ISSN 0378-4347

VOL. 311 NO. 2 NOVEMBER 28, 1984

(Biomedical Applications, Vol. 36, No. 2)

THIS ISSUE COMPLETES VOL. 311

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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JOURNAL OF CHROMATOGRAPHY

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0378-4347/84/$03.00

Printed in The Netherlands

VOL. 311, NO. 2 JOURNAL OF CHROMATOGRAPHY NOVEMBER 28, 1984

(Biomedical Applications, Vol. 36, No. 2)

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(Abstracts/Contents List published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Index Medicus, Mass Spectrometry Bulletin, Pharmaceutical Abstracts, Referativnyi Zhurnal, and Science Citation Index)

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edited by
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ISBN 0-444-42015-0.
Price: US $ 89.25 (U.S.A. & Canada)
Dfl. 210.00 (Rest of the World)

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CHROMATOGRAPHY

Fundamentals and Applications of Chromatographic and Electrophoretic Methods

Part A: Fundamentals and Techniques
Part B: Applications

ERICH HEFTMANN, U.S. Department of Agriculture, Berkeley, CA, U.S.A. *(editor)*

Journal of Chromatography Library Vol. 22 A + B

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February 1983
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US $221.25 (in USA & Canada)
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Journal of Chromatography, 311 (1984) 239—248
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2258

DETERMINATION OF SERUM AND PLASMA CONCENTRATIONS OF RETINOL USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 30th, 1984; revised manuscript received July 6th, 1984)

SUMMARY

An isocratic high-performance liquid chromatographic method specifically developed to allow simple and rapid determination of retinol concentrations in serum and plasma is reported. Retinol and retinol acetate (the internal standard) are extracted into butanol—ethyl acetate, with no subsequent evaporation step. Separation is achieved on a reversed-phase C-18 column, with a mobile phase consisting of acetonitrile—1% ammonium acetate (89:11), and UV detection at 313 nm. Recoveries of both retinol and the internal standard were 100%, and both compounds were stable in the extraction solvent for at least 2.5 h. Three anticoagulants (oxalate, citrate, EDTA) and perchloric acid (used in some methods to denature protein) all caused losses of retinol. Each run required 9 min; same-day coefficient of variation (C.V.) for identical samples averaged 2.5%; between-day C.V. was 6.4%; sensitivity was better than 10 ng/ml, while clinical concentrations were 400—1200 ng/ml. This method permits simple, rapid, sensitive, precise, and accurate determination of retinol using 0.5 ml serum or heparinized plasma.

INTRODUCTION

Compounds with "vitamin A activity" such as retinol and β-carotene may be clinically important anti-cancer agents [1—4]. This family of compounds has been used in experimental animals to prevent or delay the development of a wide range of cancers. While the mechanism(s) of this chemopreventive effect has not been firmly established, epidemiologic data in human populations have suggested a relationship between dietary intake or blood levels of these compounds, and lower risk of cancer. Our institution has recently begun a prospective, randomized, cooperative study to evaluate the chemopreventive effect of β-carotene upon the recurrence rate of non-melanoma skin cancers. This study requires the yearly determination of plasma concentrations of both

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β-carotene and retinol in all study patients (approximately 2000 determinations of each compound each year). A simple, rapid, precise, accurate, sensitive, and inexpensive assay was required for each compound.

Until the late 1970's, retinol (R) blood concentrations were determined by spectrophotometric methods, colorimetric assays or fluorescence assays. However, all three of these methods were relatively time-consuming and non-selective, even though they did achieve adequate sensitivity [5]. High-performance liquid chromatography (HPLC) seemed to be an ideal method, since sample preparation and the analysis itself allowed rapid, sensitive, and selective quantitation of R in blood based upon retinol's high intrinsic UV absorption. Indeed, several HPLC methods for determination of serum or plasma R levels have been recently published. However, none of them has been ideal.

Many of the methods required extraction of R from serum or plasma into an organic solvent, which was then evaporated to dryness [6—14]. This step would make the analysis of 2000 samples per year considerably more time-consuming; in addition, the evaporation step, even if performed under nitrogen, can lead to excessive loss of retinol [15]. Thus, extraction methods which do not require this evaporation step would be preferable. One of the earliest HPLC methods reported met this requirement [16], but the internal standard employed was not commercially available, the peaks of R and internal standard were not totally separated, and the type of HPLC column used did not have sufficient longevity. More recently, a method was published which also avoided an evaporation step [17]. However, this report did not mention percent recovery of R. In addition, the method as reported did not include the use of an internal standard, and gave non-reproducible results in our hands. Most recently, a method has been reported which avoided solvent evaporation, included the use of a commercially available internal standard, and had excellent precision [18]. However, 1 ml of serum was required, standard curves were not performed, each run required 16 min, and recovery rates of both R and the internal standard in different plasma samples were not determined. In addition, there was no information about the equivalence of R levels in serum and heparinized plasma.

