whether Koe 1801 glucuronide was not excreted in urine. It can be considered that Koe 1801 glucuronide may be hydrolyzed by glucuronidase existing in the urine.

Fig. 5 shows the plasma levels of Koe 1366 and Koe 1801 in rabbits. The plasma levels of Koe 1366 and free Koe 1801 reached a maximum within 30 min after administration. The maximum plasma level of total Koe 1801 reached was about 100 times higher than that of free Koe 1801. This indicates that the metabolic ability of Koe 1366 is much higher in rabbits than in man.

CONCLUSIONS

A sensitive and selective HPLC method was developed for the determination of Koe 1366 and its major metabolite, Koe 1801, in plasma and urine. This simple extraction method permits the analysis of the large number of samples required for pharmacokinetic studies. About 40 samples can be analyzed by this method in a day.

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CHROMBIO. 1356

DIE QUANTITATIVE BESTIMMUNG VON FEPRAZON UND EINEM FEPRAZON-METABOLITEN IN HUMANPLASMA NACH HOCHLEISTUNGSFLÜSSIGKEITSCHROMATOGRAPHISCHER ODER DÜNNSCHICHTCHROMATOGRAPHISCHER TRENNUNG*

HILDEGARD SPAHN und ERNST MUTSCHLER*

Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Gebäude 75A, D-6000 Frankfurt am Main 70 (B.R.D.)

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SUMMARY

Determination of feprazone and one of its metabolites in human plasma after high-performance liquid chromatographic or thin-layer chromatographic separation

Two procedures suitable for pharmacokinetic routine analysis are described for the simultaneous determination of feprazone and one of its metabolites (DA 3505) in plasma samples. After extraction from acidified plasma feprazone and DA 3505 are determined by measuring UV absorbance after thin-layer chromatographic (TLC) separation (reversed-phase TLC plates; methanol—water—formic acid) or high-performance liquid chromatographic (HPLC) separation (silica gel column; hexane—tetrahydrofuran—acetic acid). Limits of detection are 0.1 μg feprazone per ml plasma and 0.2 μg of its metabolite per ml plasma using the HPLC method. Concentrations down to about 0.5 μg/ml plasma of both compounds can be determined using the TLC method.

EINLEITUNG

Bei der Testung von 1,2-Diphenyl-3,5-dioxopyrazolidinen, die in 4-Stellung einen oder mehrere Isopren-Reste enthalten, wurde als antiinflammatorisch und analgetisch gut wirksame Substanz das 4-Prenyl-1,2-diphenyl-3,5-dioxopyrazolidin (Feprazon; Fig. 1a) gefunden, das im Vergleich zu Phenylbutazon bei ähnlicher Wirksamkeit einen schwächeren ulzerogenen Effekt zeigen soll [1—4].

Aus Humanurin wurden als wichtigste Metaboliten von Feprazon das 3'-Hydroxymethylbutenylderivat (DA 3505, M I; siehe Fig. 1b) und das C-Glucu-

*Teilergebnisse der Dissertation von H. Spahn, 1981.

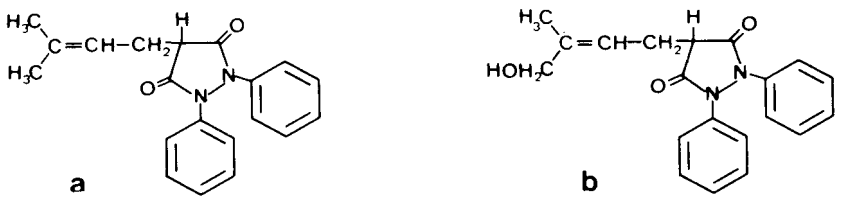

Fig. 1. Strukturformeln von Feprazon (a) und dem Feprazon-Metaboliten M I (b).

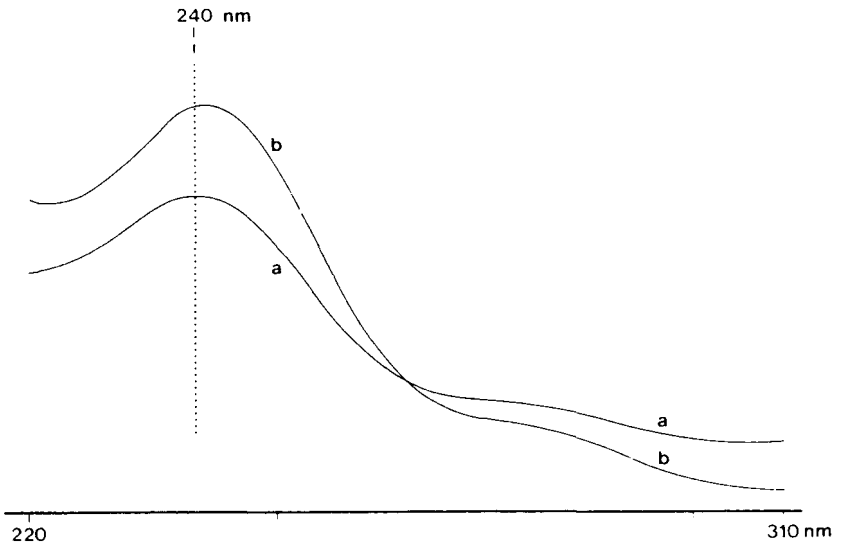

Fig. 2. UV-Absorptionsspektren von Feprazon (a) und dessen Metaboliten DA 3505 (b) in *n*-Hexan—Tetrahydrofuran—Eisessig (780:220:0.5, v/v/v). Konzentrationen der aufgetragenen Lösungen: 10 µg/ml.

ronic (C4-β; M II) von Feprazon isoliert; über 50% des applizierten Feprazon werden beim Menschen als C-Glucuronid über die Nieren ausgeschieden [5, 6]. Von Bruchhausen und Geimer [7] untersuchten mit Hilfe einer gaschromatographischen Bestimmungsmethode die Pharmakokinetik von Feprazon nach peroraler Applikation Feprazon-haltiger Lösungen und Kapseln. Dabei wurden Plasmahalbwertszeiten von ca. 22 h gefunden.

In dieser Arbeit werden zwei Methoden zur quantitativen Bestimmung von Feprazon in Plasmaproben beschrieben, bei denen auch die gleichzeitige Bestimmung des 3'-Hydroxy-Derivats möglich ist. Feprazon und auch dessen Metabolit DA 3505 können sowohl mit einem Cyclohexan—Diethylether- als auch mit einem Chloroform—Diisopropylether-Gemisch aus der angesäuerten Plasmaphase extrahiert werden. Mit Chloroform—Diisopropylether ist ferner eine "Mikrophasenextraktion", d.h. eine Extraktion mit einem geringen Lösungsmittelvolumen, möglich. Eine chromatographische Trennung der Substanzen selbst sowie von mitextrahierten Plasmabestandteilen ist sowohl mit hochleistungsflüssigkeitschromatographischen (HPLC) als auch mit dünnschichtchromatographischen (TLC) Methoden zu erreichen. Die hochleistungsflüssigkeitschromatographische Bestimmung von Feprazon und DA 3505 er-

folgt nach Trennung auf einer Kieselgel 60-Säule mit einem essigsauren *n*-Hexan—Tetrahydrofuran-Gemisch als mobiler Phase durch Messung der UV-Absorption bei 240 nm (Fig. 2). Die dünnschichtchromatographische Trennung wird auf RP-8-HPTLC-Platten mit einem sauren Wasser—Methanol-Gemisch als Fliessmittel durchgeführt. Die Detektion erfolgt über Absorptionsmessung in Remission bei einer Wellenlänge von 241 nm.

EXPERIMENTELLER TEIL

Geräte

Flüssigkeitschromatograph LC 601, variabler Wellenlängen-Detektor LC 55, Minigrator M2 (Perkin-Elmer); Servogor Sb-Schreiber. Die Aufnahme der Spektren erfolgte mit einem Beckman-Spektralphotometer (Modell 24).

Chromatogramm-Spektralphotometer KM 3 der Fa. Zeiss; Perkin-Elmer-Recorder Modell 56; Linomat III (Camag) mit Hamilton-Spritze; Tecam®-Heizblock mit Stickstoffbegasung.

Chemikalien

Die Substanzen Feprazon und DA 3505 wurden von der Fa. Boehringer Ingelheim zur Verfügung gestellt. Die Lösungsmittel und Reagenzien (p.a.-Qualität; nur *n*-Hexan, Tetrahydrofuran und Chloroform in LiChrosolv®-Qualität) wurden von der Fa. Merck (Darmstadt, B.R.D.) bezogen.

Als innerer Standard (HPLC) wird das 2,4-Dinitrophenylhydrazon des 3,4-Dimethoxybenzaldehyds [8, 9], als Extraktionsmittel eine Lösung des inneren Standards (25 µg/ml) in einem Gemisch von Chloroform—Diisopropylether (1:3, v/v) verwendet. Vom inneren Standard wird eine Stammlösung (5 mg in 10 ml Chloroform) hergestellt, die kühl aufbewahrt wird. 0.5 ml der Stammlösung wird mit 2 ml Chloroform gemischt; mit Diisopropylether p.a. wird auf 10 ml aufgefüllt (= "Extraktionsmittel" für die HPLC-Bestimmung).

Extraktion

HPLC-Bestimmung. 0.5 ml Citratplasma wird mit 0.5 ml 1 *N* Salzsäure und 0.3 ml Extraktionsmittel versetzt. Nach 20-minütigem Schütteln werden die Phasen durch Zentrifugation getrennt. Die organische Oberphase wird sofort chromatographiert oder von der Plasmaphase abgetrennt und in einem gut verschliessbaren Gefäss bis zur Analyse aufbewahrt. 20 µl der überstehenden Phase werden chromatographiert.

TLC-Bestimmung. (a) 0.5 ml Plasma wird mit 0.5 ml 1 *N* Salzsäure und 300 µl eines Gemisches von Chloroform—Diisopropylether (1:3, v/v; ohne Zusatz eines inneren Standards) versetzt. Nach 20-minütigem Schütteln wird 10 min zentrifugiert. 50 µl der überstehenden organischen Phase werden strichförmig auf die TLC-Platte aufgetragen.

(b) 0.5 ml Plasma wird in einem Sovirel®-Glas mit 0.5 ml 1 *N* Salzsäure und 4 ml eines Gemisches von Cyclohexan—Diethylether (1:1, v/v) versetzt, 20 min geschüttelt und danach zentrifugiert. Die organische Phase wird abgetrennt und in ein zweites Glas überführt. Der Extrakt wird unter Stickstoffbegasung bei 60°C eingedampft; die Wände des Eindampfgefässes werden dabei mit

wenig Diethylether abgespült. Der verbleibende Rückstand wird mit 300 µl Dichlormethan aufgenommen. 40 µl der resultierenden Lösung werden auf die TLC-Platte aufgetragen.

Chromatographie

HPLC. Chromatographische Bedingungen: analytische Säule der Fa. Knauer (Länge 250 mm, Innendurchmesser 4.5 mm, Füllmaterial: LiChrosorb® Si 100 (Merck), 7 µm; *n*-Hexan—Tetrahydrofuran—Essigsäure reinst (780:220:0.5, v/v/v) als mobile Phase; Temperatur: 50°C; Flussrate: 3 ml/min (entsprechend einem Druck von 3.45—4.14 MPa); Detektion durch Messung der UV-Absorption bei 240 nm (Fig. 2).

Als Retentionszeiten wurden 102 sec für Feprazon und 450 sec für DA 3505 gefunden, d.h. der Peak des Metaboliten erscheint erst nach 7.5 min. Zur Verkürzung der Analysenzeiten bei Reihenuntersuchungen ist es möglich, jeweils zwei Proben in kurzem Abstand hintereinander zu injizieren (siehe Fig. 3), was allerdings die Werte für die Standardabweichung bei der Bestimmung von DA 3505 geringfügig erhöht.

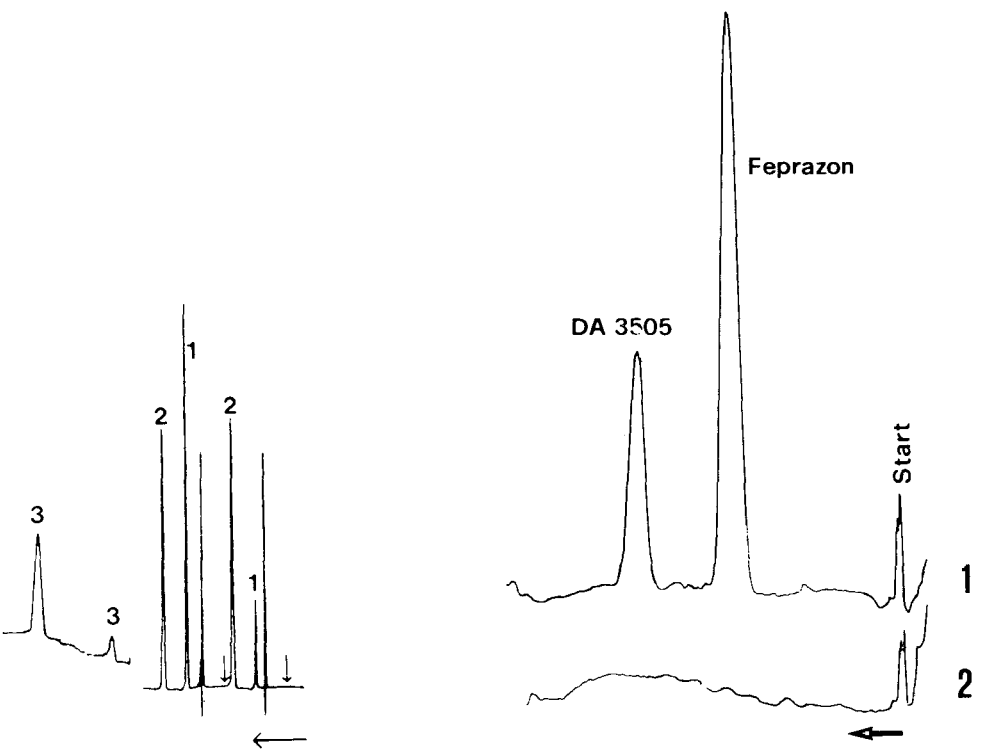

Fig. 3. Chromatogramme von Feprazon und DA 3505 nach Extraktion aus Plasma eines Probanden, dem 400 mg Feprazon oral appliziert wurden, und HPLC-Trennung. Analyse von zwei Plasmaproben. Peaks: 1 = Feprazon, 2 = innerer Standard, 3 = DA 3505 (Schreiberverstärkung gegenüber 1 und 2 verzehnfacht).

Fig. 4. Absorptions—Orts-Kurve eines Plasmaextrakts nach dünnschichtchromatographischer Trennung. 1 = Plasmastandard mit 20 µg Feprazon und 5 µg DA 3505 pro ml Plasma, 2 = Leerplasma.

TLC. Die Lösungen werden mit dem Linomaten III auf reversed-phase-Platten aufgetragen.

Chromatographische Bedingungen: RP-8-Platten der Fa. Merck, 5 mm Bandbreite beim Auftragen; Methanol—Wasser (dest.)—Ameisensäure (80:20:2, v/v/v) als Fliessmittel (TLC-Kammer gesättigt); 6 cm Laufstrecke; Detektion durch Absorptionsmessung in Remission bei 241 nm (Deuteriumlampe) bei einer Spaltgrösse von 0.5 × 6 mm.

Fig. 4 zeigt die Absorptions—Orts-Kurve nach Chromatographie eines Plasmaextrakts.

Auswertung

Bei der HPLC-Bestimmung erfolgt die Auswertung bei Feprazon über die ausgedruckten Integratoreinheiten (Quotient Feprazon/innerem Standard); die nach einmaliger Feprazon-Applikation auftretenden sehr niedrigen Konzentrationen von DA 3505 werden graphisch über die Fläche unter der Kurve (Höhe mal Halbwertsbreite) ermittelt.

Nach TLC-Trennung werden die Feprazon-Konzentrationen und die Konzentrationen des Metaboliten ebenfalls über die Fläche unter der Kurve ermittelt. Für jede TLC-Platte wird eine Eichgerade mit mindestens zwei Plasmastandards erstellt.

Präzision und Richtigkeit

Zur Untersuchung der Wiederfindung bei einmaliger "Mikrophasenextraktion" wurde die Plasmaphase ein zweites Mal mit dem Extraktionsmittel extrahiert. Ferner wurden auf die TLC-Platte methanolische Lösungen der Substanzen aufgetragen.

Die relativen Standardabweichungen und die Linearität der Eichgeraden wurden mit Plasmastandardlösungen geprüft.

Bestimmung der Plasmaspiegel von Feprazon und DA 3505 nach einmaliger oraler Gabe von Feprazon durch quantitative TLC

Einem männlichen Probanden wurden 400 mg Feprazon oral appliziert. Über einen Zeitraum von 72 h wurden Blutproben entnommen. Die Plasmagewinnung erfolgte durch Zentrifugation. Die Plasmaproben wurden bis zur Analyse bei −18°C gelagert. Die Plasmaspiegel von Feprazon und dessen Hydroxylierungsprodukt DA 3505 wurden dünnschichtchromatographisch nach der oben beschriebenen Methode bestimmt.

Vergleich der TLC- und der HPLC-Methode

Der Feprazon-Gehalt von Plasmaproben wurde jeweils dünnschichtchromatographisch und hochleistungsflüssigkeitschromatographisch bestimmt. In einem Koordinatensystem wurden die mit den beiden Methoden gefundenen Konzentrationen gegeneinander aufgetragen.

ERGEBNISSE

HPLC-Bestimmung

Bei Feprazon besteht Linearität zwischen dem ermittelten Quotienten und

den eingesetzten Konzentrationen in dem Bereich von 0—80 μg/ml Plasma. Die Eichgerade für Feprazon wurde mit fünf Plasmastandardlösungen der Konzentrationen 70, 50, 10, 5 und 1 μg/ml ermittelt. Die Gerade verläuft nahezu durch den Nullpunkt, der Korrelationskoeffizient (r) beträgt 0.999.

Da bei dem Feprazon-Metaboliten DA 3505 nur sehr niedrige Plasmaspiegel zu erwarten sind, wurde die Linearität der Eichgeraden bei dieser Substanz nur bis zu einem Höchstwert von 20 μg/ml Plasma nachgewiesen (r = 0.98). Die Nachweisgrenze aus Plasma liegt für Feprazon bei 0.1 μg/ml, für DA 3505 bei 0.2 μg/ml. Die Ergebnisse der Bestimmung der relativen Standardabweichung sind in Tabelle I aufgeführt.

TABELLE I

ANGABE DER RELATIVEN STANDARDABWEICHUNG (n — 1) BEI DER HPLC-BESTIMMUNG VON FEPRAZON UND DA 3505

	Konzentration (μg/ml)	n	Standardabweichung (%)
Feprazon	50	11	1.9
	10	12	2.8
	2	13	1.9
	0.8	7	3.0
DA 3505	10	10	3.5
	5	10	4.9
	1	8	6.9

Die Wiederfindungsraten bei einmaliger Extraktion betragen für Feprazon und DA 3505 89.9 bzw. 80.5%. (Durch eine zweite Extraktion lassen sich in der Plasmaphase noch 10.2% des eingesetzten Feprazon und 19.5% des Metaboliten nachweisen.) Der innere Standard verbleibt zu 98.2% in der organischen Oberphase.

TLC-Bestimmung

Die Flächen unter den Kurven sind den vorhandenen Substanzkonzentrationen direkt proportional; die Eichkurve verläuft im Bereich von 0—70 μg Feprazon/ml Plasma annähernd linear (r = 0.999).

Die mit der Extraktionsmethode (a) erzielten Wiederfindungsraten entsprechen den bei der HPLC-Bestimmung für die Mikrophasenextraktion ermittelten Werten. Bei Methode (b) beträgt die Wiederfindungsrate für beide Substanzen nahezu 100% (für Feprazon 99.2% und für DA 3505 98.6%).

Für Feprazon und DA 3505 liegt die Grenze der quantitativen Erfassung bei dieser Methode bei etwa 20 ng/Peak (entsprechend 0.45 μg/ml Plasma).

In Tabelle II sind die bei verschiedenen Substanzkonzentrationen gefundenen relativen Standardabweichungen aufgeführt.

Die gefundenen Plasmakonzentrationen sind in Fig. 5 halblogarithmisch gegen die Zeit aufgetragen. Jeder Punkt ist ein Mittelwert von mindestens zwei getrennten Bestimmungen. Die beim Vergleich der beiden Methoden ermittelten Konzentrationen sind in Fig. 6 gegeneinander aufgetragen. Die gefundenen Werten sind alle um die theoretische Gerade ($y = x$) verteilt.

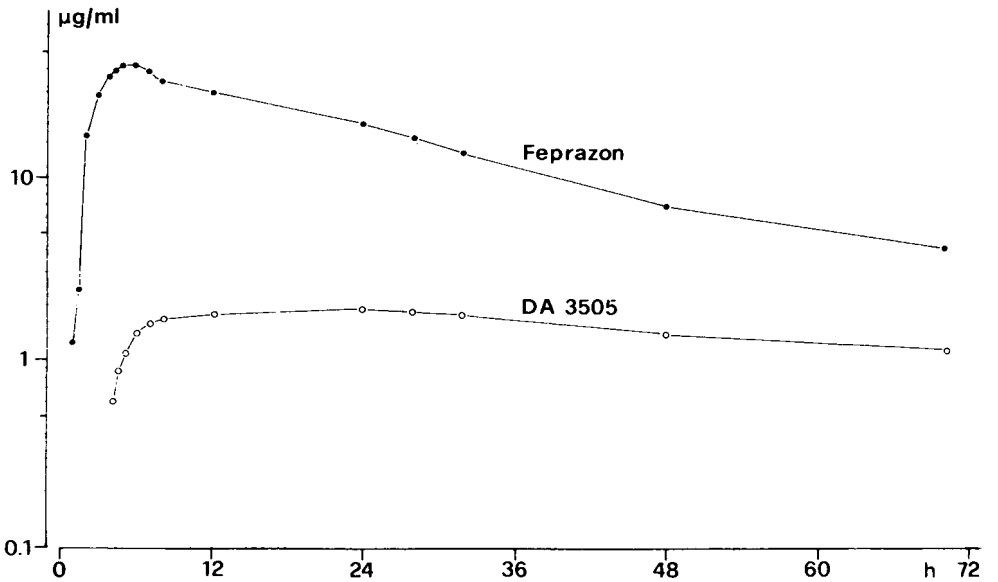

Fig. 5. Dünnschichtchromatographisch bestimmte Plasmaspiegel von Feprazon und DA 3505 nach einmaliger oraler Gabe von 400 mg Feprazon (semilogarithmische Auftragung).

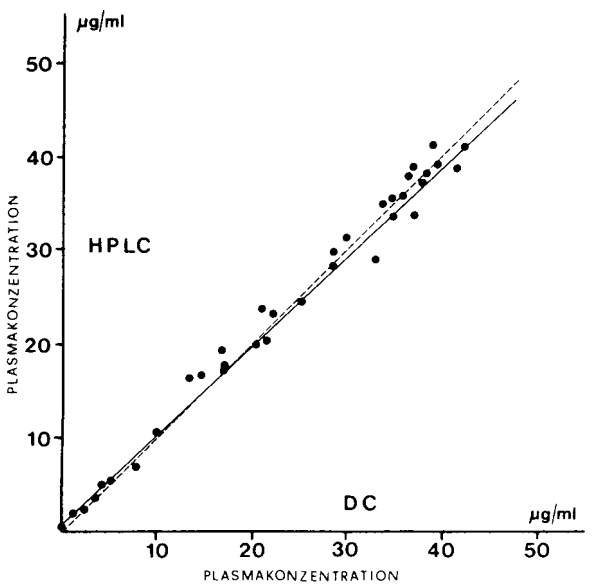

Fig. 6. Vergleich der HPLC- und der TLC-Methode bei der Bestimmung von Feprazon-Plasmaproben. Die durchgezogene Gerade stellt die ermittelte Regressionsgerade $y = 0.94x + 2.04$ dar; die gestrichelte Linie ist die theoretische Gerade $y = x$. Regressionsrechnung: $n = 35; r = 0.989$.

TABELLE II

RELATIVE STANDARDABWEICHUNG BEI DER TLC-BESTIMMUNG VON FEPRAZON UND DA 3505

Mikrophasenextraktion = Extraktionsmethode (a).

	Konzentration (µg/ml Plasma)	*n*	Standardabweichung (%)
Feprazon	50	12	2.1
	25	12	1.9
	10	12	2.7
	5	12	3.1
	1	12	4.9
DA 3505	20	12	2.3
	10	12	3.1
	5	12	4.2
Standardabweichung auf einer TLC-Platte			
Feprazon	50	8	0.4
DA 3505	10	8	2.2

DISKUSSION

Die Empfindlichkeit und die Genauigkeit der beschriebenen Methoden ermöglichen eine schnelle und exakte Bestimmung des Feprazon-Gehalts von Plasmaproben auch bei einmaliger therapeutischer Dosierung. Werden Plasmaproben untersucht, bei denen höhere Konzentrationen zu erwarten sind (chronische Gabe), ist vor allem bei der Mikrophasenextraktion eine Verminderung des eingesetzten Plasmavolumens oder der Zusatz eines grösseren Volumens des Extraktionsmittels erforderlich. Die Vorteile der Mikrophasenextraktion liegen in der sehr schonenden Aufarbeitung, da das Eindampfen des Extrakts entfällt, sowie einem geringen Zeitaufwand bei der Aufarbeitung von Plasmaproben.

Da die Retentionszeit des Metaboliten verhältnismässig hoch ist, ergibt sich bei der HPLC-Trennung eine Verbreiterung des Peaks, die bewirkt, dass dieser in geringen Konzentrationen bei normaler Verstärkung kaum auf dem Registrierpapier zu erkennen ist und meist auch kein Integratorsignal mehr auslöst. Die Konzentration des Metaboliten in Plasmaproben kann aber graphisch (Höhe mal Halbwertsbreite) ermittelt werden, wenn der Schreiberausschlag ungefähr um das Zehnfache verstärkt wird. Da der Metabolit bei normaler Verstärkereinstellung keine nennenswerte Veränderung der Basislinie hervorruft, ist es möglich, den Feprazon-Gehalt von Plasmaproben rasch zu bestimmen; der Metabolit stört die Bestimmung nicht, sofern er nicht in ungewöhnlich hohen Konzentrationen auftritt. Auf diese Weise steht wegen der kurzen Retentionszeit von Feprazon und dem inneren Standard für Reihenuntersuchungen eine Methode zur Verfügung, bei der Feprazon-Gehaltsbestimmungen sehr schnell durchgeführt werden können. Der Möglichkeit der sehr schnellen Aufarbeitung durch Mikrophasenextraktion sowohl bei der HPLC-

als auch bei der TLC-Methode steht eine — verglichen mit der Extraktion mit einem grösseren Lösungsmittelvolumen — geringere Wiederfindungsrate gegenüber. Beide Extraktionsverfahren (a und b) und beide chromatographischen Verfahren liefern jedoch reproduzierbare Ergebnisse und sind für Routineanalysen einsetzbar.

ZUSAMMENFASSUNG

Es werden zwei für pharmakokinetische Untersuchungen geeignete Verfahren für die gleichzeitige Bestimmung von Feprazon und einem seiner Metaboliten (DA 3505) in Plasma beschrieben. Nach Extraktion aus angesäuertem Plasma werden Feprazon und DA 3505 nach dünnschichtchromatographischer (RP-8-Platten; Methanol—Wasser—Ameisensäure) oder hochleistungsflüssigkeitschromatographischer Trennung (Kieselgel-Säule; Hexan—Tetrahydrofuran—Essigsäure) durch Messung der UV-Absorption bestimmt.

Die Nachweisgrenzen für Feprazon und DA 3505 liegen bei dem hochleistungsflüssigkeitschromatographischen Verfahren bei 0.1 bzw. 0.2 μg/ml Plasma, bei der dünnschichtchromatographischen Methode für beide Verbindungen bei etwa 0.5 μg/ml Plasma.

DANK

Der Dr. Robert-Pfleger-Stiftung danken wir für eine Sachbeihilfe.

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Note

Assay of ethylestrenol in urine by isotope dilution—mass spectrometry

INGEMAR BJÖRKHEM*, HÅKAN EK and OLLE LANTTO

Department of Clinical Chemistry and the Research Centre, Karolinska Institutet, Huddinge University Hospital, Stockholm (Sweden)

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Ethylestrenol is an anabolic steroid which may be illegally used with the aim of increasing performance in different power sports. To our knowledge, this compound or its metabolites have as yet not been detected in urine samples from competing athletes. In spite of that, the battery of tests used for the detection of illegal use of anabolic steroids should include analysis for ethylestrenol.

There is little detailed knowledge concerning the metabolism of ethylestrenol. It was recently shown in the rat that only about 17% of an intragastric dose was excreted in the urine, mainly as unmetabolized ethylestrenol [1]. In vitro studies with rat liver homogenates [2] as well as in vivo studies in man [3, 4] and monkeys [5] have shown that ethylestrenol may be hydroxylated in the 3-position. It has been reported that the major urinary metabolite of ethylestrenol in monkey and man is a 19-norpregnanetriol of unknown stereochemistry [3—5]. In the latter studies only small amounts of unmetabolized ethylestrenol could be detected in the urine.

In view of the severe legal consequences of the presence of anabolic steroids in the urine of competing athletes, the analytical method used must be highly accurate and specific. In previous works [6, 7] we demonstrated the usefulness of isotope dilution—mass spectrometry for such analyses. In the present work this technique was used also for the assay of ethylestrenol.

EXPERIMENTAL PROCEDURE

Preparation of [$^2H_5$]ethylestrenol

A solution of 4-estren-17-one (obtained from Steraloids Inc., Wilton, NH, U.S.A.), 50 mg in 2 ml of dried benzene, was added to a Grignard solution prepared from 37 mg of magnesium and 1.7 mmol of $C_2{}^2H_5I$ (obtained from

0378-4347/82/0000—0000/$02.75

CEA, Gif-sur-Yvette, France; 99% pure with respect to ^2H). The mixture was refluxed for 3 h. After cooling to 5°C, the complex was decomposed by the slow addition of 1 ml of ice water and 2 ml of 50% aqueous acetic acid solution. The mixture was then diluted with water and extracted with ethyl acetate. The organic phase was washed with water until neutral and dried over anhydrous sodium sulfate. The product was isolated by preparative thin-layer chromatography, using chloroform—ethyl acetate (1:1, v/v) as solvent. The product (27 mg) was pure as judged by thin-layer chromatography and gas—liquid chromatography—mass spectrometry (see Results).

Unlabelled ethylestrenol was obtained from Organon, Oss, The Netherlands, and was pure as judged by thin-layer chromatography with the same solvent as above.

Urine samples

Urine was collected from healthy males and females at the laboratory. The samples were kept frozen at −20°C until analyzed.

Radioimmunoassay

The kit for analysis of anabolic steroids was obtained from Prof. Brooks at St. Thomas's Hospital, London, Great Britain [8]. Instead of using the dextran-coated charcoal pellet for gamma counting, we used an aliquot of the supernatant.

The antibodies are directed towards 19-norsteroids, but react only to a small extent with ethylestrenol [8]. In spite of that, it has been reported that administration of ethylestrenol can be detected with these antibodies, probably due to the occurrence of metabolites with an oxygen function in the 3-position [8].

Preparation of samples for isotope dilution—mass spectrometry

The $^2H_5$-labelled ethylestrenol, 100 ng, was added to 5 ml of urine. The standard curve was prepared by adding 0—200 ng of unlabelled ethylestrenol to 100 ng of the internal standard and 5 ml of urine from an untreated subject. Sodium acetate buffer (0.15 *M*, pH 4.6) was added to the mixture and the conjugated steroids were hydrolyzed by adding 50 μl of *Helix pomatia* digestive juice. After mixing on a Vortex mixer, the solution was incubated at 37°C for 24 h. The free steroids were extracted with ethyl acetate, 10 ml, on a Rotary mixer for 30 min. The two phases were separated and the organic phase was washed twice with 0.1 *M* NaOH, 5 ml, and once with water, 5 ml. After treatment with anhydrous Na_2SO_4, the solvent was evaporated to dryness at 50°C under a stream of nitrogen. The residue was subjected to preparative thin-layer chromatography, using chloroform—ethyl acetate (1:1, v/v) as solvent. In order to locate the chromatographic zone containing ethylestrenol, [4-^{14}C]oestrone (15,000 dpm, obtained from the Radiochemical Centre, Amersham, Great Britain) was added to the mixture prior to the chromatography.

The radioactive zone (R_F = 0.88) was detected by radioscanning using a Berthold Dünnschicht Scanner II (Wildbad, G.F.R.). This zone was located just below the zone containing ethylestrenol (R_F = 0.93). The latter was scraped off and extracted with 5 ml of methanol. The solvent was evaporated under a stream of nitrogen, and the residue converted into a trimethylsilyl derivative

with trimethylsilylimidazole [9]. The reaction mixture was extracted with 50 μl of hexane and cooled in a freezer to separate the two phases.

Mass spectrometry

About 5 μl of the hexane phase were analysed by gas chromatography—mass spectrometry (GC—MS) using an LKB 9000 instrument equipped with a multiple ion detector and a 1.5% SE-30 column (Chromosorb W, 80—100 mesh, 1.4 m × 2 mm). The temperature of the column was 220°C whereas the flash heater and the ion source both had a temperature of 260°C. The carrier gas was helium and a flow-rate of 30 ml/min was used. The electron energy was 20 eV and the trap current 60 μA. The electron multiplier sensitivity was set to 240 and the amplification of both channels was 300 ×. The first channel was focused on the ion *m/e* 270 and the second channel at *m/e* 275.

RESULTS

The mass spectra of the trimethylsilyl (TMS) derivatives of unlabelled and deuterium-labelled ethylestrenol are shown in Fig. 1. The base peak in the mass spectrum of unlabelled ethylestrenol was at *m/e* 157 (cf. ref. 10). This ion is derived from the D-ring of the molecule, and corresponds to the ion at *m/e* 143 in the mass spectrum of the TMS derivative of methandienone [3]. In accordance with data given in ref. 10, prominent peaks were also seen at *m/e* 241 (corresponding to loss of the TMS group and the ethyl group from the molecular ion) and at *m/e* 270 (corresponding to loss of the TMS group from the molecular ion). In the mass spectrum of the TMS ether of deuterium-labelled ethylestrenol, the corresponding peaks were at *m/e* 162, *m/e* 241 and *m/e* 275. It may be concluded that five atoms of deuterium had been introduced and also that the common ion at *m/e* 241 is formed by loss of the ethyl group.

In the quantitative analysis of urine extracts, the ions at *m/e* 270 and *m/e* 275 were chosen. Contaminating compounds sometimes interfered when using the more intense ions at *m/e* 157 and *m/e* 162.

Fig. 2A shows the multiple ion detector recordings obtained in the analysis of a purified urine extract from an untreated subject. Deuterium-labelled ethylestrenol, 100 ng, had been added to the urine sample as an internal standard. A prominent peak was obtained in the recording of the ion at *m/e* 275, corresponding to the presence of internal standard. Only a very small peak was obtained in the recording at *m/e* 270, corresponding to unlabelled ethylestrenol. After ingestion of a small dose of Orgabolin®, 6 mg, by the same subjects as above, a corresponding analysis of a urine sample gave a prominent peak also in the recording of the ion at *m/e* 270, indicating the presence of unlabelled unmetabolized ethylestrenol (Fig. 2B). The concentration of ethylestrenol could be calculated from a standard curve, obtained by analysis of standard mixtures of different amounts of unlabelled ethylestrenol together with a fixed amount of deuterium-labelled ethylestrenol. It was shown that the ratio between the peaks of the tracings at *m/e* 270 and *m/e* 275 was linear with the concentration of ethylestrenol in the range 0—40 ng/ml of urine.

The possibility that endogenous compounds may interfere in the present

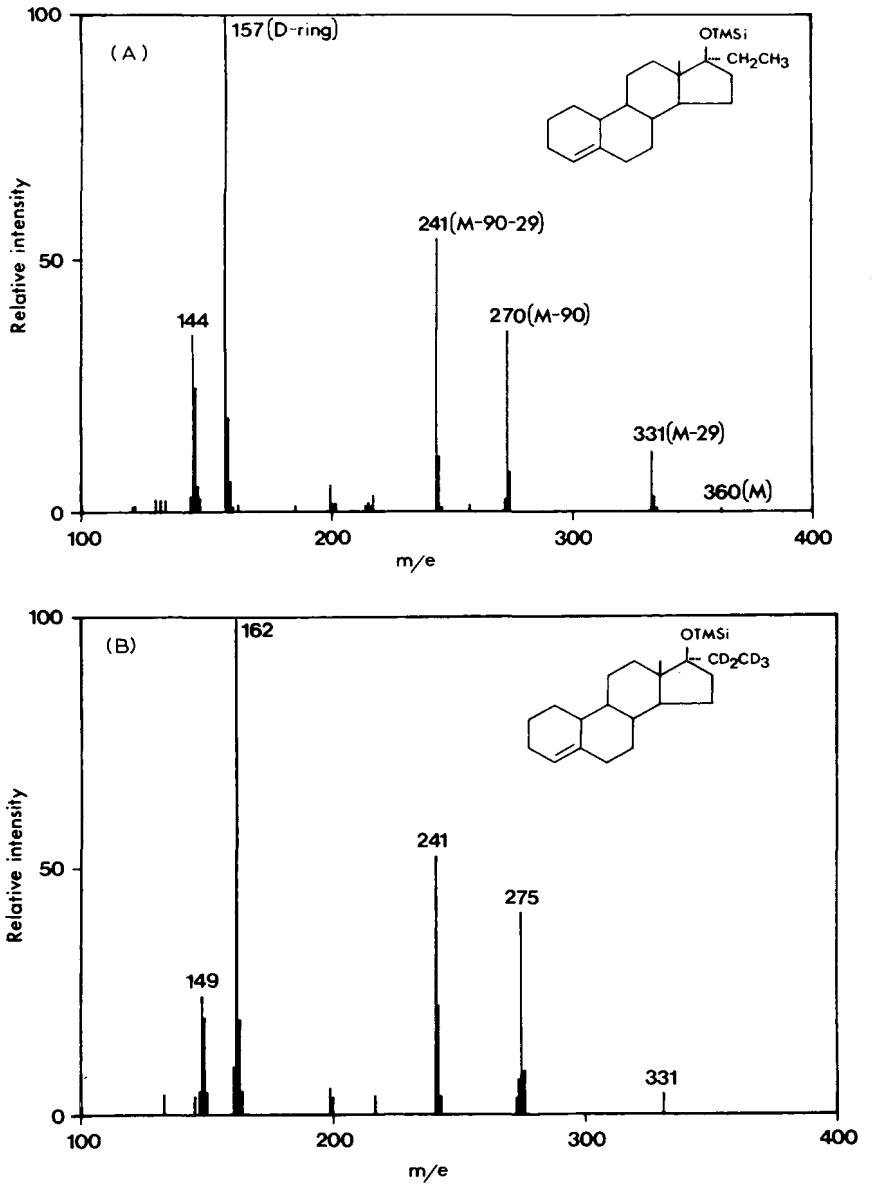

Fig. 1. Mass spectra of the TMS ethers of unlabelled (A) and deuterium-labelled (B) ethylestrenol.

assay was investigated by analysis of urine samples from ten untreated subjects. The apparent concentration of ethylestrenol in these urine samples never exceeded 1 ng/ml of urine. The concentration of ethylestrenol in the first 24-h portion of urine after ingestion of 6 mg of Orgabolin® was 16 ng/ml. It was not possible to detect significant amounts of ethylestrenol in the second 24-h portion of urine. Even after ingestion of 12 mg of Orgabolin, it was only pos-

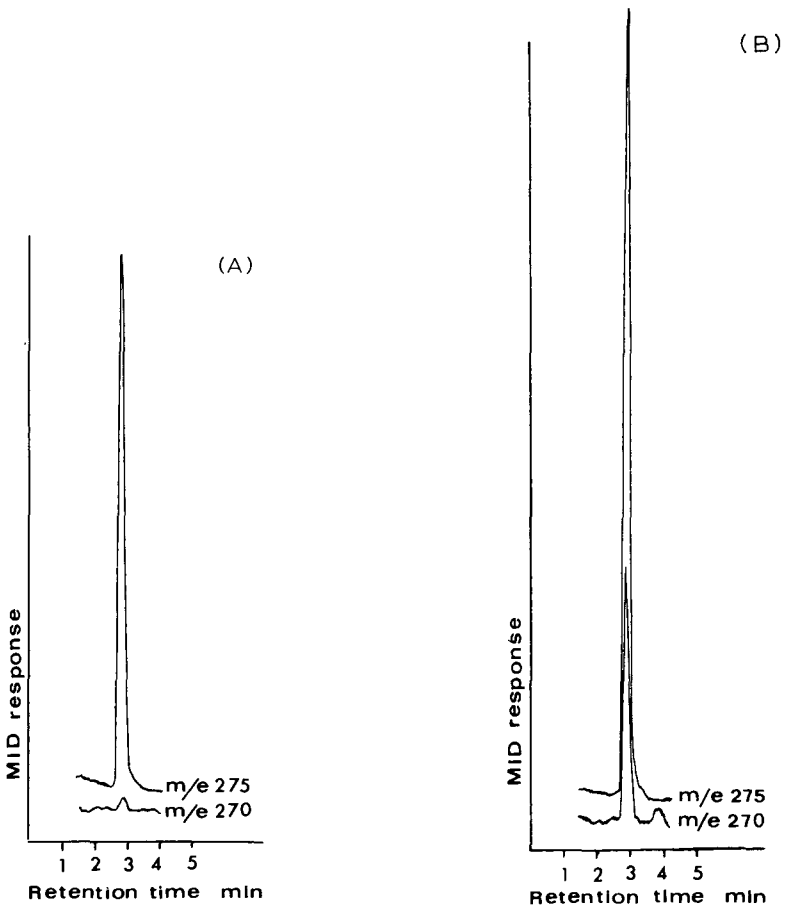

Fig. 2. Multiple ion detector recording of derivatives of a purified extract of urine from an untreated subject (A) and a subject treated with 6 mg of Orgabolin® (B). Deuterium-labelled ethylestrenol had been added as internal standard. For experimental details, see Experimental procedure.

sible to trace ethylestrenol in the first 24-h portion of urine. This was the case also when using radioimmunoassay with antibodies directed towards 19-norsteroids [1].

The coefficient of variation of the isotope dilution method, calculated from five replicate measurements of urine samples containing 10 and 50 ng/ml of ethylestrenol, was about 6% at each concentration.

DISCUSSION

In most doping laboratories, radioimmunoassay is used as a screening test for presence of anabolic steroids in urine. The presence of ethylestrenol or its metabolites in urine may be detected with use of antibodies directed towards 19-norsteroids [8]. In combination with a positive radioimmunoassay test, a positive identification with the present method should give sufficient evidence

that the athlete has taken ethylestrenol. Preferably, however, the identity should be confirmed by use of the other "diagnostic" ions at *m/e* 157 and *m/e* 144. In this low mass range there is always a risk of the presence of interfering compounds. In such cases it is advisable to analyse the urine sample also without the addition of internal standard. Under such conditions also the ions at *m/e* 241 and *m/e* 331 are "diagnostic" for the presence of ethylestrenol.

It may be added that Brooks et al. [10] have used the ion at *m/e* 157 for quantitation of the TMS ether of analogous 17α-ethylated steroids by single ion monitoring. Brooks and Middleditch [11] have also described the principles of an assay of the chloromethyl(dimethyl)silyl ether of ethylestrenol based on single ion monitoring of the ion at *m/e* 270.

In view of the extensive metabolism of ethylestrenol, a sensitive assay for the detection of its illegal use should preferably be directed towards the metabolites rather than towards unmetabolized ethylestrenol. Development of an isotope dilution assay for the major metabolite of ethylestrenol is now in progress.

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Note

Rapid identification of *Klebsiella* by gas chromatography

K.S. MANJA* and K.M. RAO

Department of Microbiology, Defence Research and Development Establishment, Gwalior - 474002 (India)

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Klebsiella, an opportunistic pathogen, is found in cases of burns, pneumonia [1, 2], urinary tract infections [3] and a variety of other infections in man and animals [4]. Recently, a primary *Klebsiella* identification method has been reported [5] employing MacConkey inositol carbenicillin agar, which takes 24 h for identification. A quicker method would help in the early diagnosis and therapy of these infections. A possible application of gas chromatography (GC) in diagnostic microbiology has been reported by many workers [6—9]. The GC identification of *Klebsiella* based on its characteristic products is done by the analysis of glycoprotein components in liver samples of infected rats [10]. GC analysis of acids and amines produced by *Klebsiella* in a chemically defined medium has also been made use of [11, 12]. Derivatisation of the sample is required in these procedures.

We report here a simple GC method for rapid identification of *Klebsiella*. Adonitol, a sugar alcohol, is fermented by all the strains of *Klebsiella* [13], yielding ethanol [14] which can be detected by GC.

MATERIALS AND METHODS

The bacteria used in this study were isolated from the natural water sources and urine samples of patients suffering from urinary tract infections. The identity of the culture specimens was confirmed using the procedure reported in ref. 13. The bacteria were cultured in M-9 salt mixture medium as described earlier [15], the lactose being replaced by adonitol (Difco). Carbenicillin, 50 μg/ml, was also incorporated in the medium. Organisms, $1 \cdot 10^5$, of 62 strains comprising *Klebsiella*, *Salmonella*, *Escherichia coli*, *Citrobactor* and *Arizona*

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were inoculated to the modified M-9 salt mixture medium and incubated at 37°C for 5 h. Six strains of *Klebsiella* were incubated with the medium for 18 and 24 h.