Therefore, we decided to attempt to develop an optimal HPLC method specifically designed to determine serum or plasma R concentrations simply and rapidly, incorporating the positve features outlined above. This paper concerns a method which has the required ease, speed, sensitivity, selectivity, precision, and accuracy for routine high-volume use.

MATERIALS AND METHODS

Chemicals

Retinol (R) and retinol acetate (RA) were the highest grade available, and were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium heparin (10,000 units/ml) was purchased from Elkins-Sinn (Cherry Hill, NJ, U.S.A.). All solvents were HPLC grade, manufactured by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium acetate HPLC grade, 70% perchloric acid, and potassium dihydrogen phosphate were obtained from Fisher Scientific

(Fairlawn, NJ, U.S.A.). Water used for HPLC mobile phase preparation was house distilled, then passed through a Milli-Q Purification System (Millipore, Bedford, MA, U.S.A.).

Blood samples

Vacutainer glass tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.) were used to collect serum and four types of plasma (anticoagulated with lithium heparin, oxalate, citrate, or EDTA). Serum and plasma samples were protected from direct sunlight and fluorescent light by wrapping the tubes in aluminum foil, and were stored frozen at −35°C in Nunc polypropylene tubes (AH Thomas Company, Philadelphia, PA, U.S.A.) until analysis.

Standard solutions

Retinol and retinol acetate were dissolved in acetonitrile. Serial dilutions of each were made until R solutions were obtained with approximate concentrations of 10, 6.7, and 3.3 μg/ml (stock solutions), and RA solutions were obtained with approximate concentrations of 10 (stock solution) and 3 μg/ml. Exact concentrations were calculated by measuring the UV absorption of the most dilute solutions at 325 nm with a Varian Techtron Model 635 spectrophotometer (Springfield, NJ, U.S.A.). Extinction coefficients for 1% (w/v) solutions of R (1850) and RA (1565) in ethanol were used, once it was demonstrated that both compounds had equal absorption in pure acetonitrile and pure ethanol. Standard solutions for extraction were prepared fresh daily by adding 50 μl of the R and RA stock solutions to 500 μl water.

Extraction procedures

All extractions were performed in a dark room, illuminated with a 25-W incandescent bulb. In method A, 500 μl of serum, plasma, or water were placed in a 1.5-ml polypropylene microcentrifuge tube (Fisher Scientific). To this were added 50 μl of the RA stock solution (approximately 10 μg/ml) (or 50 μl the R stock solution), and 50 μl of acetonitrile. After vortexing for 15 sec, 250 μl of butanol—ethyl acetate (1:1) were added, with further vortexing for 60 sec. Finally, 150 μl of an aqueous solution of potassium dihydrogen phosphate (1.2 g/ml) were added, the solution vortexed for 30 sec, and then centrifuged at 13,000 *g* for 1 min (Fisher Micro-Centrifuge Model 235B). The organic upper layer was transferred by pipet to a 0.5-ml polypropylene microcentrifuge tube (Fisher Scientific) and centrifuged at 13,000 *g* for 1 min. Fifty μl of this organic solution were then injected directly onto the HPLC system.

Method B required the addition of 100 μl of 5% perchloric acid to 500 μl of plasma in a 1.5-ml microcentrifuge tube. After vortexing for 45 sec, 500 μl of butanol—ethyl acetate (1:1) or 500 μl of ethyl acetate were added, with subsequent vortexing for 1 min. Centrifugation was performed, as in method A. An extraction solvent consisting of butanol—ethyl acetate (1:1) was finally chosen because it produced the best peak shapes, greatest peak heights, optimal viscosity, and optimal eluotropic value on alumina, when compared to either pure ethyl acetate or pure butanol.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 510 dual-piston pump (Waters Assoc., Milford, MA, U.S.A.), an SSI 0.5-μm in-line filter (Rainin Instruments, Woburn, MA, U.S.A.), a Rheodyne Model 7125 injector with a 100-μl loop (Rainin), a Brownlee pre-column (30 × 4.6 mm) packed with 5 μm diameter RP-18 material (Rainin), an Altex Ultrasphere-ODS column (250 × 4.6 mm, Beckman Instruments, Wakefield, MA, U.S.A.), a Beckman Model 160 UV detector equipped with a 313-nm filter, and a one-channel strip-chart recorder (Model D-5119-1, Houston Instruments, Austin, TX, U.S.A.). Detector sensitivity was set at 0.030 absorbance units full scale (aufs). The mobile phase was acetonitrile—1% ammonium acetate (89:11), flowing at 2.5 ml/min (pressure 166 bars).

Calculations

R and RA peak heights were measured, and the R/RA ratio calculated. A standard curve was generated each day from the three extracted standard solutions, by plotting the R/RA ratio on the ordinate versus R concentration (μg/ml) on the abscissa. The best fit linear regression line was calculated using the method of least squares. In experiments in which recoveries were tested under different conditions, the significance of differences between means was explored using one-way analysis of variance. When significant differences occurred, they were further investigated using the Student—Newman—Keuls test [19].