Gas chromatography

A Toshniwal gas chromatograph Model ROL 4 with a flame-ionisation detector was used. Stainless-steel tubing (180 cm × 0.31 cm I.D.) was packed with 15% EGS on Chromosorb W HP 80—100 mesh. Nitrogen at a flow-rate of 40 ml/min was the carrier gas. The operating temperatures of the column, injection port and detector were 110, 150 and 170°C, respectively. The electrometer amplifier was operated at $1 \cdot 10^{-9}$ A. A Heath-kit multispeed servo chart recorder was operated at 1 mV sensitivity and a chart speed of 7.5 cm/min. Five microlitres of 62 5-h old strains of various cultures of bacteria, and of 18- and 24-h old cultures of six strains of *Klebsiella* were directly injected into the chromatograph. The peak height recorded for ethanol in the medium was measured. Twelve lots of culture media were injected into the GC system to determine if there were any background peaks produced by the medium. Standard peaks were also recorded for ethanol of various concentrations, ranging from 50 to 900 ppm in the medium (Fig. 1).

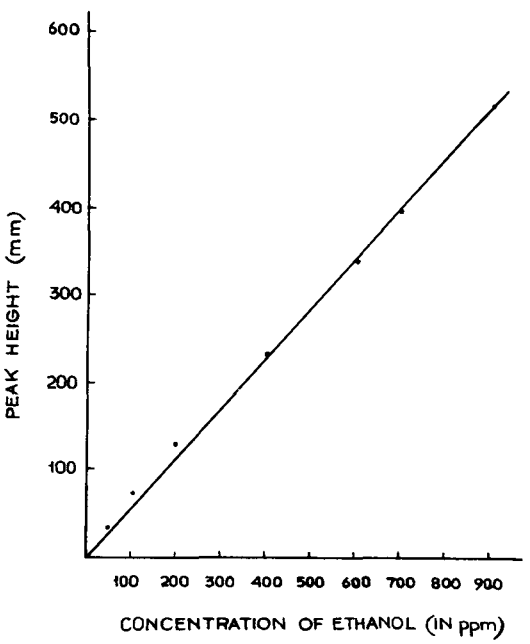

Fig. 1. GC response of various concentrations of ethanol.

RESULTS

Fig. 2 shows the GC peak for ethanol produced by *Klebsiella* in the medium and the absence of the ethanol peak in other bacterial cultures. The results are presented in Table I. The GC peak for ethanol was very sharp, making it

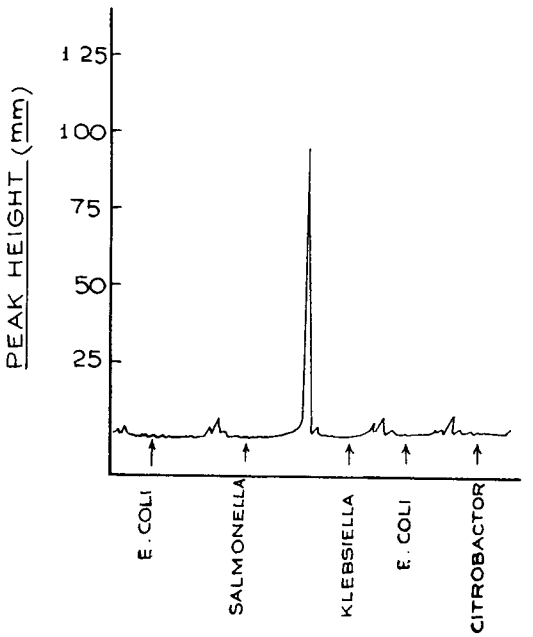

Fig. 2. GC profiles of culture media.

TABLE I

RESULTS OF GC ANALYSIS OF VARIOUS BACTERIA IN ADONITOL MEDIUM

Bacteria screened	No. of strains	No of strains giving peak for ethanol	Peak height (mm)
Klebsiella	30	30	78*
E. coli	15	—	<5
Citrobactor	10	—	<5
Salmonella	5	—	<5
Arizona	2	—	<5
Control medium lots	12	—	<5

*Average.

TABLE II

PRODUCTION OF ETHANOL BY *KLEBSIELLA*

Klebsiella strains	Peak height (mm)		
	5 h	18 h	24 h
MB/14	65	475	525
MB/19	73	500	500
MB/20	87	500	538
MB/21	100	500	500
MB/22	85	475	535
MB/23	70	500	535

difficult to measure the peak area; the peak height was found to be directly proportional to the amount of ethanol produced in the medium. *Klebsiella* produced sufficient ethanol to give a peak height of 78 mm in 5 h, corresponding approximately to 100 ppm ethanol. The amount of ethanol in the medium increased to 900 ppm when cultures were incubated for 18 h, but not beyond that time (Table II). None of the other bacteria produced ethanol from adonitol (Fig. 2). Twelve lots of culture media and cultures of other bacteria gave no significant peak for ethanol.

DISCUSSION

Virtually all *Klebsiella* strains ferment adonitol [13], and ethanol in the adonitol-based medium in which bacteria are grown indicates the presence of *Klebsiella*. Further, carbenicillin in the medium selectively suppresses the growth of other bacteria and permits the multiplication of *Klebsiella* [5]. In the conventional method for primary identification of *Klebsiella*, inositol is used by many workers [5, 16, 17]. A few strains of *Salmonella* and *Citrobactor* also ferment inositol but they do not attack adonitol [13]. Hence adonitol as substrate for *Klebsiella* is superior to inositol.

The GC procedure described above could help as a rapid identification method for *Klebsiella*. Another advantage of the method is that only 0.5 ml of culture medium is required as compared to 15 ml in the conventional method. We injected 1500 samples directly without the gas chromatograph losing any analytical efficacy.

In this study we used 10^5 organisms as the initial inoculum. A typical urinary tract infection as described by Kass [18] is the presence of bacteriurea in a concentration of 10^5 or more organisms per ml of urine. It is evident that bacteriurea caused by *Klebsiella* can be diagnosed by the GC method in 5 h. We are investigating on these lines and the results will be reported later.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. P.K. Ramachandran, Director, for the encouragement given in this study. We also thank Mr. K.N. Kaul for secretarial assistance.

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CHROMBIO. 1346

Note

High-performance liquid chromatography of tryptophan metabolites

Applications in biosynthesis and kinetics

P.L. FRANCIS and IVOR SMITH*

Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 7PN (Great Britain)

(First received February 5th, 1982; revised manuscripts received April 9th, 1982)

In 1958 Lerner et al. [1] isolated melatonin from the pineal gland. Shortly afterwards Axelrod and Weissbach [2] described its biosynthesis showing that hydroxytryptamine was first acetylated by means of N-acetyltransferase and then methylated by hydroxyindole-O-methyltransferase. Lerner et al. [1] also showed that methoxyindole acetic acid was present in the gland and McIsaac et al. [3] showed that methoxytryptophol was present. Since that time many workers have incubated pineal glands with radioactive tryptophan, methyl (S-adenosylmethionine) and acetyl (acetyl coenzyme A) in order to study various aspects of the biosynthesis and biology of melatonin (for reviews see ref. 4). Originally it was assumed that melatonin was the main if not the sole radioactive product when labelled S-adenosylmethionine was used and the radioactive material extractable with chloroform or isoamyl alcohol—toluene was counted and equated with melatonin synthesis. Subsequently one dimensional thin-layer chromatography was used to separate the components of the chloroform extract and radioactivity running coincident or in parallel with synthetic melatonin was, again, equated with melatonin. Although various workers had noted other unidentified radioactive spots on the chromatograms they were entirely ignored until Smith and co-workers [5,6] showed that one such compound was the tryptophol analogue of melatonin, namely O-acetyl, 5-methoxytryptophol. They postulated a biosynthetic pathway also starting from hydroxytryptamine through hydroxytryptophol, O-acetylhydroxytryptophol to O-acetyl, 5-methyltryptophol. Balemans et al. [7] have independently suggested the existence of the same pathway. Subsequently Balemans et al. [8] have shown there is an O-acetyl transferase circadian rhythm in the rat. The existence of both melatonin and acetylmethoxytryptophol must indicate

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that all their precursors are present in the pineal to a greater or lesser degree although only N-acetyl, hydroxytryptamine has been granted any significance. Studies on the circadian rhythm of melatonin in the pineal have been carried out by many (for reviews see ref. 4) but the most comprehensive have been by Balemans et al. [7]. The growing realisation that a number of tryptophan metabolites were present in the pineal suggested to us that it would be well worthwhile to develop high-performance liquid chrromatographic (HPLC) methods suitable for separating the various metabolites.

A limited number of previous studies on indole HPLC have been carried out. As these were designed either for urinary compounds (Richards [9]; Graffeo and Karger [10]) or indole amine metabolites formed in the lung (Crooks et al. [11]), there is relatively little overlap between that work and ours.

The acyltryptophols are all hydrolysed by choline esterase (see Fig. 4). Concurrent with the above studies we have developed a much simpler isocratic HPLC method for studying the kinetics of these reactions.

MATERIALS AND METHODS

In all, tryptophan and eighteen indole metabolites have been examined. The compounds are listed together with their abbreviations shown in brackets; the abbreviations have been fully elaborated and discussed elsewhere [4]. Tryptophan (W), N-acetyltryptophan (aW), 5-hydroxytryptophan (HW), 5-methoxytryptophan (MW), N-acetyl-5-hydroxytryptamine (aHT, acetylserotonin), melatonin (aMT), tryptophol (L), 5-hydroxytryptophol (HL), 5-methoxytryptophol (ML), 5-hydroxyindole acetic acid (HA) and 5-methoxyindole acetic acid (MA) were all obtained from Sigma. N-Acetyl-5-methoxytryptophan (aMW), O-acetyl-5-methoxytryptophol (aML), N-acetyl-5-hydroxytryptophan (aHW), O-acetyltryptophol (aL), O-acetyl-5-hydroxytryptophol (aHL), N-acetyltryptamine (aT), 5-hydroxyskatole (HS) and 5-methoxyskatole (MS) were prepared as previous described [12].

The complete chromatography system (Anachem, Luton, Great Britain) consisted of an Altex Model 332 programmable gradient system, a Model 210 Universal sample injector fitted with a 20-μl loop and a Model 115-40 variable-wavelength UV—VIS spectrophotometer. A 50 mm × 2.1 mm I.D. LiChroprep RP-18 pre-column was inserted between the injection valve and the 150 mm × 4.5 mm 5-μm Hypersil ODS analytical column (HPLC Technology Ltd.). The primary solvent was unbuffered (pH ca. 6) and the secondary solvent was methanol (Rathburn Chemicals, Walkerburn, Great Britain). Gradient from 0—100% methanol were used as shown in the figures. Both solvents were filtered through a SintAGlass Filter, porosity 5 and degassed under vacuum. A 10-μl volume of a standard solution of indoles was injected onto the column. The eluent was monitored at 280 nm at 0.1 a.u.f.s. Chromatography was carried out at room temperature and at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

We first devised a method for separation of the methoxyindoles as these are obtained radioactive after incubation of the gland with radioactive S-adenosylmethionine (see Fig. 1). The rationale for this is that non-radioactive standards

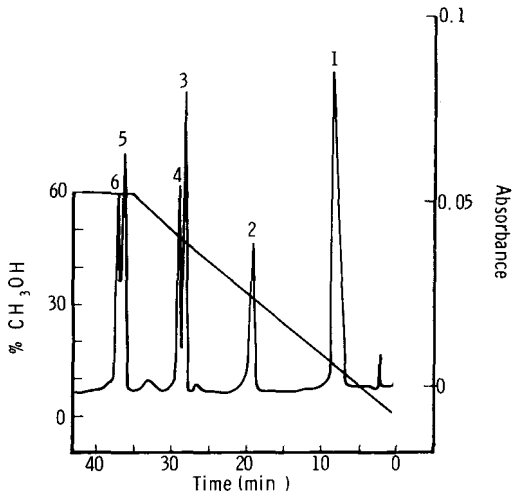

Fig. 1. The separation of methoxyindoles. General chromatographic conditions are described in the text and the methanol gradient is shown in the figures. Peaks: 1 = aMW +MA, 2 = MW, 3 =ML, 4 = aMT, 5 =MS, 6 = aML. MS has not yet been found to occur naturally but was included because HS has been found in urine. Specific conditions were: flow-rate 1 ml/min; pressure 83 bar (1250 p.s.i.); chart speed 0.2 cm/min; detection 280 nm UV at 0.2 a.u.f.s. Sample: 10 μl of standard mixture containing 100 μg/ml of each compound.

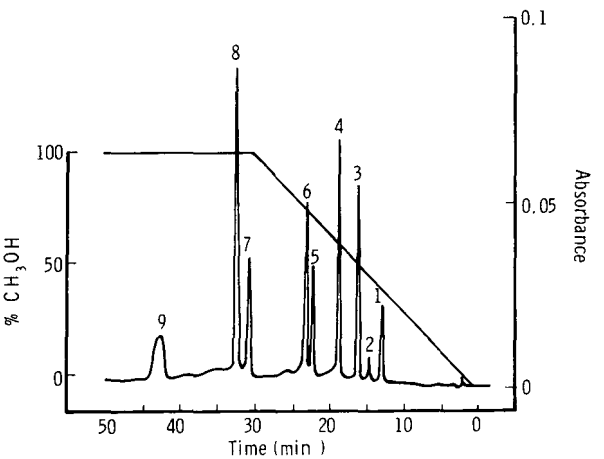

Fig. 2. The separation of acetylindoles. Peaks: 1 = aW, 2 = aHW, 3 = aHT, 4 = aMW, 5 = aMT, 6 = aT, 7 = aML, 8 = aL, 9 = aHL. Specific conditions as in Fig. 1.

can be added to the solution to be chromatographed and the peaks located and separated by linking the UV output to a Gilson CPR fraction collector so that each peak is run into a different collector tube. We then devised a method suitable for the separation of acetylindoles derived from radioactive acetyl coenzyme A (see Fig. 2). Finally we devised a method which is suitable for the separation of radioactive metabolites derived from tryptophan or hydroxytryptamine and, although all the possible metabolites are not completely resolved, separation is sufficient for fractions to be collected for scintil-

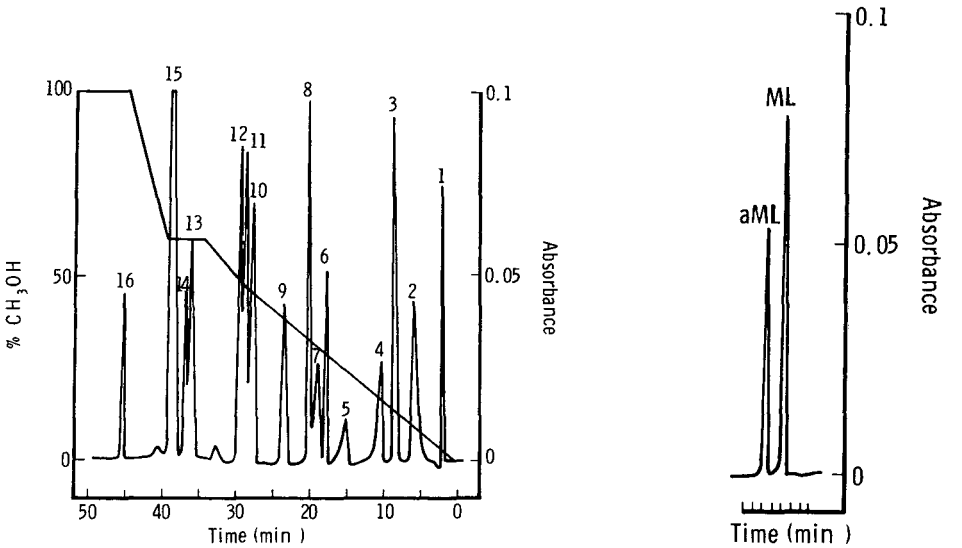

Fig. 3. Separation of nineteen indoles. Peaks: 1 =HA, 2 = aW + aMW +MA, 3 = HW, 4 = aHW, 5 =W, 6 = HL, 7 = MW, 8 = aHT, 9 = HS, 10 = ML, 11 = aMT + L, 12 = aT, 13 = MS, 14 = aML, 15 = aL, 16 = aHL. Specific conditions as in Fig. 1.

Fig. 4. Rapid isocratic separation of acyltryptophols from the parent compound. In this example, aML was incubated in buffer with choline esterase when ML is formed. Both compounds were extracted with chloroform and an aliquot was applied to the column. Separation was effected with 60% methanol. Relative peak heights plus molar extinctions or, alternatively, calibration curves with known amounts of aMl and ML, can be used to determine the degree of hydrolysis for each timed sample.

lation counting or further separation of individual pairs of compounds (see Fig. 3). Clearly resolution could be improved by selective solvent extraction prior to the HPLC. Thus, from neutral aqueous solution, chloroform [1] and ethyl acetate [3,5] extracts 90—100% of melatonin, methoxytryptophol, acetylmethoxytryptophol and other neutral compounds but isoamyl alcohol—toluene [13] extracts a much wider range of indoles [14].

Choline esterase hydrolysis of the acyltryptophols yields the parent tryptophol itself. Hence, it is necessary to separate only two compounds for each kinetic study. One example is illustrated in Fig. 4 which shows the separation of acetylmetroxytryptophol from methoxytryptophol and, allowing for the different extinction coefficients, the peak heights enable the degree of hydrolysis to be calculated for each time of hydrolysis.

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CHROMBIO. 1360

Note

Measurement of branched chain amino acids and branched chain α-ketoacids in plasma by high-performance liquid chromatography

STEVEN L. NISSEN, CAROL VAN HUYSEN and MOREY W. HAYMOND*

Department of Pediatrics, Endocrine Research Unit, Mayo Clinic and Medical School, 4-407 Alfred Building, Rochester, MN 55905 (U.S.A.)

(First received February 19th, 1982; revised manuscript received April 6th, 1982)

The metabolism of the branched chain amino acids (leucine, isoleucine and valine) is closely linked to that of their respective branched chain α-ketoacids (α-ketoisocaproate, α-ketomethylvalerate and α-ketoisovalerate, respectively), often requiring quantitation of both the amino and α-ketoacids [1, 2]. The first practical method for the measurement of plasma branched-chain α-ketoacids (BCKAs) utilized gas—liquid chromatography [3], but was laborious and time consuming. Subsequently, rapid high-performance liquid chromatographic (HPLC) methods for the quantitation of BCKA [4—7] were published, but none were able to quantitate all three BCKA in physiological fluids.

Quantitation of the branched-chain amino acids (BCAAs) in plasma has usually been accomplished by automated ion-exchange chromatographic methods [8]. While HPLC methods have the potential for decreasing the time and expense involved in amino acid analysis, most are not suitable for routine quantitation of plasma BCAA due to poor peak resolution [9]. Amino acid derivatization improves the chromatographic resolution by HPLC, but their instability makes automated analysis of large numbers of samples impractical [10]. The present paper describes an HPLC method for the quantitation of all three BCKAs and BCAAs in small plasma samples. In addition, this method is adaptable to automated HPLC analysis and can be used to isolate compounds of interest.

EXPERIMENTAL

Materials

HPLC grade solvents were obtained from Fisher Chemical. Norleucine

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(Pierce) and calibrated amino acid standards (Hamilton) were obtained from commerical sources. α-Ketoisocaproate, α-ketomethylvalerate, α-ketoisovalerate, α-ketocaproate, amino acid oxidase, catalase and all other chemicals were obtained from Sigma.

HPLC was accomplished using a 5-μm C_{18} silica column (Altex) and a Varian liquid chromatograph (Model 5060) interfaced with an integrator (Varian 401) and UV detection (Model 441, Waters Assoc.) at 214 nm. The HPLC running buffer (1.4 ml/min) consisted of 0.05 *M* sodium phosphate, pH 7.0—acetonitrile (90:10). Between each sample the column was flushed for 1 min with methanol and re-equilibrated with running buffer. All injections were made with an automatic sample injector (WISP, Waters Assoc.). Plasma BCAA concentrations were independently determined using a Beckman 119 CL amino acid analyzer [8].

Methods

Plasma samples (1 ml) are adjusted to pH ≈ 1 with 1 *M* hydrochloric acid (ca. 200 μl) and the internal standards, 20 nmol of α-ketocaproate (for keto-acid analysis) and 50 nmol of norleucine (for amino acid analysis) are added to each tube. Aliquots (1 ml) of standard solutions of the BCAAs (50—500 μ*M*) and BCKAs (5—50 μ*M*) are processed along with each set of plasma samples. BCAAs and BCKAs are initially fractionated by transferring the plasma to a 5 × 1 cm column (Isolab) containing 2 ml of a 50% aqueous solution of cation-exchange resin (H^+ from Bio-Rad Labs.) . The columns are washed with four 1.0-ml aliquots of 0.01 *M* hydrochloric acid and the effluent plus washings collected in 25 mm × 150 mm glass screw-capped tubes for BCKA analysis. The amino acids are eluted from the washed column with 4 ml of 4 *M* ammonium hydroxide into 60 × 17 mm screw-cap vials (Kimble), and frozen for subsequent analysis (see below).

The effluent from the columns containing the BCKA is extracted once with 35 ml of methylene chloride. After centrifugation for 5 min at 800 *g*, the supernate (aqueous layer) is aspirated and discarded. The methylene chloride layer (infranate) is transferred to a clean 150 × 25 mm tube and back-extracted with 350 μl of 0.1 *M* sodium phosphate buffer (pH 7.0). After centrifugation for 5 min at 800 *g*, the aqueous layer is transferred to 250-μl centrifuge tubes, briefly centrifuged (Beckman Microfuge) and 200 μl of the aqueous solution injected into the HPLC system. BCKA concentrations are calculated using a standard curve constructed from the peak height ratios of the standard solutions of BCKA and the internal standard, α-ketocaproate.

The frozen amino acid fraction was taken to dryness by lyophilization. To each each sample, 1 ml of a solution containing amino acid oxidase and catalase (0.1 ml mino acid oxidase and 0.05 mg catalase in 1 ml of 0.5 *M* Tris buffer, pH 7.6) is added. The sample is flushed with oxygen, capped, and placed in a shaking water bath at 37°C for 1.5 h. 1 *M* Hydrochloric acid (ca. 150 μl) is then added to lower the pH to < 1.0, and subsequently processed as described above for the BCKAs. BCAA concentrations are calculated using a standard curve derived from the peak height ratios of the BCAA standards and the internal standard, norleucine.

RESULTS AND DISCUSSION

BCKA analysis

The chromatograms derived from standard BCKA solutions (broken line) and human plasma (solid line) are presented in Fig. 1. All three BCKAs can be resolved easily in this system and plasma BCKA concentrations quantitated on the basis of recovery of the internal standard, α-ketocaproate. Using this

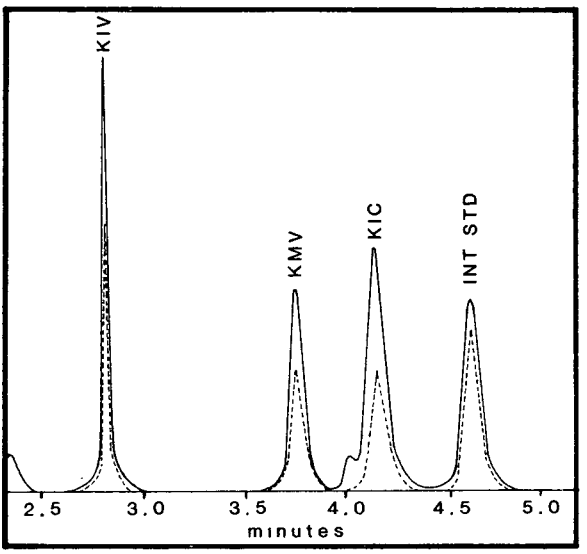

Fig. 1. Chromatogram of α-ketoacid standards (broken line) and a plasma extract (solid line). KIV = α-Ketoisovalerate; KMV = α-ketomethylvalarate; KIC = α-ketoisocaproate; INT STD = α-ketocaproate (internal standard). Extraction procedures and HPLC conditions are given under Experimental.

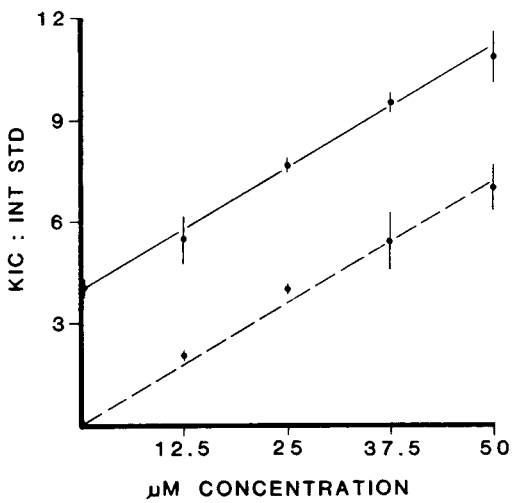

Fig. 2. Peak height ratio of α-ketoisocaproate (KIC) to internal standard (α-ketocaproate) plotted against the concentration of KIC added to saline (broken line) and to plasma (solid line). Each point represents the mean ± S.E.M. of triplicate determinations.

method, a sample can be analyzed every 10 min from as little as 50 μl of plasma.

In Fig. 2, triplicate samples from the standard curve of α-ketoisocaproate (broken line) and from plasma samples to which the standards were added (solid line) is presented. The two curves are linear and parallel, indicating quantitative recovery of α-ketoisocaproate added to plasma. α-Ketomethylvalerate and α-ketoisovalerate exhibited similar linear standard curves and were quantitatively recovered from plasma (data not shown). The coefficient of variation for replicate analyses (n = 10) of the same sample was 3% for each of the α-ketoacids. Using this method, the venous plasma concentrations of α-ketoisocaproate, α-ketomethylvalarate and α-ketoisovalerate were 28, 18 and 17 μM, respectively, which are in close agreement with published values using gas chromatographic techniques [11].

BCAA analysis

Fig. 3 illustrates the HPLC chromatograms derived from a standard solution of BCAAs (broken line) and plasma after treatment with amino acid oxidase and analysis as α-ketoacids. Without amino acid oxidase treatment, no peaks were detected, indicating no contamination of the amino acid fraction with plasma BCKAs. In addition to quantitation of the BCAAs, it appears methionine can also be analyzed as its α-ketoacid (α-ketomethiolbutyrate).

The leucine standard curve (broken line) and recovery of leucine standards added to plasma (solid line) are presented in Fig. 4. The standard curve and the standard added to plasma were parallel, indicating quantitative recovery of leucine from plasma. The standard curve and recovery for isoleucine and valine were similar to that of leucine (data not shown). The coefficient of variation of replicate analyses (n = 10) of leucine, isoleucine and valine in human plasma was 3%.

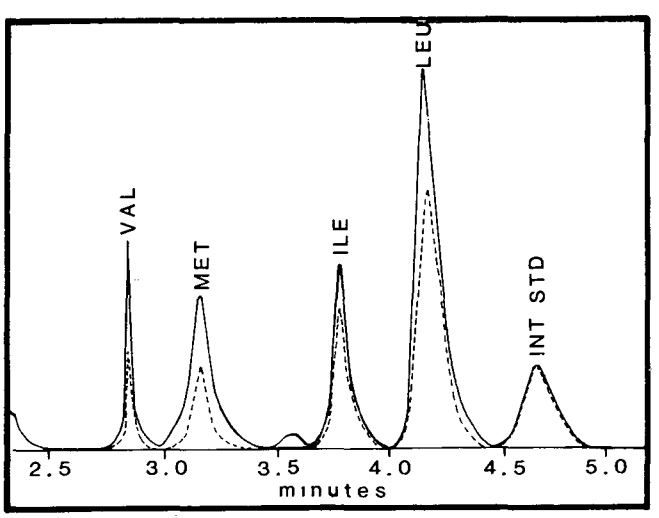

Fig. 3. Chromatograms of the BCAA standards (broken line) and a plasma extract (solid line). VAL = Valine; MET = methionine; ILE = isoleucine; LEU = leucine INT STD = norleucine (internal standard). Samples are deaminated with amino acid oxidase and subsequently chromatographed as their respective α-ketoacids. See Experimental section for details.

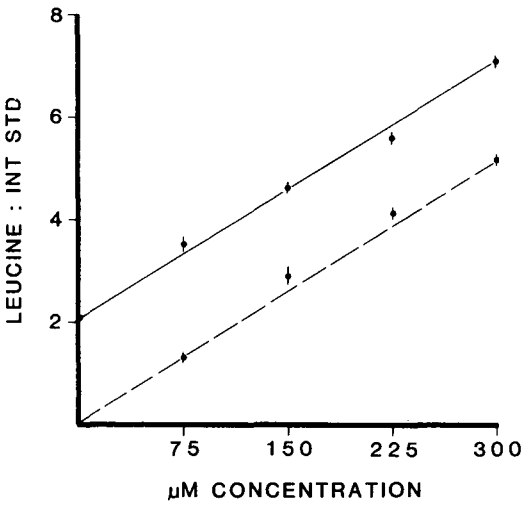

Fig. 4. Peak height ratio of leucine to internal standard (norleucine) plotted against the concentration of leucine added to saline (broken line) and to plasma (solid line). Each point represents the mean ± S.E.M. of triplicate determinations.

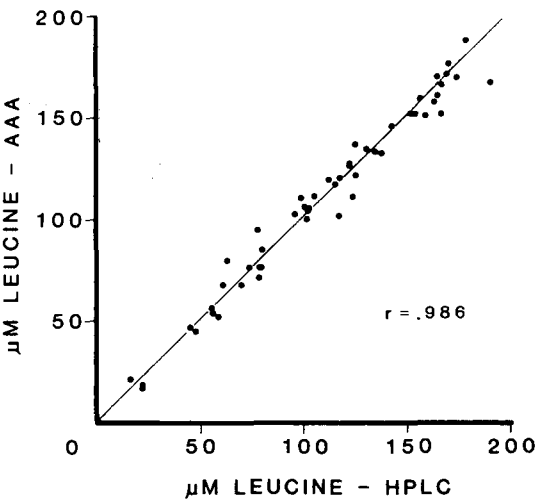

Fig. 5. Comparison of leucine concentrations in plasma measured by ion-exchange chromatography (amino acid analyzer, AAA) and by the HPLC method described under Experimental.

Fig. 5 presents the correlation between leucine concentration measured by the amino acid analyzer and the HPLC procedure described here, indicating close agreement (r = 0.986). Isoleucine and valine have similar correlation (r = 0.92, r = 0.85, respectively).

Analysis of the BCAAs by HPLC requires approximately 10 min per sample compared to a minimum of 2 h by conventional amino acid analyzer techniques. In addition to being as accurate as the amino acid analyzer, this HPLC technique can also be automated. The α-ketoacids derived directly from plasma or from the BCAAs were stable for at least 8 h at room temperature in the automatic injector sample deck.

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We are indebted to Collette Schmidt for technical assistance and to Marylee Campion for her secretarial help. This investigation was supported by the Wassie and Mayo Foundations, and U.S. Public Health Service Grant AM-26989. S.L.N. is supported by Diabetes Training Grant AM-7352C.

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Note

Simple method for the separation of monohydroxy fatty acid metabolites of arachidonate metabolism

GUNDU H.R. RAO*

Department of Laboratory Medicine and Pathology, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)

K. RATNAMMAL REDDY

Department of Pediatrics, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)

and

JAMES G. WHITE

Departments of Laboratory Medicine and Pathology and Pediatrics, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)

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Prostaglandins play a major role in cardiovascular physiology [1]. Arachidonic acid is the most abundant of the polyunsaturated fatty acids in platelet membranes and is transformed to biologically active intermediates by two different pathways [2,3]. Active compounds of the cyclooxygenase pathway are prostaglandin endoperoxides (PGG_2, PGH_2) and thromboxane A_2 [4]. Hydroperoxy acids (HPETE), hydroxy acids (HETE) and leukotrienes are generated by the lipoxygenase pathway [5,6]. Methods have been developed to monitor several of the stable metabolites of the labile compounds as an index of the enzyme activity in various tissue. Methods of choice have been thin-layer chromatography (TLC), high-performance liquid chromatography, radioimmunoassay, gas chromatography (GC) and massspectrometry (MS) [7]. Recently, Vincent et al. [8] have suggested using ratios of thromboxane B_2 (TxB_2); 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT); and 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), as an index of arachidonic acid metabolism by platelets in health and disease. By-and-large, the method used to separate HETE from HHT involve methylation followed by TLC [7,9,10]. In addition, separation achieved by this method may not be complete. In the

present paper we describe a simple procedure in which total separation of the two hydroxy acids could be achieved without derivatization.

MATERIALS AND METHODS

Blood for these studies was obtained from normal volunteer donors. The procedures used to obtain blood, mix the samples with trisodium citrate—citric acid—dextrose (CCD) buffer, (0.1 *M* citrate, 7 m*M* citric acid, 0.14 *M* dextrose, pH 6.5), in a ratio of nine parts of blood to one part anticoagulant and isolate platelet rich plasma (PRP) by centrifugation at room temperature have been described in several publications [11—13]. To obtain platelets free of plasma, PPR was centrifuged, 0.34 *M*—PRP EDTA (9:1), with EDTA as anticoagulant in a cold centrifuge for 20 min (860 *g*). Plasma was discarded and platelets resuspended in Hanks' balanced salt solution (HBSS) free of calcium and magnesium ions. Each sample was mixed with equal volumes of CCD and the procedure was repeated twice to obtain washed platelets. Platelets thus prepared were suspended in normal HBSS and cell counts were made with a Coulter counter.

[^{14}C] Arachidonic acid metabolism by intact platelets

For measurement of arachidonic acid metabolism each reaction mixture containing $1.5 \cdot 10^9$ cells suspended in 1 ml of regular HBSS was stirred on an aggregometer for 5 min at 37°C with 1 μg of labelled arachidonic acid ([1-^{14}C]-AA; New England Nuclear, Boston, MA, U.S.A.). At the end of the experiment 1 ml of ethyl acetate was added to each reaction mixture and acidified with 10 μl of 0.5 *M* citric acid. After thorough mixing the ethyl acetate layer was separated and the reaction mixture was once more extracted with an equal volume of ethyl acetate [14]. Fractions of the organic phase were pooled, concentrated over nitrogen and saved for further analysis.

Separation of thromboxane B_2 and hydroxy acids

Pooled concentrates were plated on a silica gel G plate. The solvent system used for the separation of TxB_2 was diethyl ether—methanol—acetic acid (135:3:3). Hydroxy acids (HETE,HHT) were separated by using a different solvent system consisting of petroleum ether (60—70°C)—diethyl ether—acetic acid (60:39:1). Radioactivity was monitored with a Berthold radiolabel scanner and quantitation was achieved by separation of the spots and counting for radioactivity in a Beckman LS3133T scintillation counter. For characterization of the compounds identified as peaks I and II, the extracts from the plate containing the hydroxy acids were concentrated, derivatized to methyl esters by using diazomethane. Methyl esters were silated by reacting with 25 μl of TRI-SIL/TBT (Pierce, Rockford, IL, U.S.A.) at 50°C for 10 min. Derivatives of the hydroxy acids were subjected to electron impact MS using an LKB 9000 mass spectrometer with a digital PDP-8e data processor. The GC separation of the hydroxy acids were achieved on a 3% OV-1 column with an initial temperature of 180°C programmed for linear increase of 6°C/min.

RESULTS AND DISCUSSION

[1-^{14}C] AA was transformed to TXB_2 and hydroxy acids using intact washed

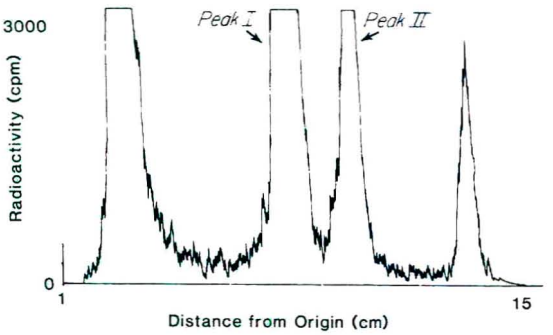

Fig. 1. Radiochromatogram showing the separation of the two hydroxy fatty acids generated by intact human platelets.

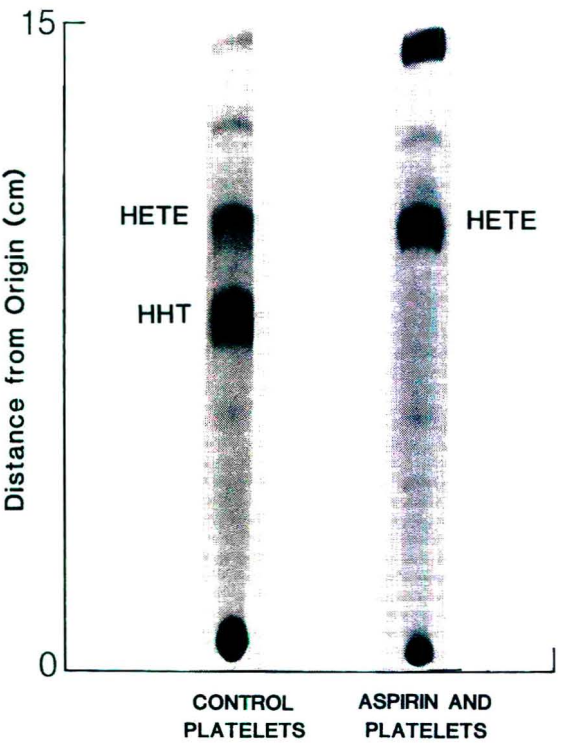

Fig. 2. Photograph of an X-ray film developed on a thin-layer plate. The two hydroxy fatty acids generated by intact blood pletelets are separated completely from one another. Aspirin treated placelets generated only 12L-hydroxy 5,8,10,14-eicosatetraenoic acid (HETE).

platelets as the source of cyclo-oxygenase and lipoxygenase enzymes. The reaction mixtures were extracted, concentrated, plated on a TLC plate without any derivatization step and the metabolites separated. The solvent system selected for the separation of metabolites of arachidonic acid, separated the hydroxy acids completely from one another (Figs. 1 and 2). Retention times for the peaks I and II were 5.8 and 8.4 min, respectively. The mass spectrum of peak I showed ions of high tensity at *m/e* 366, 351, 335, 295, 276, and 225

whereas the major fragments for peak II were at *m/e* 406, 391, 375, 295, 229, 205, and 173. The fragmentation pattern matched the expected pattern for HHT and HETE, respectively. There was less than 1% cross contamination of these products in the individual peaks obtained by this technique. The method is very simple, needs no derivatization of the metabolites and gives complete separation of the two hydroxy acids.

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Note

Lidocaine determination in human plasma with application to single low-dose pharmacokinetic studies

DARRELL R. ABERNETHY*,* and DAVID J. GREENBLATT

Division of Clinical Pharmacology, Departments of Psychiatry and Medicine, Tufts University School of Medicine and New England Medical Center Hospital, Boston, MA 02111 (U.S.A.)

and

HERMANN R. OCHS

Medizinische Universitätsklinik, Bonn-Venusberg (G.F.R.)

(First received February 9th, 1982; revised manuscript received March 26th, 1982)

Lidocaine, a widely used local anesthetic [1] and antiarrhythmic [2], has been measured by specific gas—liquid chromatography (GLC) using flame ionization detection [3—5], GLC using mass spectroscopy (MS) [6, 7], liquid chromatography [8—10], and by enzyme immunoassay (EMIT) [5, 11]. Though specific, with the exception of GLC—MS determinations which are limited by the number of analyses that can be performed, these methods lack adequate sensitivity to detect very low lidocaine plasma levels. This is of importance since lidocaine kinetics are dose-dependent, with only very small doses exhibiting first-order processes, and higher doses becoming zero-order as metabolizing enzyme saturation occurs, thus being described by Michaelis—Menten kinetics [12]. To evaluate lidocaine pharmacokinetics between populations and identify potential differences in the first-order processes, we describe here a method that is specific, separating lidocaine from metabolites and plasma contaminants, and sensitive to 2.5 ng/ml using GLC with a nitrogen—phosphorus detector (NPD). In addition, when coupled with an automated injection system, up to 125 samples per 24 h may be analyzed after a straightforward plasma extraction which requires only 3—4 h technical time.

*Address for correspondence: Division of Clinical Pharmacology, Box 1007, Tufts—New England Medical Center, 171 Harrison Avenue, Boston, MA 02111, U.S.A.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytical instrument is a Hewlett-Packard Model 5840A gas chromatograph equipped with an NPD and an electronic integrator. The column is coiled glass, 1.83 m × 2 mm I.D., packed with 5% OV-101 on 80—100 mesh Chromosorb W HP (Lot No. 17186; Supelco, Bellefonte, PA, U.S.A.). The carrier gas is ultra-high-purity helium (Matheson Gas Products, Gloucester, MA, U.S.A.) at a flow-rate of 30 ml/min. The detector purge is ultra-high-purity hydrogen (Matheson) at 3 ml/min mixed with dry air (Matheson) at 50 ml/min. Operating temperatures are: injection port, 310°C, column, 190°C, detector, 275 °C. Before being connected to the detector, a new column is conditioned at 270°C for 48 h with a carrier flow-rate of 30 ml/min.

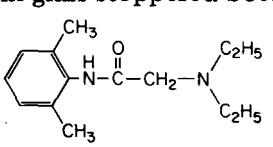
At the beginning of each working day, the column is primed with 2 μg phospholipid (asolectin) in benzene.

Reagents

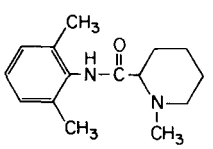
The following reagents are used: certified 99% pure *n*-hexane (Fisher Scientific, Fair Lawn, NJ, U.S.A.), analytical-reagent grade toluene (Mallinckrodt, St. Louis, MO, U.S.A.), certified isoamyl alcohol and analytical-reagent grade methanol (Fisher); analytical-reagent grade concentrated hydrochloric acid, analytical-reagent grade sodium hydroxide and analytical-reagent grade sodium carbonate and sodium bicarbonate all from Mallinckrodt. Isoamyl alcohol is glass distilled prior to use. Other organic solvents are used without further distillation. All aqueous solvents (0.25 *M* sodium hydroxide, 0.1 *M* hydrochloric acid, 1 *M* carbonate—bicarbonate buffer) are washed five times with hexane—isoamyl alcohol (98:2) prior to use.

Reference standards

Pure standards of lidocaine hydrochloride (Fig. 1) and metabolites monoethylglycinexylidide (MEGX) and glycinexylidide (GX) were kindly provided by Astra Pharmaceutical, Worcester, MA, U.S.A.). Mepivacaine hydrochloride (Fig. 1) was supplied by Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). Standards of each are prepared by dissolving the appropriate quantity of the hydrochloride salt to yield 100 mg base in 100 ml methanol. Sequential dilutions to 1 μg/ml are made. The solutions are stored in the dark in glass-stoppered bottles at 4°C and are stable for at least four months.



LIDOCAINE



MEPIVACAINE

Fig. 1. Structural formulae of lidocaine and the internal standard, mepivacaine.

Preparation of samples

Mepivacaine is used as the internal standard for all analyses. A 50-μl volume of stock solution (10 μg/ml), containing 500 ng mepivacaine, is added to a series of 15-ml round-bottomed glass culture tubes, with PTFE-lined screw-top caps. A 0.25—2.0 ml sample of unknown plasma is added to each tube. Calibration standards for lidocaine are prepared by adding 5, 10, 25, 50, 100, 200, 300, 400 and 500 ng of drug to consecutive tubes. Drug-free control plasma is added to each of the calibration tubes. One blank sample, taken from the subject prior to drug administration, is analyzed with calibration standards and each set of unknown samples.

Extraction procedure

A 1-ml volume of 0.25 *M* sodium hydroxide solution is added to each tube. To this is added 5 ml hexane—isoamyl alcohol (98:2) and the tubes are agitated gently in the upright position on a vortex mixer for 15 min. The samples are centrifuged at room temperature for 5 min at 400 *g* (Portable Refrigerated Centrifuge Model PR-2; Head No. 269, International Equipment, Boston, MA, U.S.A.). The organic layer is transferred to another 15-ml glass culture tube which contains 1.2 ml 0.1 *M* hydrochloric acid. This mixture is agitated gently in the upright position on a vortex mixer for 10 min. The samples are again centrifuged at room temperature for 5 min at 400 *g*. The upper, organic layer is discarded. The aqueous layer is transferred by a 9-in. pipet to a conical 13-ml screw-top centrifuge tube. Great care is taken to transfer only the aqueous layer uncontaminated with organic residue. To this 0.5 ml of 1 *M* carbonate—bicarbonate (pH 9.8) buffer is added. The final organic extraction is done by adding 300 μl toluene—isoamyl alcohol (85:15) to the conical centrifuge tube. This mixture is agitated gently in the upright position on a vortex mixer for 15 min. The samples are again centrifuged at room temperature for 5 min at 400 *g*. Using a 9-in. disposable pipet passed through the organic layer, the entire aqueous layer is removed leaving the small volume (300 μl) of organic phase containing lidocaine, metabolites, and internal standard. This is transferred to a 2-ml Wheaton automatic sampling vial (Wheaton Scientific, Millville, NJ, U.S.A.). A 6-μl aliquot of this is injected into the gas chromatograph using the automatic injection sampling system.

Single-dose pharmacokinetic study

Four healthy, young volunteers participated after giving written informed consent. While being electrocardiographically monitored, 25 mg lidocaine hydrochloride (Xylocaine) was administered by intravenous bolus infusion. The drug solution (10 mg/ml) was administered through a glass syringe. Multiple venous blood samples were drawn into Venoject heparin-containing tubes over the following 8 h. Concentrations of lidocaine were determined by the method described above.

Plasma lidocaine concentrations were analyzed by iterative weighted nonlinear least-squares regression analysis [13, 14]. After correction of the dose for the quantity of free base given, the following pharmacokinetic variables were determined: distribution half-life, elimination half-life, total volume of distribution, and total clearance.

RESULTS

Evaluation of the method

Under the described conditions, retention times for lidocaine and metabolites MEGX and GX are shown in Table I. The chromatogram of a plasma sample obtained 2 h after a subject received 25 mg lidocaine hydrochloride intravenously is shown in Fig. 2.

The relation between lidocaine concentrations and the area ratio (versus internal standard) is linear at least to 500 ng/ml. Analysis of more than 50 standard curves over a 6-month period indicates that the correlation coefficient is always 0.99 or greater. Day-to-day coefficient of variation in the slopes of the calibration curves was 8.1%.

The sensitivity limit of the method is 2.5 ng/ml of a 2-ml extracted plasma sample. Within-day coefficients of variation for identical samples were: at 500 ng/ml, 4.0% (n = 6); 200 ng/ml, 6.5% (n = 6); 100 ng/ml, 4.1% (n = 6); 50 ng/ml, 3.9% (n = 6); 25 ng/ml, 5.7% (n = 6); and 5 ng/ml, 1.0% (n = 4).

TABLE I

RETENTION TIMES OF LIDOCAINE, METABOLITES MONOETHYLGLYCINEXYLIDIDE (MEGX), GLYCINEXYLIDIDE (GX), AND MEPIVACAINE, USED AS THE INTERNAL STANDARD

Drug	Retention time (min)
Lidocaine	4.73
MEGX	3.98
GX	3.15
Mepivacaine	8.39

Fig. 2. Gas—liquid chromatograms of (A) extract of 1 ml plasma obtained from subject prior to receiving lidocaine and (B) 2 h after receiving 25 mg lidocaine hydrochloride intravenously. Peaks: L = lidocaine, M = mepivacaine (internal standard).

Residue analysis indicated the extraction of lidocaine is greater than 95% at low (25 ng/ml) and relatively high (200 ng/ml) lidocaine plasma concentrations.

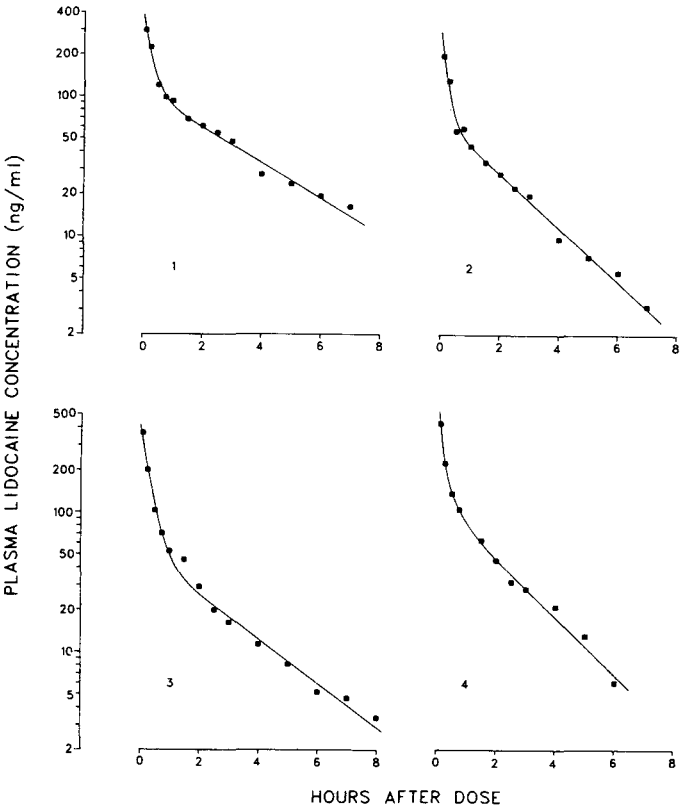


Fig. 3. Plasma lidocaine concentrations and pharmacokinetic functions following intravenous lidocaine administration to four healthy, young subjects. See Table II for derived kinetic variables.

TABLE II

DERIVED LIDOCAINE PHARMACOKINETIC PARAMETERS AFTER A SINGLE 25-mg INTRAVENOUS DOSE ADMINISTERED TO FOUR HEALTHY, YOUNG SUBJECTS

	Subject No.			
	1	2	3	4
Subject characteristics				
Age/sex	31/M	25/F	42/F	24/M
Weight (kg)	70.5	54.5	71.4	70.5
Lidocaine kinetic variables				
Distribution half-life (min)	4.4	12.6	10.6	7.4
Elimination half-life (h)	1.47	1.91	2.37	1.58
Central compartment volume (l/kg)	0.442	0.948	0.785	1.05
Total volume of distribution (l/kg)	1.86	4.29	2.38	3.64
Total metabolic clearance (ml/min/kg)	14.60	25.91	11.58	26.69

Pharmacokinetic study

Fig. 3 shows plasma lidocaine concentrations and pharmacokinetic functions for the subjects. Derived pharmacokinetic parameters are listed in Table II.

DISCUSSION

This report describes a reliable, specific method for the quantitation of lidocaine in plasma using GLC—NPD. Sensitivity is adequate to carry out single-dose pharmacokinetic studies with very low doses to permit study of factors which may influence first-order elimination processes of lidocaine in humans. A basic extraction from plasma, acidic back-extraction, subsequent adjustment of the aqueous phase to a basic pH, and final organic extraction into a small volume for direct injection into the gas—liquid chromatograph is the method employed. This method produces blank plasma samples that are consistently free of contaminants in the areas corresponding to the retention time for lidocaine and efficiently separates the known metabolites, MEGX and GX, from the parent drug.

The value of this GLC—NPD method includes the reasonable time required for sample preparation, as well as its sensitivity which is adequate for single low-dose pharmacokinetic studies.

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Note

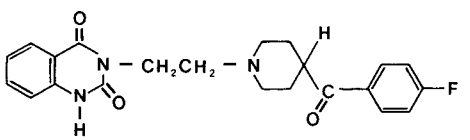
Rapid method for determination of ketanserin, a novel antiserotonin drug, by high-performance liquid chromatography

VERNE SIMON and PITAMBAR SOMANI*

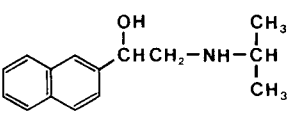
Division of Clinical Pharmacology, Departments of Pharmacology and Medicine, Medical College of Ohio, C.S. 10008, Toledo, OH 43699 (U.S.A.)

(First received February 16th, 1982; revised manuscript received May 3rd, 1982)

Ketanserin (R-41,468) is a novel synthetic drug (Fig. 1) which has been reported to be a highly selective antagonist of the peripheral serotonergic[2] receptors [1]. In the isolated preparations of either arterial or venous vascular segments, ketanserin competitively blocked the direct vasoconstrictor effects of serotonin (5-hydroxytryptamine) thus suggesting its antagonist effect on both resistance and capacitance blood vessels. This drug also caused a dose-dependent reduction in arterial blood pressure of spontaneously hypertensive rats. The exact role of serotonin in the pathophysiology of hypertension, congestive heart failure or other cardiovascular diseases is presently not known, but recent experimental data suggest that serotonin potentiates the vasoconstrictor response of norepinephrine on the alpha adrenergic receptors [1] and it may play some role in these diseases. Ketanserin also inhibits this



KETANSERIN



PRONETHALOL

Fig. 1. Chemical structures of ketanserin and the internal standard, pronethalol.

potentiating effect of serotonin$_2$ receptors since it does not inhibit the response mediated via the adrenergic, cholinergic or other receptors [1]. Ketanserin is currently being investigated for clinical management of hypertension [2] and congestive heart failure [3]. We have developed a simple and rapid method for measurement of ketanserin in human plasma using reversed-phase high-performance liquid chromatography (HPLC) in order to enable us to correlate pharmacodynamic response with plasma drug levels in patients receiving this drug.

EXPERIMENTAL

Chemicals and reagents

Ketanserin, {3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1H,3H)-quinazoline dione} was provided by Janssen Pharmaceutical (New Brunswick, NJ, U.S.A.). The internal standard, pronethalol {α-[isopropylamino)-methyl]-2-naphthalenemethanol}, selected because its chemical structure suggested the possibility of similar extraction and identification characteristics by the HPLC techniques, was obtained from Ayerst Labs. (New York, NY, U.S.A.). Pronethalol is not used clinically and therefore no interference would be expected in patients who may receive ketanserin. HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Methylene chloride was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade.

Instrumentation

All HPLC components were manufactured by Waters Assoc. (Milford, MA, U.S.A.) and included: 6000A solvent delivery system, U6K injector, and Model 440 UV absorbance detector with the wavelength fixed at 254 nm. The recorder was a Houston OmniScribe with a 10-mV output.

Chromatographic conditions

The column was a Waters μBondapak phenyl reversed-phase, 30 cm × 3.9 mm I.D., with particle size of 10 μm. The flow-rate was set at 1 ml/min which produced a precolumn pressure of 69.0 bar. Detection was at 254 nm with sensitivity at 0.02. The chart speed was 0.5 cm/min.

Mobile phase preparation

For preparation of 2 l of mobile phase, 5.44 g of potassium dihydrogen phosphate was dissolved in ca. 1200 ml of water and 608 ml of acetonitrile was added. The pH was adjusted to 2.3 by adding approximately 19 ml of 42% phosphoric acid. The resulting solution was diluted with water to 2 l in a volumetric flask. A type FH 0.5-μm Millipore filter was wetted with acetonitrile, and the mobile phase was filtered, discarding the first small portion.

Extraction procedure

Plasma (1 ml) was added to glass tubes with PTFE-lined screw caps followed by addition of pronethalol hydrochloride (1 μg) in aqueous solution. After vortexing, a solution containing 2 *M* sodium hydroxide and 4 *M* sodium

chloride (200 μl) was added, followed by methylene chloride (5 ml). The tubes were placed on a wheel tilted at a 45° angle and rotated for 20 min at 40 rpm. The tubes were then centrifuged at 2120 *g* (bench top centrifuge) for 10 min and the upper plasma layer was removed by aspiration. If the plasma layer extended into the organic layer, it was removed by gentle shaking and, if necessary, repeated centrifugation. The organic layer was transferred to a conical centrifuge tube and evaporated to dryness in water which was gradually warmed from 45°C to 80°C. To the cooled tubes were added 0.8 *M* phosphoric acid (150 μl) and *n*-pentane (500 μl). The tubes were vortexed vigorously for 90 sec and centrifuged at 2120 *g* for 5 min. The aqueous layer was frozen by immersion in dry ice—ethanol for 1 min and the *n*-pentane layer was removed by aspiration. The pH of the aqueous layer was adjusted to 6.6 by addition of 3 *M* sodium hydroxide (50 μl). A 100-μl aliquot of the resulting 200 μl of aqueous extract was injected into the HPLC apparatus. The steps involved in the extraction of ketanserin from the plasma are summarized in Fig. 2.

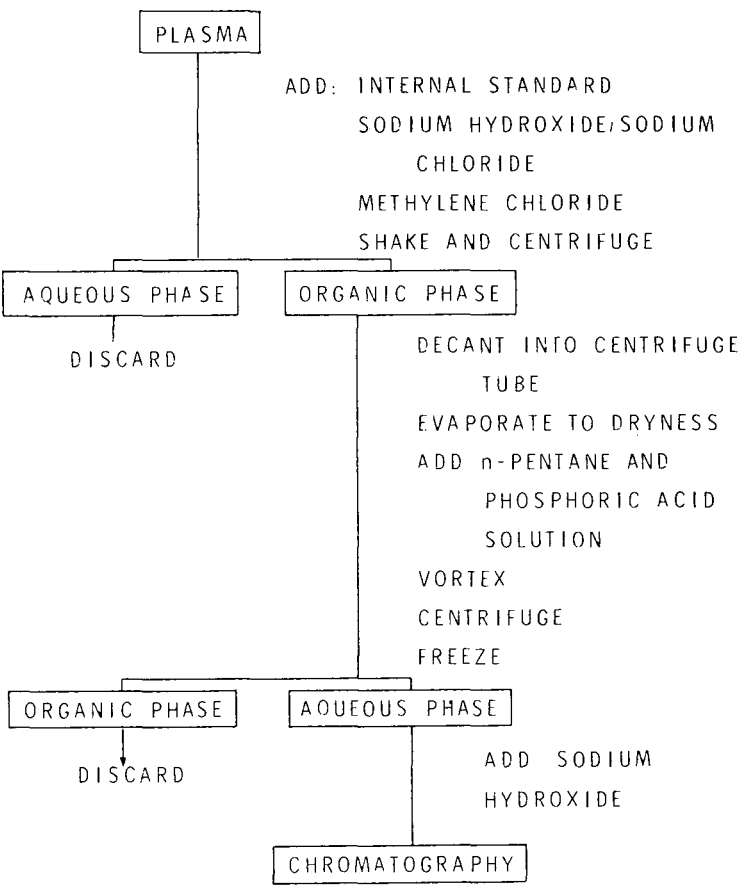


Fig. 2. Flow diagram of the procedure for the extraction of ketanserin from plasma.

Preparation of calibration standards

Ketanserin (free base) was dissolved in 0.8 *M* phosphoric acid and diluted with distilled water to produce a solution containing 2 ng/μl ketanserin and $5.8 \cdot 10^{-5}$ *M* phosphoric acid. Aqueous pronethalol·HCl at a concentration of 20 ng/μl was used as the internal standard. Although fresh solutions of ketanserin and pronethalol were prepared daily to generate the data presented in this report, both solutions appear to be stable for at least one month when stored at 4°C.

RESULTS AND DISCUSSION

In the procedure described above, the retention times for pronethalol and ketanserin on this column were found to be 7.0 and 9.8 min, respectively. The chromatogram of a plasma sample obtained from a patient 0.5 h following the intravenous injection of 10 mg of ketanserin is shown in Fig. 3. This

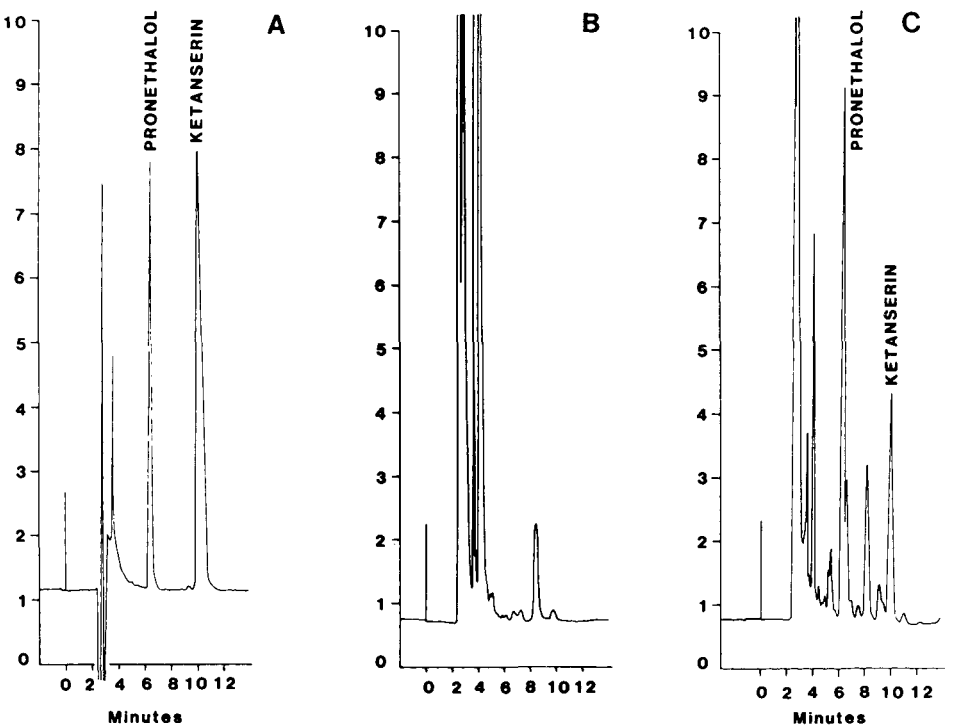


Fig. 3. High-performance liquid chromatograms of ketanserin and pronethalol (internal standard) extracted from a plasma sample obtained from a patient. Blood was drawn before and at 0.5 h after intravenous infusion of 10 mg ketanserin. In each trace, the absorbance is shown along the vertical axis and the retention time along the horizontal axis. (A) Aqueous solution of pronethalol and ketanserin; (B) control plasma and (C) post-ketanserin plasma of a patient who was given 10 mg of the drug intravenously. The peaks for ketanserin and pronethalol can be easily distinguished from other unidentified peaks in this patient (see blank, B). The calculated concentration of ketanserin in this patient was found to be 0.235 mg/l.

patient was a 45-year old white male suffering from congestive heart failure secondary to cardiomyopathy of unknown etiology. He also was concurrently receiving digoxin, furosemide, valium and acetaminophen. It may be seen (Fig. 3) that both pronethalol and ketanserin peaks were easily identified in the plasma of this patient and these peaks correlated well with those obtained with an aqueous solution containing ketanserin and pronethalol. It should be pointed out that the retention times of both ketanserin and pronethalol were found to increase if either the pH or the water content of the mobile phase was increased.

The standard curve for ketanserin was obtained in a series of experiments in which varying amounts of ketanserin ranging from 0.05 to 0.25 μg/ml and a fixed concentration of the internal standard (1 μg/ml pronethalol) were added to the plasma obtained from normal human volunteers. After the extraction procedure described above, the samples were injected into the HPLC apparatus, the peak heights of both ketanserin and pronethalol were carefully determined, and the peak height ratio, H_k/H_p, was calculated for each concentration. Linear regression of the drug concentration versus peak height ratio showed an excellent fit ($r^2 = 0.98$) and yielded the equation:

$$[K] = (0.5046 \pm 0.0053)\, H_k/H_p \quad (n = 24) \tag{1}$$

where $[K]$ = calculated concentration of ketanserin free base (μg/ml), H_k = height of the ketanserin peak and H_p = height of the pronethalol peak. The coefficient of the peak height ratio in eqn. 1 was based on the addition of a fixed amount (1 μg/ml) of pronethalol to the plasma samples. However, it may be pointed out that this coefficient is proportional to the quantity of pronethalol added, and therefore if a different amount of the internal standard were used, a new coefficient should be derived.

The calculated values of ketanserin ($[K]_{calcd.}$) from the measured peak height ratio shown in eqn. 1 were correlated with the actual ketanserin con-

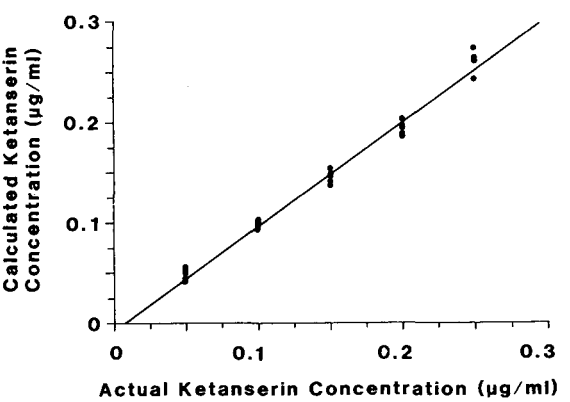


Fig. 4. Linear regression analysis of the actual ketanserin concentration added to the plasma (horizontal axis) versus calculated concentration from the peak height ratios (vertical axis). Each point represents one sample. The calculated regression line showed an excellent fit with $r^2 = 0.99$.

centration ($[K]_{actual}$) added to the plasma by the linear regression analysis (Fig. 4) and the following equation was obtained:

$$[K]_{calcd.} = 0.007 \pm 1.036\ [K]_{actual} \quad (2)$$

Data obtained by this technique showed an excellent correlation of the measured and actual ketanserin concentration ($r^2 = 0.99$).

Reproducibility of the results obtained by this technique was evaluated by replicate analysis of five plasma samples containing ketanserin at each of the following five concentrations: 0.05, 0.1, 0.15, 0.2 and 0.25 μg/ml. The amount of calculated vs. actual plasma concentration was again found to show an excellent correlation and reproducibility, since the coefficients of variation were 9.5, 1.6, 4.3, 3.0 and 8.8%, respectively.

The extraction efficiency was determined by comparing the peak height of the entire aqueous extract of ketanserin with the peak height of the same quantity of ketanserin in the aqueous solution. The recovery of ketanserin was 82 ± 8% ($n = 25$).

The entire procedure including sample preparation, extraction and HPLC determination of ketanserin can be easily carried out by a single person and it may be possible to perform as many as 15—20 drug assays per day. In order to permit batch analysis, we routinely carry out the extraction procedure the day prior to the drug assay and we have found the results to be consistent with the above data. These results suggest that the HPLC assay for ketanserin is simple, rapid, reproducible and sensitive in the range of expected plasma concentrations in patients receiving this new drug and the method reported here should prove to be useful for determination of the clinical pharmacokinetics of ketanserin.

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Note

Determination of a novel 5-fluorouracil derivative in rat and human plasma by high-performance liquid chromatography

KANJI TAKADA*, HIROSHI YOSHIKAWA and SHOZO MURANISHI

Kyoto College of Pharmacy, Yamashina, Kyoto 607 (Japan)

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Although 5-fluorouracil (5-FU) remains an important antitumor agent for the treatment of solid tumors in clinical medicine [1, 2] it has severe adverse reactions such as gastrointestinal disorders [3, 4]. Therefore, it is necessary to minimize or improve its side-effects and develop a new derivative from the standpoint of biopharmaceutics and drug design. A novel 5-FU derivative (Fig. 1), 1,3-didecanoyl-2-[6-(5-fluorouracil-1-yl)carbonylamino]glyceride

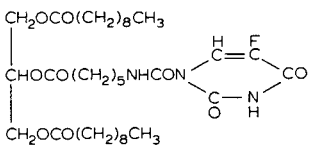

Fig. 1. Chemical structure of DFUG.

(DFUG), was designed in our laboratory. As suggested from the chemical structure, DFUG is a conjugate of 5-FU with triglyceride. The potent antitumor activity of orally administered DFUG was confirmed by basic studies using mice transplanted with tumors in our laboratory. Then, to measure not only the absorbability from the gastrointestinal tract after oral administration of DFUG but also the conversion efficiency of DFUG to 5-FU, it is necessary to develop an assay system for DFUG separate from 5-FU in plasma of both experimental animals and human.

MATERIALS AND METHODS

Reagents

The purified DFUG was provided by Dr. H. Nakao (Sankyo Pharmaceutical

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Co. Ltd., Tokyo, Japan). Methanol, ethyl acetate, and tetrahydrofuran were obtained from Nakarai Chemicals, Kyoto, Japan. Miglyol® 812 was obtained from Dynamit Nobel, Troisdorf-Oberlar, G.F.R. All other chemicals were of reagent grade.

Determination of DFUG in plasma

To 15-ml centrifuge tubes, 100 μl of rat plasma, 1 ml of 0.1 *N* hydrochloric acid, and 4 ml of ethyl acetate were added. The tubes were placed on a reciprocating shaker for 15 min. The aqueous and organic phases were then separated by centrifugation (1500 *g*, 10 min). Then 3 ml of the organic phase were removed with a Pasteur pipette and placed in a 15-ml glass tube. This ethyl acetate extraction of the plasma was repeated one more time and the organic phases were pooled in the conical glass tube. The combined ethyl acetate extracts were then evaporated at room temperature under vacuum. The residue was dissolved in 200 μl methanol of which 50 μl were injected into the chromatograph.

Analysis was performed using a Shimadzu (Kyoto, Japan) Model LC-3A pump and Model SPD-2A UV absorbance detector. The column (25 cm × 4 mm I.D., stainless steel) was packed with nominal 10-μm ODS-silica gel (LiChrosorb ODS, manufactured by E. Merck, Darmstadt, G.F.R.) using a balanced density slurry packing procedure similar to that described by Majors [5]. The mobile phase, methanol—water—tetrahydrofuran (400:10:4), was prepared fresh daily. The flow-rate was 1 ml/min and the pressure approximately 20 kg/cm^2. Detection was UV spectrophotometry at 260 nm. The detector was usually at a sensitivity of 0.01 a.u.f.s. The detector signal was processed and recorded using a Model C-R1A reporting integrator (Shimadzu). Levels were estimated by the chromatographic technique of comparing peaks obtained from rat or human plasma with curves obtained from the plasma to which were added known amounts of DFUG.

RESULTS AND DISCUSSION

The UV absorption spectrum of DFUG is shown in Fig. 2, where the peak of DFUG is at 255 nm. However, determination of DFUG was facilitated at 260 nm, since there is minimal interference by other components eluting near the retention time of DFUG, about 5.8 min in Fig. 3. Fig. 3A shows a typical chromatogram for DFUG from human plasma. For comparison, the chromatogram obtained from DFUG-free human plasma at the same sensitivity is shown in Fig. 3B. There are no peaks in this chromatogram that would interfere with DFUG. In addition, this assay method was applied to a rat plasma sample spiked with DFUG. Though the resulting chromatograms are not shown, it may be mentioned that this assay method is also applicable to rat plasma samples.

The recovery of DFUG added to rat plasma was determined by comparing the peak area from a plasma sample containing 500 ng of DFUG per ml with the results obtained from an aqueous standard of the same concentration. The results (Table I) indicate that recovery is greater than 98%. Plasma samples containing 833.3, 625.0, 416.7, 208.3, 138.9, and 83.3 ng of DFUG per ml

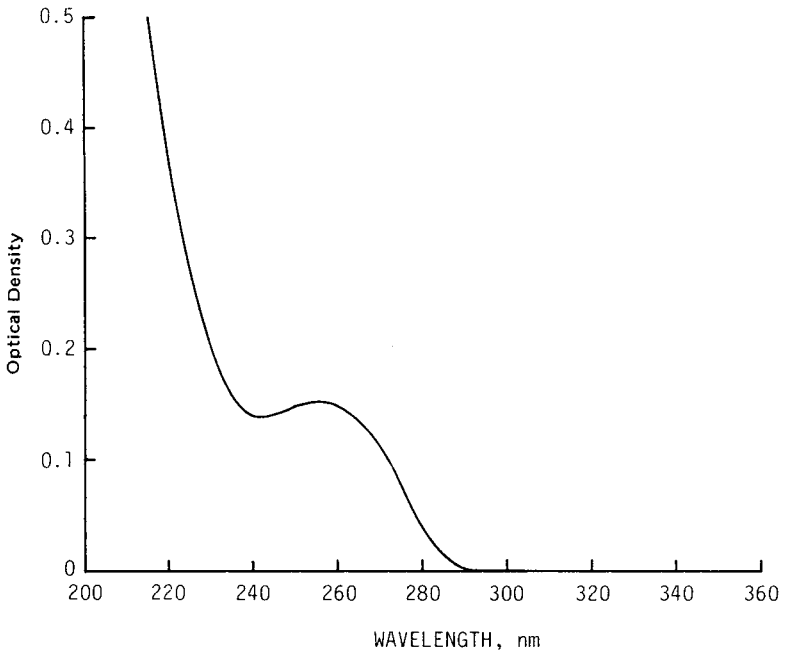

Fig. 2. The UV absorption spectrum of DFUG at a concentration of 10 μg/ml in methanol.

TABLE I

COMPARISON OF PEAK AREAS FOR FOUR ANALYSES OF A STANDARD MIXTURE OF DFUG

Injection	Peak area ($\mu\bar{V} \times$ sec)			Peak area ratio
	Mean	Standard deviation	Coefficient of variation (%)	
Serum samples	100,562	3331	3.31	98.4
Aqueous standards	102,191	3151	3.08	100

were prepared and analyzed to determine the standard curve. For each concentration, duplicate injections were made and the sums of the DFUG peak heights were averaged. A linear least-squares regression analysis gave a coefficient of correlation (r) of 0.997. The UV detector was set at its maximum sensitivity (0.005 a.u.f.s.), and 50 μl of ethyl acetate extract dissolved in methanol were injected onto the column. However, up to 100 μl can be injected before resolution of DFUG is lost. Therefore, the limit of detection of this method is about 40 ng/ml. The standard curves were made at least once a day as no internal standard was used in this assay. The standard curves were well reproducible as the standard deviation was 3.5% (n=6 at 208.3 ng/ml). This can probably be attributed to the stability of the column and the accuracy of the selection of the mobile phase. At the early stage of this assay method, methanol—water (100:5) was used as a mobile phase. However, slight inter-

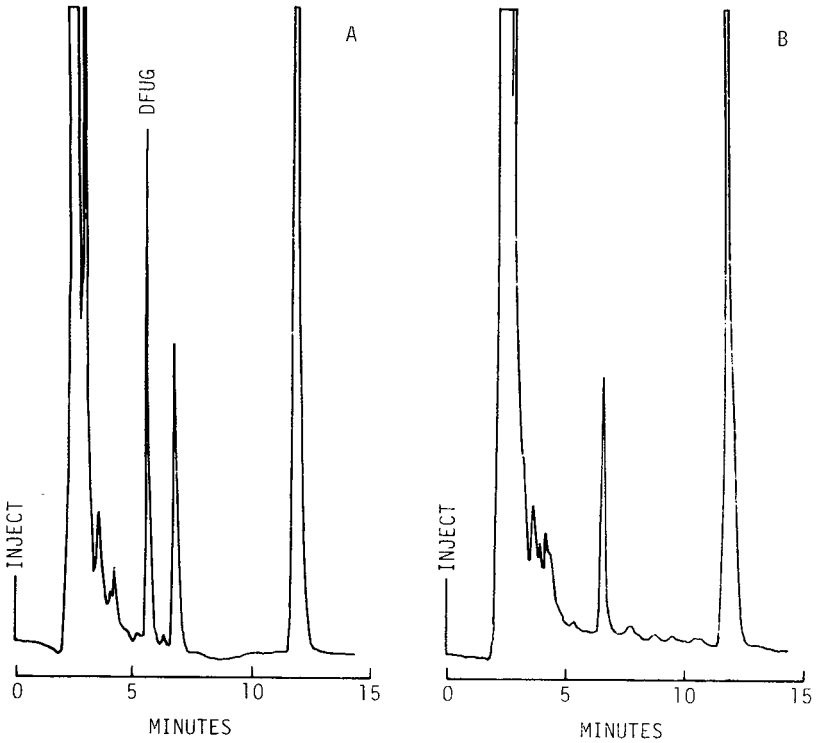

Fig. 3. A, Representative chromatogram of the HPLC of DFUG in human plasma. B, Chromatogram of a blank human plasma.

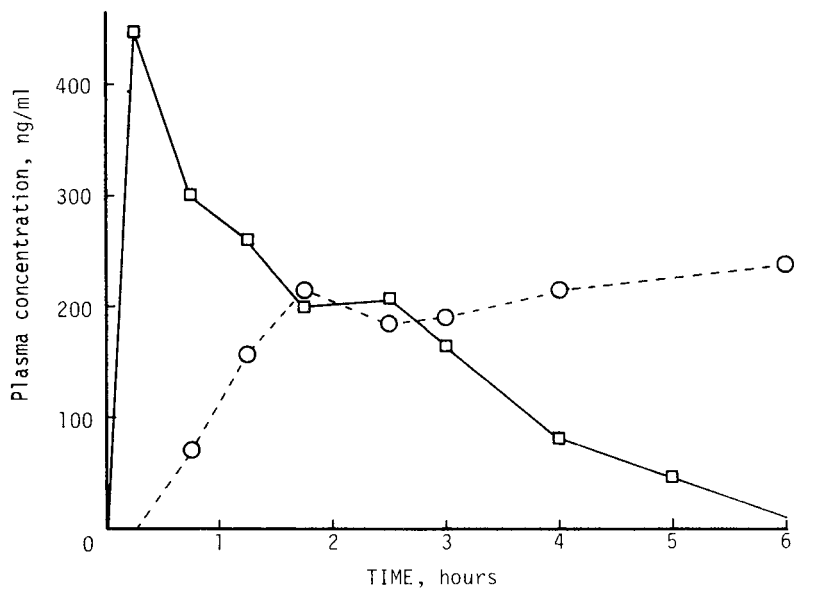

Fig. 4. Concentrations of DFUG (□) and 5-FU (○) in plasma of a rat after administration of 22 mg of DFUG per kg body weight, orally.

ference was detected by another component eluting near the retention time of DFUG. This problem was solved by adding tetrahydrofuran to the mobile phase, and the interference was eliminated, as shown in Fig. 3.

A male Wistar rat was administered DFUG, 22 mg/kg, dissolved in 0.1 ml of Miglyol 812 as an oral dose. Venous blood samples were taken for 6 h. Plasma levels of DFUG and 5-FU were both measured by the high-performance liquid chromatographic (HPLC) method (Fig. 4). With respect to the plasma level of 5-FU, several investigators [6, 7] have developed assay methods using reversed-phase HPLC. In this experiment, the same column was used for this purpose. However, as a mobile phase, 0.1% acetic acid was used. As shown in Fig. 4, the peak plasma DFUG level appeared at about 20 min after oral dose. On the other hand, a considerably high plasma 5-FU level was maintained for a long period.

These investigations will be continued with patients suffering from cancer.

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Note

Rapid method for the purification of [3,5-^{14}C]paracetamol

JAGDISH L. DEVALIA* and ANDRÉ E.M. McLEAN

Laboratory of Toxicology and Pharmacokinetics, Rayne Institute, University College London, University Street, London WC1E 6JJ (Great Britain)

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Paracetamol is a widely used analgesic which is generally well tolerated at the therapeutic dose but causes centrilobular hepatic necrosis when taken in overdose [1]. Various mechanisms for the toxicity of paracetamol have been proposed, but covalent binding of an active metabolite to vital cellular proteins [2] is widely accepted as an essential step.

In many of the studies concerned with mechanism of injury, covalent binding of [^{14}C]paracetamol to hepatocellular proteins has been measured. We have also carried out such studies, but it was found that the commercially available [^{14}C]paracetamol, of apparently high purity, as indicated by thin-layer chromatography (TLC), contained an impurity that bound strongly to proteins.

Here we report a simple and rapid high-performance liquid chromatographic (HPLC) technique for the purification to $\geqslant$99.9% of commercially available [^{14}C]paracetamol on a preparative scale using an ordinary analytical column. By this technique, a ^{14}C-labelled contaminating compound (approximately 1.5% of total preparation) which was not detected by any of the TLC techniques employed in previous work [2,3] and which bound covalently to albumin, without prior need for metabolic activation, was isolated.

MATERIALS AND METHODS

[^{14}C]Paracetamol, labelled in the ring C-3 and C-5 positions (specific activity 35 mCi/mmol) was obtained from Amersham International (Amersham, Great Britain). TLC silica gel plates, containing a fluorescent indicator F-254, were supplied by E. Merck (Darmstadt, G.F.R.). All solvents used for TLC techniques were of AnalaR grade and obtained from BDH (Poole, Great Britain). HPLC grade methanol was from Fisons (Loughborough, Great Britain) and Hypersil ODS 5 μm HPLC column packing was purchased from Magnus Scien-

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tific (Sandbach, Great Britain). Instagel scintillation cocktail was supplied by Packard (Caversham, Great Britain) and crystalline paracetamol and bovine serum albumin (BSA) were purchased from Sigma (London) (Poole, Great Britain).

TLC of [^{14}C]paracetamol

TLC analysis was carried out on silica gel 60 plates. Chromatographic separations, employing 5 μl (45 μg) of 60 m*M* paracetamol stock solution as standard, were carried out in vapour-saturated glass chromatography tanks using one of the following solvent systems: System 1: propan-2-ol–acetone–ammonia solution (Sp. gr. 0.88)–water (70:20:20:20, v/v); System 2: ethyl acetate [2, 3]; System 3: ethyl acetate–methanol–glacial acetic acid–water (12:6:0.2:1.8, v/v) [2, 3].

Following chromatographic development for 1–3 h, the plates were allowed to dry and then observed under UV light at 254 nm. Strips (1 cm) were scraped off, from the line of origin up to the solvent front, and each fraction suspended as a fine slurry in 1.5 ml water. After addition of 5.0 ml Instagel, the fractions were counted in Packard Tri-Carb liquid scintillation counter. Counts were converted to disintegrations per minute (dpm) using the automatic external standard, calibrated with an internal [^{14}C]hexadecane standard. The silica powder suspended in water did not interfere with the estimation of paracetamol radioactivity.

HPLC of [^{14}C]paracetamol

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A constant flow chromatography pump connected to a Waters Assoc. universal liquid chromatography injector, with a 2.0-ml back-fill loop, was used as the solvent delivery system. This led to a column, 25 cm × 4 mm I.D. packed with Hypersil ODS 5 μm.

Chromatographic purification of [^{14}C]paracetamol was carried out using a methanol–water (10:90, v/v) solvent system pumped at a constant flow-rate of 1.0 ml/min. The column was loaded by injection of 1.0 ml of paracetamol solution in water containing approximately 1.0 mg and about 15 μCi. Prior to the chromatographic run, this comparatively large quantity of paracetamol was concentrated down onto the column, by pumping a 100% water mobile phase, instead of methanol–water (10:90), for 2 min. During the chromatographic operation, up to 60 fractions of 0.5 ml were collected, using a LKB 2112 Redirac fraction collector.

Standardisation of the chromatographic separation was achieved using unlabelled paracetamol. The fractions collected were analysed for optical absorption at 243 nm wavelength, in a Pye-Unicam SP 30 spectrophotometer.

Prior to recovery of the radioactive paracetamol, 20-μl aliquots of each fraction were analysed for radioactivity. The seven fractions (from 19 to 25) containing the highest counts of the major peak were pooled, saturated with sodium chloride and gently mixed with re-distilled diethyl ether. Purified [^{14}C]paracetamol, which had been back-extracted into the solvent ether phase, was crystallised out by evaporating the diethyl ether to dryness under a gentle stream of oxygen-free nitrogen, and finally dissolved in water as a concentrated working solution.

The radioactivity profile of the fractions collected revealed a contaminating ^{14}C-labelled compound 2, which was also purified and recovered (fractions 34 to 43).

Between runs the column was cleaned by pumping 100% methanol through the system. This led to recovery of a very small fraction (0.5% of total) of radioactive material.

Covalent binding study

Preliminary experiments to study non-enzyme catalysed covalent binding of both [^{14}C]paracetamol and the ^{14}C-labelled contaminant (compound 2) were carried out using either a Hepes—Ringer buffer system [4], containing 2.5% bovine serum albumin, or an aqueous suspension of rat liver hepatocytes which had been prepared by a modification of the method of Seglen [5]. For the purposes of this study the hepatocytes were made non-viable, by suspension and vigorous mixing in water.

Flasks containing 2.5-ml final volumes of incubation medium containing albumin or the approximately 300 mg/ml cell suspension, plus either purified radioactive paracetamol (approx. 0.5 μCi, and 25 μmol) or compound 2 (approx. 0.12 μCi and 3.4 nmol, if the specific activity is that of the original [^{14}C]paracetamol as bought) were incubated at 37°C in a shaking water bath. Aliquots (0.5 ml) were withdrawn from the flasks at 0, 1, 2 and 4 h of incubation, and were analysed for total covalently bound ^{14}C-labelled material to protein. The cellular preparation was precipitated with 10% (w/v) trichloroacetic acid (TCA) in water and washed four times with the TCA and then twice with methanol—water (80:20, v/v). Each washing stage was carried out by suspension of the protein pellet followed by centrifugation at 1500 *g* for 3 min.

The albumin precipitates were also washed as described for the protein pellets, except that at the methanol washing stages, the precipitates were initially dissolved in 5.0 ml of methanol—water (80:20) followed by re-precipitation with 5.0 ml of 10% TCA.

The final washed protein pellets were suspended in 2.0 ml 1 *M* sodium hydroxide solution and digested overnight at 45°C. Aliquots (1 ml) of the digests were suspended in 5.0 ml. Instagel, neutralised with 1.0 ml 1 *M* hydrochloric acid and subjected to liquid scintillation counting.

Protein was also estimated by the method of Lowry et al. [6], in the alkaline digests.

RESULTS

TLC analysis

All TLC separations, irrespective of the development system used, displayed a single major [^{14}C]paracetamol spot, which corresponded to the reference sample spot in R_F and fluorescence quenching when viewed under the UV light at 254 nm. The R_F values and percent recovery of total radioactivity for each system are as shown in Table I.

Radioactivity on the TLC plates corresponded, in all cases, with the major spot visualised by its UV fluorescence quenching.

TABLE I

R_F VALUE OF AND PERCENT RADIOACTIVITY RECOVERY FROM [^{14}C]PARACETAMOL SPOT VISUALISED IN DIFFERENT SOLVENT SYSTEMS

Chromatographic methods and solvents are as described in the Materials and methods section. A 5-μl volume of [^{14}C]paracetamol solution containing 136,000 dpm was loaded onto the TLC plates. A 1.5% impurity spot containing about 2000 dpm, an easily countable amount, could not be detected. TLC of the isolated compound 2 also showed that the R_F value of this compound was very similar to that of paracetamol and *p*-aminophenol (PAP) in the three TLC systems chosen. A further TLC system [7] could distinguish paracetamol, PAP and the impurity, compound 2. PAP and compound 2 both gave a long tailing spot (R_F 0.18—0.35), that overlapped the distinct paracetamol spot (R_F 0.24). However, this does not suffice to give a distinct spot for a 1.5% impurity of compound 2 in paracetamol.

Solvent system	R_F	Percent of total activity
1	0.74	99.7
2	0.29	99.6
3	0.80	99.4

HPLC analysis

Analysis, by absorption spectroscopy, of the fractions collected after loading 1.0 mg of non-radioactive paracetamol indicated that the retention time of paracetamol on the column under the specified conditions was 9—10 min and only one peak came off the column.

The radioactivity profile of the fractions collected after loading 1.0 mg of [^{14}C] paracetamol displayed two major peaks, of which the first peak had both a retention time and a profile equivalent to the absorption profile of authentic paracetamol (Fig. 1). The second peak had a longer retention time of 16—18 min and did not show up on the UV absorption monitor set at 243 nm (Fig. 1).

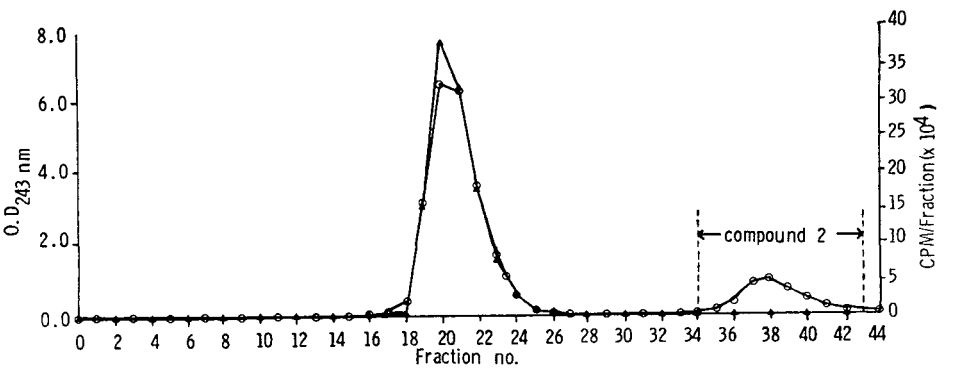

Fig. 1. Spectrophotometric absorption profile (▲) and radioactivity profile (○) of the fractions collected after separate HPLC runs are shown. Unlabelled paracetamol (approx. 1.0 mg) was chromatographed and the absorbance profile was determined for each 0.5-ml fraction. The radioactivity profile was determined by counting 20-μl aliquots of each fraction. For compound 2 only, the cpm/fraction is calculated as a multiplier factor of 10^3 instead of 10^4.

Calculation of total radioactive counts from each HPLC run indicated that the [^{14}C]paracetamol accounted for approximately 98.5% and the ^{14}C-labelled compound 2 accounted for about 1.5% of the total counts recovered.

When the separated paracetamol was re-run only one peak was found, indicating that peak 2 was not a breakdown product in equilibrium with paracetamol. Similarly when peak 2 material was re-run, it re-appeared at the same retention time. Preliminary observations of the ^{14}C-labelled compound 2 material suggest that this compound may be *p*-aminophenol (PAP), which is used in the preparation of [^{14}C]paracetamol. Like PAP it forms a dark brown compound on standing in air.

Non-enzyme catalysed covalent binding

It was noticed that very little purified [^{14}C]paracetamol bound covalently to albumin in the Hepes—Ringer buffer. In contrast, the binding of the hepatocellular proteins was slightly elevated initially and then quite markedly by the end of the 4-h incubation period (Table II).

The ^{14}C-labelled contaminating compound 2 on the other hand bound extensively, in a cumulative fashion to both albumin and hepatocellular protein, in spite of being present at far lower concentration.

TABLE II

NON-ENZYME CATALYSED COVALENT BINDING OF PURIFIED [^{14}C]PARACETAMOL AND [^{14}C]COMPOUND 2 TO PROTEIN

Approximately $1.3 \cdot 10^6$ dpm of [^{14}C]paracetamol was added to flasks 1 and 3, and $0.3 \cdot 10^6$ dpm of [^{14}C]compound 2 was added to flasks 2 and 4. Samples were taken, washed and analysed as described in the Materials and methods section.

Incubation mix	Incubation time (h)	dpm bound/ flask	dpm bound/ mg protein	dpm bound/ 10^3 dpm added
(1) Purified	0	24.8	0.7	0.02
[^{14}C]paracetamol	1	99.4	3.5	0.08
Hepes—Ringer	2	125.8	3.5	0.10
albumin	4	176.1	3.9	0.14
(2) Purified	0	12.4	0.4	0.04
[^{14}C]compound 2	1	1125.0	33.5	3.75
Hepes—Ringer	2	1543.2	47.6	5.14
albumin	4	2780.0	60.4	9.27
(3) Purified	0	49.7	3.0	0.04
[^{14}C]paracetamol	1	600.0	36.6	0.46
Hepatocytes	2	1321.0	94.4	1.02
in water	4	4762.5	270.6	3.66
(4) Purified	0	87.5	4.8	0.29
[^{14}C]compound 2	1	3879.0	262.1	12.93
Hepatocytes	2	8773.2	577.2	29.24
in water	4	20037.5	1138.5	66.79

DISCUSSION

The drug paracetamol shows a combination of usefulness in normal dose and risk of injury in overdose that makes it imperative to understand its mechanism of toxicity. In order that this goal be achieved, it is also important that the compound under study is both authentic and of highest purity available so that mis-interpretation of experimental results can be avoided.

The covalent binding studies described here show that the purified [^{14}C] paracetamol used does not bind to albumin to any significant extent. However, the binding of [^{14}C] paracetamol to cellular proteins may be due to the presence of endogenous haem and co-factors even in the non-viable cell preparation.

The contaminating ^{14}C-labelled compound 2 binds to both albumin and cellular proteins far more rapidly and extensively than [^{14}C] paracetamol, even though present at only a quarter of the activity of the paracetamol. This again emphasises the need for working with compounds of both very high radiochemical and chemical purities.

In some previous studies where covalent binding of paracetamol was thought to be the cause of paracetamol hepatotoxicity, radioisotope preparations of apparent radiochemical purities of $\geqslant$99.9%, as assessed by TLC techniques, were used. Results presented in the present study clearly show that TLC techniques, as a check for radiochemical purity, may not be enough and that other methods of much higher resolution may have to be employed for this purpose. The HPLC technique described is suitable since it incorporates the high sensitivity required for analytical work. In addition, the technique is suitable as a preparative method, because of its simplicity, rapidity and the relative ease with which very highly purified preparations of [^{14}C] paracetamol may be obtained.

ACKNOWLEDGEMENTS

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CHROMBIO. 1359

Note

Determination of 2-oxo-pyrrolidine-1-acetamide (piracetam) in human plasma using high-performance liquid chromatography

W. RIECK and D. PLATT*

Institute of Gerontology, University of Erlangen-Nürnberg and 2nd Internal Medicine, Nürnberg (G.F.R.)

(First received March 9th, 1982; revised manuscript received April 6th, 1982)

The high-performance liquid chromatographic (HPLC) determination of piracetam proved to be desirable in the course of a pharmacokinetic study [1].

Research into the gas chromatographic detection of piracetam can be found in the literature [2, 3]; however, until recently, there was no mention at all of a method for determining piracetam using HPLC [4, 5].

The method described below is distinguished by its simplicity, selectivity and sensitivity. Preliminary results concerning the pharmacokinetics of piracetam in man using this assay are also given.

EXPERIMENTAL

Chemicals

Piracetam was kindly provided by the UCB Company (Batch No. 4070), the methanol for chromatography was supplied by Merck.

Instruments and chromatographic conditions

The analyses were carried out isocratically in an HPLC system consisting of a Kontron LC pump (Model 410), Kontron autosampler (Model MSI 660), Kontron ultraviolet (UV) detector (Model Uvikon 720LC), and a Spectra-Physics integrator (Model SP 4100).

A LiChrosorb RP-18 column (particle size 10 μm, 25 cm $\times$ 4.9 mm I.D.) manufactured by Kontron was used. The flow-rate of the methanol saturated with helium was 1 ml/min. A 20-μl volume of the prepared sample solution was used for each injection, piracetam being detected at 206 nm.

The external standard method was chosen for calibration.

Sample preparation

Venous blood was taken (containing no piracetam) and left to stand in a heparinized test tube for 1 h at room temperature and subsequently centrifuged at 3000 *g* for 10 min. The stock solution was obtained by adding 71.0 mg of piracetam to 5 ml of the plasma, mixing for 1 h in a reciprocating impeller agitator (IKA-Vibrax VXR) and then removing samples of 250, 150, 50, 25, and 10 μl from the solution, pouring them into small measuring flasks and filling to 1 ml with pure plasma. (Plasma concentrations: 3.55, 2.13, 0.71, 0.355, and 0.142 mg/ml.) Then 50 μl were taken from each of the five solutions and 1 ml of methanol was added to each 50-μl sample.

After centrifugation (Eppendorf Centrifuge, Model 3200) for 3 × 2 min at 17,000 *g*, the remaining liquid was filtered through a methanol-preconditioned C_{18} Sep-Pak cartridge (Waters Assoc.) and the eluate was collected in a 5-ml measuring flask.

The centrifuged residue was extracted twice with 1 ml of methanol each time and the extracts filtered once again through the C_{18} Sep-Pak cartridge. Finally, the cartridge was rinsed with 1 ml of methanol and the measuring flask was filled to a final volume of 5 ml with methanol.

The patients' plasma samples were processed using the same method.

Each sample was injected four times.

RESULTS

The calibration curve of piracetam from plasma was linear in the concentration range concerned (Table I).

The detection limit of piracetam with plasma components is approximately 0.7 μg/ml, corresponding to approximately 14 ng per injection, and without plasma components approximately 0.3 μg/ml, corresponding to 6 ng per injection.

The typical chromatographic behaviour of piracetam on the LiChrosorb RP-18 column is shown in Fig. 1.

The recovery rate for piracetam in the concentration range 3.55 and 0.142 mg/ml is 99.8% (relative S.D. = 10.5%) (average value from 20 injections).

In contrast to the paper of Daldrup et al. [4], the piracetam detection presented here is distinguished by the fact that neither a gradient system nor expensive acetonitrile need be used. Furthermore, the decisive advantage of our

TABLE I

CALIBRATION CURVE FOR PIRACETAM

Plasma concentration (mg/ml)	Area (arbitrary units)	Relative S.D. (%)
3.55	1.679	2.004
2.13	1.000	1.719
0.71	0.336	1.037
0.355	0.139	2.073
0.142	0.052	5.050

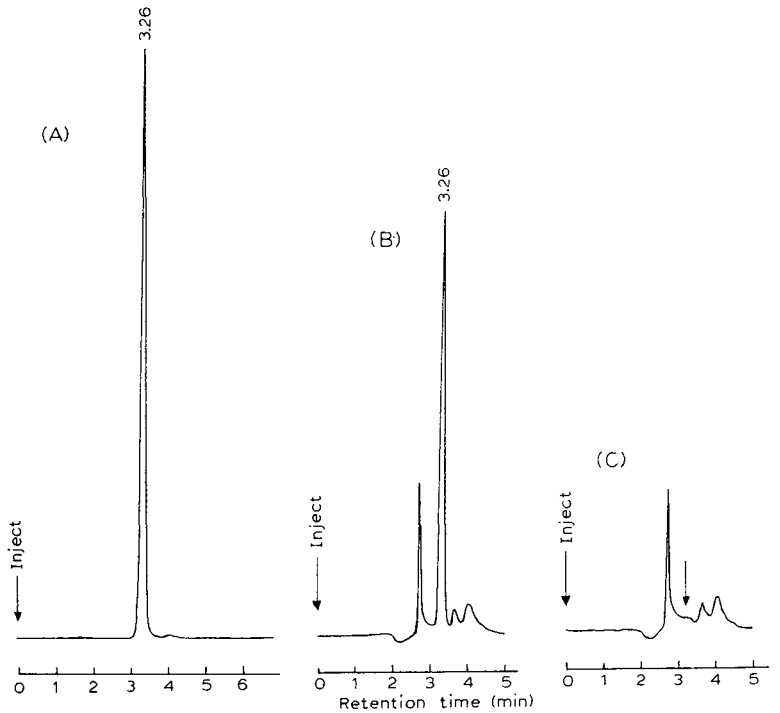

Fig. 1. Chromatograms of piracetam (A) without plasma components, (B) with plasma components, (C) blank plasma. For chromatographic conditions see text.

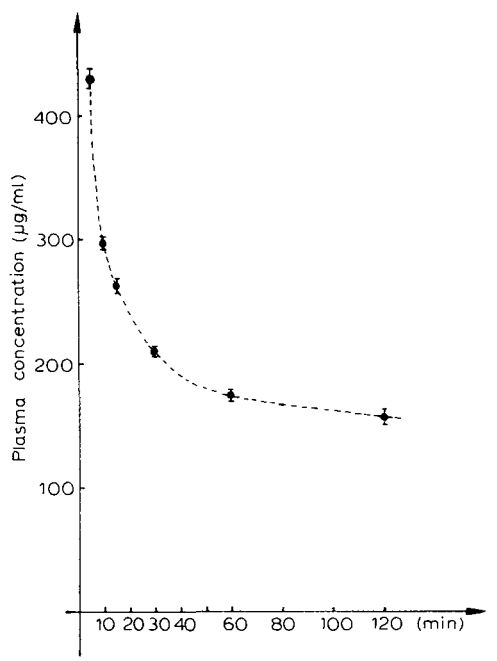

Fig. 2. Profile of piracetam plasma concentration versus time after an intravenous injection of 6 g. Each point is the mean of the plasma concentration of three geriatric patients.

method is that UV detection of piracetam at 206 nm can be increased by a factor of approximately four in comparison to that at a wavelength of 220 nm.

The time course of the plasma concentration of piracetam after a single intravenous injection in each of three geriatric patients with coronary heart disease and cardiac insufficiency is shown in Fig. 2.

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CHROMBIO. 1358

Note

Determination of etomidate in human plasma by high-performance liquid chromatography

EDWIN OWEN ELLIS and PETER RICHARD BECK*

Department of Clinical Biochemistry, Rotherham District General Hospital, Moorgate Road, Oakwood, Rotherham S60 2UD (Great Britain)

(First received February 16th, 1982; revised manuscript received April 14th, 1982)

Etomidate is a short acting imidazole hypnotic which is commonly used for induction of anaesthesia. More recently, there is increasing interest in the use of etomidate as a sedative for ventilated patients. This application, which is currently under investigation in this hospital [1, 2], has required a precise assay for plasma etomidate. A number of procedures have been described for measurement of plasma etomidate [3—6]. Each of these has disadvantages in terms of methodological complexity or instrumental requirements.

Thus, a simple method was developed to monitor etomidate levels in these patients using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

Etomidate [(R)-(+)-ethyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate] and propoxate hydrochloride [(+)-propyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate hydrochloride] were supplied by courtesy of Janssen Pharmaceutica (Beerse, Belgium). The solvents acetonitrile, methanol and water were HPLC grade from BDH (Poole, Great Britain). The pentane (BDH) was analytical grade further purified with activated charcoal.

Instrumentation

A Perkin-Elmer series IIIB pump solvent delivery system was used in conjunction with a Perkin-Elmer LC75 variable-wavelength ultraviolet detector (Perkin-Elmer, Beaconsfield, Great Britain). The sample was injected via a 7105 sample injector maximum loading 175 μl (Rheodyne, Berkeley, CA,

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U.S.A.). The octyl (C_8) 5-μm chromatographic column, 25 cm × 4.6 mm I.D., was manufactured by Altex Scientific (U.S.A.) and obtained from Anachem (Luton, Great Britain). A Smith Venture Mk II digital integrator was connected directly to the ultraviolet detector. Peaks were recorded on a 10-mV recorder.

Preparation of standard solutions

A stock standard solution of etomidate (1 mg/ml) is prepared in methanol. A stock internal standard solution of propoxate hydrochloride (1 mg/ml) is similarly prepared. The stock solutions are stable for more than 12 months at 4°C. A working standard solution of etomidate is prepared by diluting the stock solution 1 in 500 with water (2 μg/ml) and a working solution of propoxate hydrochloride by diluting the stock solution 1 in 250 with water (4 μg/ml). The working standards are prepared fresh for each set of assays; but they may be kept for at least three days at 4°C.

Procedure

A 10-ml volume of blood is collected into a heparinised tube containing 10 μl of saturated potassium fluoride to inhibit esterase activity [7]. The plasma is separated by centrifugation and stored at −20°C prior to assay. No significant change in plasma etomidate concentration has been detected over a 12-month period under such conditions. Add 200 μl of working internal standard to 2 ml of plasma sample, extract with 10 ml of pentane by vortex-mixing for 30 sec. Centrifuge at 1500 *g* for 5 min, then transfer the upper organic layer to a 10-ml conical centrifuge tube. Evaporate to dryness at 40—45°C in a water bath, and dissolve the residue in 200 μl of mobile phase consisting of acetonitrile—methanol—water (35:32.5:32.5).

Inject 75 μl of the aliquot onto the column and elute with degassed mobile phase at a flow-rate of 1.2 ml/min giving a pressure of 14 MPa (2000 p.s.i.). A calibration curve is prepared by adding 50, 100, 200, 400 and 500 μl of working etomidate standard to 2-ml aliquots of horse serum, corresponding to etomidate concentrations of 50, 100, 200, 400 and 500 ng/ml. These tubes are then treated as per plasma samples.

The chromatogram was recorded at 248 nm on a 10-mV recorder at 0.02 a.u.f.s. and 10 mm/min chart speed. The etomidate to propoxate peak height ratios are calculated, and these ratios are used for construction of the calibration curve and calculation of plasma etomidate concentrations.

RESULTS

Figs. 1 and 2 show typical chromatograms from a patient before and 6 min after commencement of etomidate infusion.

Recovery and linearity

Extraction efficiency was determined by comparing a standard curve for etomidate using propoxate as external standard with a standard curve using propoxate as internal standard. Within the range 0—50 ng/ml the mean

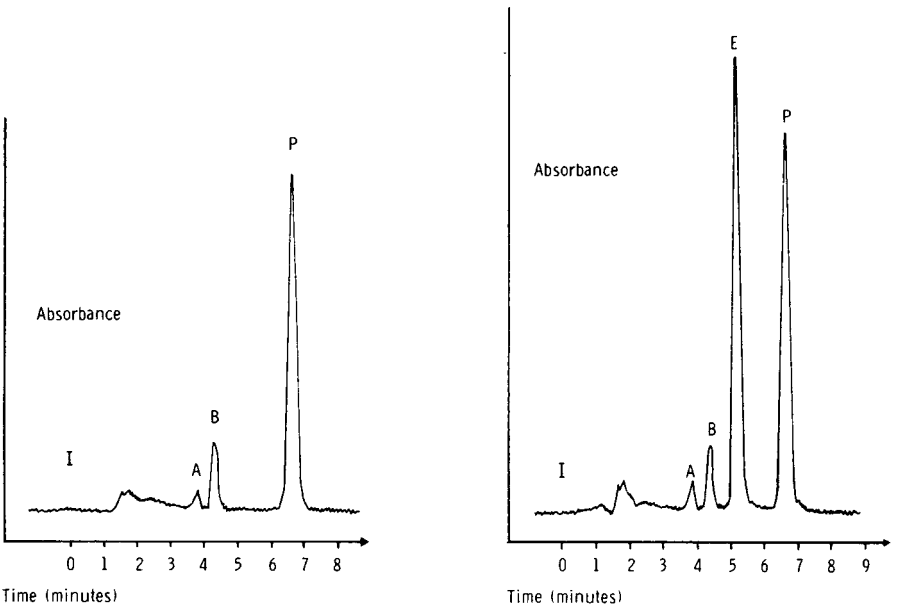

Fig. 1. Chromatogram of plasma before etomidate infusion. Peaks: P = propoxate; I = point of injection; A and B = unknown. Etomidate not detected.

Fig. 2. Chromatogram of plasma 6 min after the beginning of etomidate infusion. Bolus dose 0.3 mg/kg followed by an infusion rate of 25 μg/kg/min. Peaks: E = etomidate; P = propoxate; I = point of injection; A and B = unknown. Etomidate concentration, 306 ng/ml.

recovery was 65%, and 60% within the range 50—500 ng/ml. The standard curve was linear over the range 0—500 ng/ml.

Precision

The precision of the method was determined by calculating the coefficient of variation (C.V.) at two levels of etomidate concentration. The C.V. was 8.3% (n = 10) for 50 ng/ml and 2.8% (n = 10) for 500 ng/ml. Repeated standard curves gave similar etomidate:propoxate ratios.

Drug interference

The following drugs which have been used in conjunction with etomidate in our clinical studies were found not to interfere with the method in vitro: fentanyl, dopamine, frusemide, cimetidine and diazepam.

DISCUSSION

A number of methods have been described for the estimation of plasma etomidate. The first method, Wynants et al. [3], used gas—liquid chromatography (GLC) with a nitrogen detector. A laborious extraction technique was involved, and to achieve reasonable detection limits very pure solvents were required.

An alternative GLC method was described by De Boer et al. [5], using an

open capillary column, solid injection technique and a nitrogen detector. This used a simple extraction procedure and gave good detection limits and precision. The authors stress that a solid injection system is necessary to prevent rapid column deterioration. The short retention times (2 min for both etomidate and propoxate) would enable rapid throughput of samples but no reference is made as to whether the method is subject to drug interference.

Van Hamme et al. [4], described a mass fragmentographic method for plasma etomidate which gave excellent detection limits and precision. However, the complexity of the equipment probably precludes its use as a routine procedure.

An HPLC method has been described [6] utilising a silica gel column. However, we have been unable to obtain either the separation or sensitivity claimed by these authors.

The method described in the present paper is suitable for any laboratory possessing basic HPLC equipment. The simple extraction technique and retention times for etomidate and propoxate of approximately 5 and 7 min, respectively, allows a throughput of 15—20 samples per day.

The lack of interference in the measurement of plasma etomidate by endogenous substances is evident from the flat baseline illustrated in a patient prior to etomidate infusion (Fig. 1). It is impractical to check for interference from all drugs which could potentially be used in conjunction with etomidate, but no interference has been found by those drugs in common use in our intensive care unit. These studies were carried out by adding the drug to plasma samples in vitro. This will not detect potential inteference by drug metabolites in vivo. However, samples taken from patients receiving these drugs have not demonstrated any detectable interference, although some unidentified peaks have appeared in the chromatogram (Fig. 2).

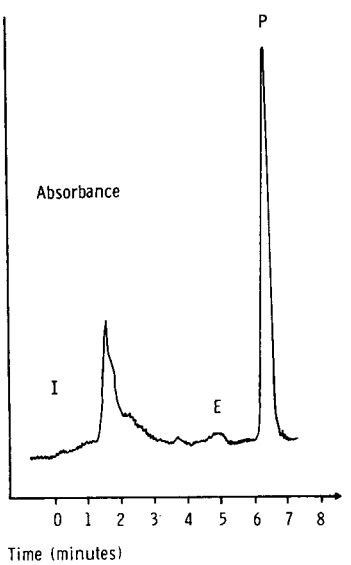

Fig. 3. Chromatogram of plasma 22 h after stopping etomidate infusion, 4 ml of plasma was extracted. Peaks: E = etomidate; P = propoxate; I = point of injection. Etomidate concentration, 4 ng/ml.

With the standard procedure as described, the detection limit is about 8 ng/ml etomidate in plasma. This detection limit may be lowered by increasing plasma volume, by further extraction of the plasma with pentane, and by increasing the percentage of extract which is applied to the column. For instance by using 4 ml of plasma and by applying 150 μl of extract to the column a detection limit of 2 ng/ml may be achieved. In our application of the method 4-ml plasma samples were used where etomidate concentrations were expected to be less than 50 ng/ml. An example of such a chromatogram with an etomidate concentration of 4 ng/ml is shown in Fig. 3.

Over the last year approximately 400 patient samples have been assayed and the method has proved suitable for routine application in the laboratory.

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CHROMBIO. 1363

Note

Determination of gentamicin components C_{1a}, C_2 and C_1 in plasma and urine by high-performance liquid chromatography

J.D'SOUZA* and R.I. OGILVIE*

Department of Pharmacology and Therapeutics, McGill University, and the Division of Clinical Pharmacology, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec H3G 1A4 (Canada)*

(First received Feburary 25th, 1982; revised manuscript received April 13th, 1982)

Aminoglycoside antibiotics are used extensively in medicine but have serious drawbacks with respect to ototoxicity and nephrotoxicity, particularly when used in patients with renal failure. Consequently, drug doses need to be carefully adjusted in these patients. The mechanisms for this toxicity are unknown, and no single factor has been shown to be clearly responsible. Frequent and accurate monitoring of drug concentrations in biological fluids is necessary to enable proper dose adjustments in order to achieve optimum therapeutic levels compatible with safety.

Gentamicin is a widely used aminoglycoside antibiotic, having a broad spectrum of activity against both gram-positive and gram-negative bacteria. It is a complex of three major components designated C_{1a}, C_2 and C_1 [1, 2] possessing similar antibacterial activities [3]. In a commercial preparation such as Garamycin® these components are present in roughly equal proportions [4].

Information available on gentamicin is mainly data for the complex as a whole, as the kinetic disposition of the individual fractions has been incompletely elucidated [5—7]. A variety of methods are available for the quantitative estimation of gentamicin in plasma [8]. Recently, sensitive high-performance liquid chromatographic (HPLC) procedures have also been reported. These methods involve derivatization with either *o*-phthalaldehyde [9—11], Dns chloride [12, 13] benzene sulfonyl chloride [14], 1-fluoro-2,4-dinitrobenzene [15] or fluorescamine [16], and quantitation by fluorescent or UV detection. Only three of the reported procedures claim to resolve the individual

*Present address: G.D. Searle Co. of Canada Ltd., 400 Iroquois Shore Road, Oakville, Ontario, L6H 1M5 Canada.

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components of gentamicin with sample clarification being achieved either by passing samples through CM-Sephadex resin [10], silicic acid column [9] or by solvent extraction after precipitation of plasma proteins [11].

Laboratories are still in need of a rapid method for the analysis of gentamicin components, requiring small volumes of plasma or urine samples. We report here a simple, rapid and sensitive micromethod for the quantitative estimation of the individual gentamicin components in 100 μl of plasma or serum. Furthermore, an assay method for gentamicin components in urine is also presented, which employs the same HPLC system as plasma, but with a preliminary extraction procedure using Sep-Pak C_{18} cartridges. The latter procedure involves a rapid method which eliminates solvent extraction and still produces clean chromatograms.

METHODS

Chemicals and reagents

Gentamicin (USP reference standard) and the purified gentamicin components C_{1a}, C_2 and C_1 were kindly provided by Dr. J. Chiz (Schering Canada, Pointe Claire, Canada), the labelled potencies being 586, 788, 641 and 620 μg/ml of powder, respectively. *o*-Phthalicdicarboxaldehyde (OPA) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and 2-mercaptoethanol from Eastman-Kodak (Rochester, NY, U.S.A.). Water was deionized and distilled; organic solvents were of chromatographic quality and all other chemicals were of reagent grade.

Chromatographic system

Separation was performed with a liquid chromatograph (Model ALC/GPC 204; Waters Assoc., Milford, MA, U.S.A.) equipped with a Waters M6000A solvent delivery system and a Waters UK6 universal injector. The column (25 cm × 30 mm I.D.) consisted of reversed-phase LiChrosorb RP-8 5 μm packing (E. Merck, Darmstadt, G.F.R.) supplied by Brownlee Labs. (Santa Clara, CA, U.S.A.) and a guard column dry-packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used. The eluent was monitored with a Schoeffel Model FS 970 fluorometer (Schoeffel Instruments, Westwood, NJ, U.S.A.). Excitation wavelength was set at 340 nm with a Kv 418 emission cut-off filter. The mobile phase consisted of 70% acetonitrile in tris(hydroxymethyl)aminomethane (1 g/l adjusted to pH 3.0 with 1 *M* hydrochloric acid). This solution was passed through a 0.45-μm filter and degassed under vacuum; the flow-rate was maintained at 1.5 ml/min and chromatography was performed at room temperature. Extractions of urine samples were performed using Sep-Pak C_{18} cartridges (Waters Assoc.).

Derivatizing reagent

A 100-mg amount of OPA was dissolved in 1 ml methanol to which was added 0.2 ml mercaptoethanol and then made up to 20 ml with 0.4 *M* potassium borate buffer at pH 10.4. The solution was prepared every week and in an air-tight amber bottle at 4° C.

Extraction procedure and standard curve preparation

For calibration, an aqueous solution containing equal amounts of C_{1a}, C_2 and C_1 base was added to plasma and urine blank samples to provide concentrations in the range 0.25—25 μg/ml.

Plasma

To each sample of plasma or serum (100 μl in duplicate) in a 1.5-ml polypropylene micro test tube (Eppendorf) was added Tris buffer (20 g/l; 100 μl) and acetonitrile (600 μl). The solutions were mixed by vortexing (10 sec) and the precipitate sedimented by centrifugation (2000 *g*, 2 min). The proteins precipitated as a pellet at the bottom of the micro test tube and the supernatant was poured into another 1.5-ml micro test tube containing chloroform (600 μl). Following mixing and centrifugation, the top aqueous layer (100 μl) was transferred into a 500-μl micro test tube to which was added OPA solution (100 μl). The solutions were then vortexed and ethyl acetate (200 μl) was added. After mixing and centrifuging, 10—30 μl of the top ethyl acetate layer containing the derivatised gentamicin components, were injected onto the liquid chromatograph.

Urine

Sep-Pak C_{18} cartridges were conditioned by flushing with methanol (5 ml) followed by distilled water (5 ml). Urine (1 ml) was then passed through the cartridge and this was washed with distilled water (10 ml), methanol—distilled water (1:1) (10 ml) and methanol (10 ml). Flow-rate for the sample and eluting solvents through the cartridge was maintained at between 5—10 ml/min. The gentamicin components were eluted by passing 2 ml of 10% ammonia solution (28—30% NH_3, v/v) in methanol, discarding the first 0.5 ml and collecting the final 1.5 ml into micro test tubes (2 ml capacity). After evaporating off the methanol using a stream of nitrogen, the residue was reconstituted with distilled water (100 μl), derivatized with OPA solution (100 μl) and the derivatives extracted into ethyl acetate (200 μl), 10 μl of which were injected onto the column.

Radioimmunoassay

This present HPLC method was compared with a standard radioimmunoassay technique using the Beckman RIAphase Gentamicin Kit (Beckman Instruments, Fullterton, CA, U.S.A.). This liquid phase kit is based on the double-antibody test system using [^{125}I]gentamicin and specially designed for the quantitative radioimmunoassay of gentamicin.

RESULTS

Linearity and sensitivity

Fig. 1 shows typical chromatograms of human plasma and urine from a volunteer who received a single infusion dose of gentamicin (1.7 mg/kg). The three components of gentamicin, C_{1a}, C_2 and C_1 eluted as distinct peaks with retention times of 8, 10 and 14 min, respectively. There were no interfering peaks when compared to chromatograms of blank plasma and urine samples

taken prior to administration of gentamicin, and analyzed by the procedures described.

Standard calibration curves for the three gentamicin components in both plasma (range 0.5—10 mg/l) and urine (range 0.5—5 mg/l) were linear (r = 0.99). The lower limit of sensitivity in both plasma and urine was 0.5 mg/l. The upper limit in plasma was as high as 40 mg/l but urine samples above 10 mg/l did not give reasonable duplicates (with 10%). This problem was resolved by either diluting urine samples above this concentration or by reducing sample volume in the Sep-Pak extraction.

Recovery, precision and specificity

Both plasma and urine recovery when compared to corresponding aqueous standards was consistent and greater than 85% for all the three components of gentamicin. The mean intra-assay coefficient of variation for all three fractions at 0.5 mg/l was 10.1% (range 6.0—14.4%) and 2.4% (range 2.2—2.5%) at 10 mg/l. OPA solutions were used for five consecutive days and new calibrations were constructed every week using freshly prepared OPA solutions; the inter-assay coefficient of variation never exceeded 8%.

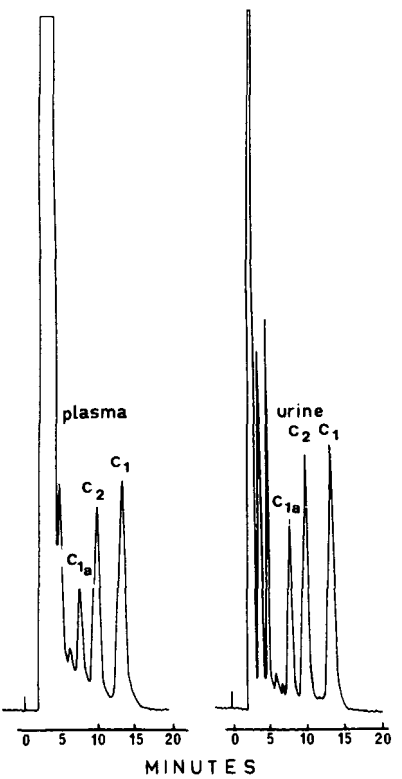

Fig. 1. Chromatograms of human plasma and urine from a volunteer who received gentamicin (1.7 mg/kg) by single infusion (20 min). The blood sample was taken 30 min after infusion and the urine sample was a 36—48-h collection.

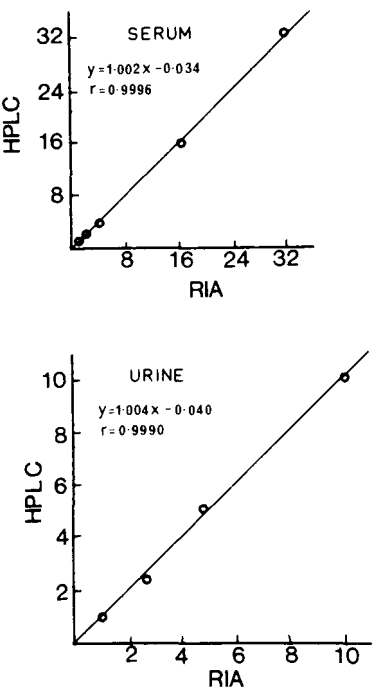

Fig. 2. Linear regression analysis of total gentamicin concentration in human serum and urine as determined by the radioimmunological and HPLC procedures.

Our HPLC procedures for plasma and urine were compared with a standard radioimmunoassay method for total gentamicin. Fig. 2 illustrates the conformity between the two procedures. Values for total gentamicin using the HPLC method are expressed as the sum of the three components of gentamicin.

Comparison with standard aqueous solutions of drugs and other antibiotics which are frequently used concomitantly with gentamicin showed that kanamycin, amikacin, tobramycin, clindamycin, chloramphenicol, cloxacillin, cefazoline, penicillin G and netilmicin did not interfere with the assay. Netilmicin upon derivatization with OPA showed a single peak with a retention time of 17 min. Since it is a dehydrogenated analogue of gentamicin C_{1a} and due to its close chemical similarity with other gentamicin components, it serves as a useful internal standard.

Effect of pH

In order to optimize chromatographic conditions, the mobile phase (70% acetonitrile in Tris 1 g/l) was adjusted with 1 *M* hydrochloric acid to various pH values. A standard aqueous solution containing equal amounts (5 mg/l) of the three components of gentamicin was derivatised with OPA and extracted into ethyl acetate as described in Methods. After equilibration of the various mobile phases, 10 μl of the ethyl acetate layer were injected onto the chromatograph. Fig. 3 illustrates the results obtained. Clearly the mobility of gentamicin C_1 was more affected by pH than either C_{1a} or C_2. For example, at pH 7.0 the retention time of gentamicin C_1 was around 40 min. Hence the mobile phase was maintained at pH 3.0 for optimal resolution.

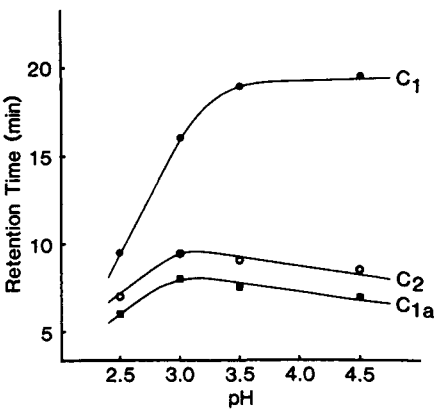

Fig. 3. Effect of pH on the mobility of gentamicin components. The mobile phase consisted of 70% acetonitrile in Tris (1 g/l) which was adjusted to the required pH with 1 *M* hydrochloric acid. Flow-rate was maintained at 1.5 ml/min.

DISCUSSION

We have described an HPLC method for the quantitative separation and analysis of gentamicin C_{1a}, C_2 and C_1 in either plasma or urine. OPA was chosen as the derivatising reagent since the reaction occurs rapidly at room temperature in an aqueous media which can be buffered appropriately for optimising reaction conditions.

There have been many HPLC methods for determination of gentamicin reported in the literature [9—16] utilizing various derivatising reagents and also differing in chromatographic conditions. The procedure reported here is simple, can be performed rapidly using relatively small sample volumes. Furthermore, it allows the simultaneous monitoring of the three major fractions of gentamicin in both urine and plasma. The time taken for elution of a complete chromatogram is less than 15 min and hence many samples can be analyzed within a day. The plasma method has an added advantage in that the assay can be conducted on a microscale level, thus requiring only small volumes of blood samples for detailed pharmacokinetic analysis in infant or adult patients.

The use of netilmicin as an internal standard in both assays minimizes variations in detector response or any chromatographic changes. It also compensates for injection and sample preparation errors.

Extraction using the Sep-Pak C_{18} cartridges has several advantages over solvent precipitation and extraction procedures. It provides chromatographically cleaner extracts and removes any material which would otherwise adsorb irreversibly to the column matrix thus prolonging the lifetime of the analytical column. Sensitivity can also be improved simply by increasing sample size.

The Sep-Pak extraction development was arbitrary and the best eluting solvent was found to be 10% ammonia solution in methanol. This combination was used previously [2] for the elution of tritiated gentamicin from urine after adsorption on an Amberlite XAD-2 resin column. By comparing subsequent extractions after 0.5-ml elutions, the first 1.5 ml after the void volume was found to elute most of the gentamicin applied to Sep-Pak cartridges. The accuracy and reproducibility was found to be within acceptable limits. However, it was necessary to evaporate this fraction to dryness in order to remove any ammonia which might react with OPA. Sep-Pak cartridges were used at least twice without loss in performance, despite the manufacturer's recommendations.

The assay is presently being used to study the kinetic disposition of the individual fractions of gentamicin in urine and plasma after single and multiple dosing in humans.

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Note

Determination of clofazimine in human plasma by thin-layer chromatography

Z. LANYI* and J.P. DUBOIS

Pharma Research, Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basle (Switzerland)

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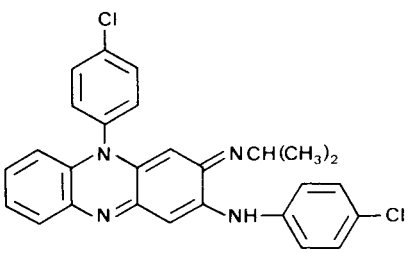
Clofazimine, 3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine (I), is effective in the treatment of leprosy.



I

To date, Barry et al. [1] and Dill et al. [2] have reported, respectively, a spectrophotometric and a fluorometric method of assay for clofazimine in biological fluids and tissue homogenates. The sensitivity of these methods is not sufficient for the determination of plasma clofazimine levels after a single oral dose in man. Gidoh et al. [3] have published a high-performance liquid chromatographic method for the simultaneous analysis of the principal antileprosy drugs and their metabolites in human serum. Meanwhile, we have developed a densitometric method for determining clofazimine in plasma. This method was applied to a healthy volunteer given single oral doses of 200 mg and 400 mg of Lamprene®.

EXPERIMENTAL

Reagents

All solvents and reagents were of analytical grade (Merck, Darmstadt,

0378-4347/82/0000—0000/$02.75

G.F.R.) and were used without further purification. The clofazimine (active substance of Lamprene) originated from Ciba-Geigy Ltd., Basle, Switzerland; acetic acid, citric acid, sodium acetate and buffers pH 4, 6, 8, 10 and 13 (Titrisol®) were from Merck.

Procedure

Plasma samples (1—3 ml, depending on the concentration of clofazimine) are acidified with 2 ml of acetate buffer (according to Walpole, 1 *M*, pH 5) and then extracted with 6 ml of toluene for 15 min using a mechanical horizontal shaker. After brief centrifugation, 5 ml of the organic phase are removed and evaporated to dryness under a stream of nitrogen at 40°C. The residues are dissolved in 100 μl of toluene and aliquots are applied on to the thin-layer chromatographic (TLC) plate.

Thin-layer chromatography

All TLC separations are carried out using 20 × 10 cm precoated HPTLC silica gel 60 plates (Merck) predeveloped in chloroform—methanol (1:1) prior to use. Concentrated extracts (30 μl) are applied to the plate using a Linomat III applicator (Camag, Muttenz, Switzerland), 19 samples being applied to one plate, including biological, calibration and control samples. The plates are developed in toluene—acetic acid—water (50:50:4) using a solvent-saturated, paper-lined tank, and then allowed to stand 30 min at room temperature. R_F value of clofazimine is 0.36.

In situ quantitation of the TLC spots is performed with a Camag scanner coupled to a W+W recorder. Measurements are made in the reflectance mode

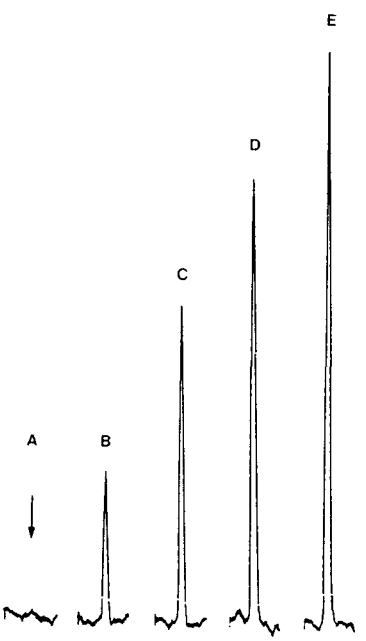


Fig. 1. Typical densitograms of (A) extract of a blank plasma sample and extract of plasma samples spiked with clofazimine (B) 23 ng/ml, (C) 46 ng/ml, (D) 70 ng/ml and (E) 93 ng/ml.

for visible absorption. A mercury light source is used with the monochromator set at 545 ± 15 nm.

Densitograms obtained for blank plasma and for plasma spiked with various amounts of clofazimine are shown in Fig. 1.

RESULTS AND DISCUSSION

Extraction

The pH dependence of the extractability of clofazimine was evaluated by adjusting spiked plasma samples to pH values ranging from 2 to 13 using acetic acid, citric acid or buffers (Fig. 2). Extraction of the biological samples with toluene resulted in good recovery with a low background.

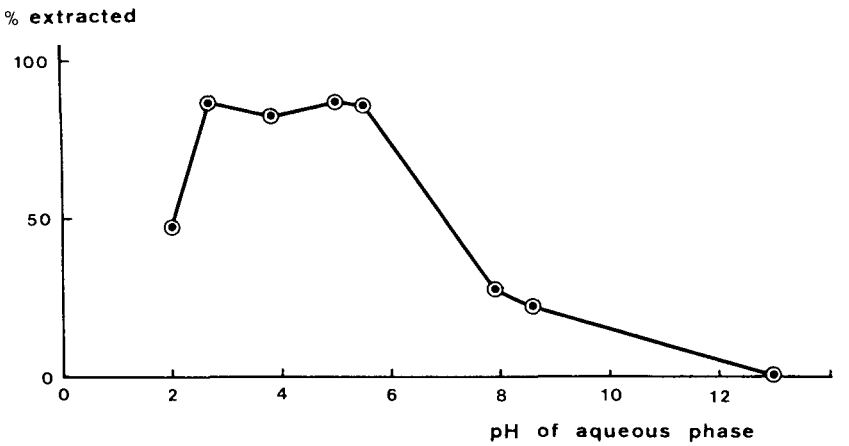


Fig. 2. pH Dependence of extractability of clofazimine with toluene from spiked plasma samples.

Calibration curves

Calibration curves were prepared with spiked plasma samples processed as described above. An almost linear relationship between the peak height and the amount of clofazimine on the plate was observed between 2 and 20 ng/spot. For a calibration range from 2 to 30 ng/spot or higher, calibration curves of the type $y = a + bx + cx^2$ (y corresponding to the peak height and x to the amount of clofazimine in the sample) were employed (Fig. 3).

Accuracy and precision

The method was tested by analysing samples spiked with various amounts of clofazimine unknown to the analyst. The mean values found differed from the initial concentrations by between −3.3% and +15.4% (Table I).

No interference from dapsone or rifampicin was observed.

Application

The method was employed for the determination of plasma clofazimine levels in a healthy volunteer following single oral doses of 200 mg and 400 mg

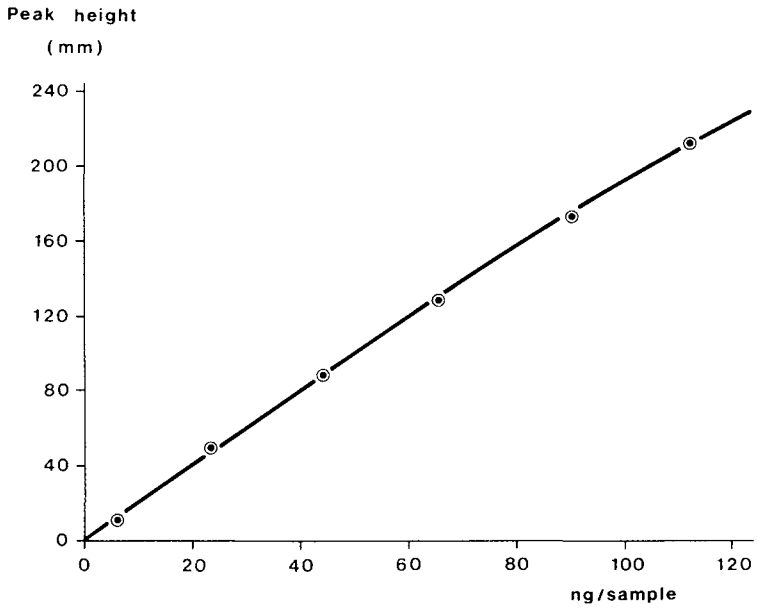


Fig. 3. Calibration curve for the entire analytical method.

TABLE I

COMPARISON BETWEEN INITIAL AND FOUND PLASMA CONCENTRATIONS OF CLOFAZIMINE IN SPIKED SAMPLES

Clofazimine concentration in plasma (ng/g)			
Initial	Found (mean)	No. of determinations	Percentage deviation of mean from initial value
13	15	2	+15.4
33	34	2	+ 3.0
40	41	8	+ 2.5
51	51	2	0.0
60	58	12	— 3.3
84	96	2	+ 2.4
100	100	4	0.0

of Lamprene (2 and 4 capsules of 100 mg of Lamprene) after an overnight fast (Fig. 4). A peak clofazimine concentration of 70 ng/g was reached 8 h after administration of 200 mg of Lamprene and one of 162 ng/g 4 h after the 400-mg dose. Following the distribution phase a slow elimination phase with an apparent elimination half-life of 8 days was observed, clofazimine concentrations still above the limit of detection of 5 ng/g being measured for up to 264 h after ingestion.

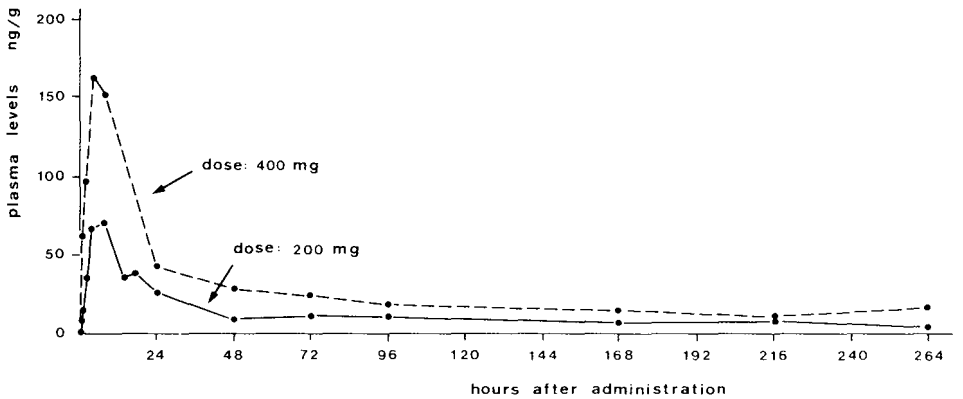


Fig. 4. Plasma levels of clofazimine in a healthy volunteer following single oral doses of 200 mg and 400 mg of Lamprene after an overnight fast.

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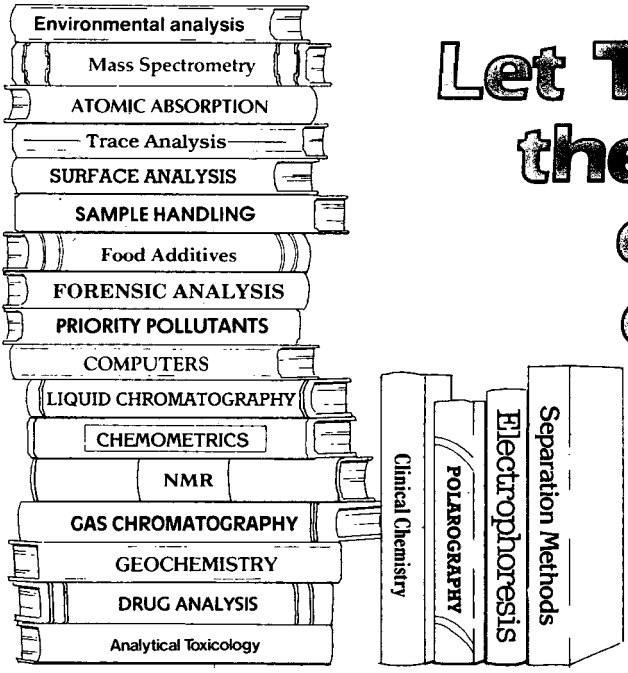

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