RESULTS

Extractions using perchloric acid

Using extraction method B with 100% ethyl acetate as originally reported [17], heparinized plasma samples from six subjects were analyzed. The RA peak heights should have been constant, since the same amount was added to each sample (approximately 1000 ng/ml). However, the measured RA peak heights showed a coefficient of variation (C.V.) of 16.3%. Comparison of R peak heights could not be made, since the samples were from six different subjects. However, extraction methods A and B (each using 500 μl of butanol—ethyl acetate) were then applied to triplicate plasma samples from the same subject. R peak heights by method B showed a C.V. = 15.8%, compared to a C.V. = 4.9% by method A. Similarly, RA peak heights showed a C.V. = 14.6% by method B, compared to a C.V. = 5.0% by method A. Since method A gave more precise results, the effect of perchloric acid upon R and RA stability was measured. An aqueous solution of 0.83% perchloric acid had R and RA added (each approximately 1000 ng/ml), and was then extracted with butanol—ethyl acetate as in method A after incubation at 22°C for 1, 4 or 15 min. The results are summarized in Table I. There was a pronounced deterioration in R peak height over 15 min (reduction to 13.9% of control), while the reduction in RA peak height was smaller (reduction to 83.5% of control). Because the R height declined more than the RA height, the R/RA ratio declined as well (reduced to 16.7% of control).

TABLE I

EFFECT OF PERCHLORIC ACID ON RETINOL AND RETINOL ACETATE OVER TIME

R and RA were added to water or 0.86% $HClO_4$, to concentrations of approximately 1000 ng/ml. Extractions were performed using method A, after timed incubations at room temperature, in the dark.

Solution extracted	Incubation time (min)	R height	RA height	R/RA ratio
Water (control)	15	0.770 ± 0.015	0.377 ± 0.014	2.04 ± 0.05
0.83% $HClO_4$	1	0.556 ± 0.044**	0.367 ± 0.007	1.51 ± 0.10**
0.83% $HClO_4$	4	0.340 ± 0.012**	0.352 ± 0.010*	0.97 ± 0.06**
0.83% $HClO_4$	15	0.107 ± 0.015**	0.316 ± 0.011**	0.34 ± 0.04**

*$p < 0.05$.
**$p < 0.01$.

Recoveries of R and RA during extraction

Samples of 500 µl of water or pooled heparinized plasma had R (approximately 0, 200, 400 or 800 ng/ml) and RA (approximately 800 ng/ml) added, and were then extracted using method A. Net R heights were determined by subtraction of the R height of the unspiked plasma. As can be seen in Table II, the values of R, RA, and the R/RA ratio were essentially identical whether the extraction was performed on a spiked water or plasma matrix, indicating that recovery from both was equal. High recovery rates for both compounds were confirmed by demonstrating that when R and RA were added to water (both at 1000 ng/ml), exactly 100% of UV absorption in the water measured with the spectrophotometer at 325 nm was removed after the water was extracted with butanol—ethyl acetate.

RA, the internal standard, was added to a final concentration of 800—1000 ng/ml. However, RA peak heights were proportional to RA concentration when

TABLE II

STANDARD CURVES (R/RA PEAK HEIGHTS RATIO VS. R CONCENTRATION) IN SPIKED WATER AND PLASMA SAMPLES

Water and plasma were spiked with R (0, 192, 384 or 768 ng/ml) and RA (approximately 800 ng/ml).

	R (µg/ml)	R (net height)	RA (height)	R/RA ratio	Best line fit	
Water	0.768	0.679	0.365	1.860	R/RA = 2.419	R + 0.003;
	0.384	0.335	0.360	0.930		$r > 0.9999$
	0.192	0.170	0.360	0.472		
	0	0.000	0.358	0.000		
Plasma	0.768	0.670	0.362	1.850	R/RA = 2.405	R + 0.003;
	0.384	0.342	0.371	0.922		$r > 0.9999$
	0.192	0.178	0.378	0.471		
	0	0.000	0.374	0.000		

RA concentrations varied from 400 to 1600 ng/ml ($r > 0.9999$ for the best fit line). Finally, it was felt necessary to demonstrate that recoveries of R and RA were equal when both were added to plasma samples from different subjects. Heparinized plasma from three subjects and water were spiked with R (800 ng/ml) and RA (800 ng/ml). In addition, the plasma samples were also analyzed with only RA added, so that the net R peak height associated with the R added could be determined. Samples for each subject were run in duplicate or triplicate. The mean RA heights (± S.D.) for the three subjects and for the water samples were 0.197 ± 0.014 (n = 6), 0.195 ± 0.008 (n = 6), 0.188 ± 0.005 (n = 5), and 0.192 ± 0.010 (n = 3), respectively. These means were not significantly different. More importantly, the R/RA ratios from the three different plasma samples and the water were 1.82 ± 0.18 (n = 2), 1.74 ± 0.23 (n = 3), 1.77 ± 0.01 (n = 2), and 1.74 ± 0.06 (n = 3). Again, these means were not significantly different, indicating that recoveries of R and RA from three different plasma samples and from water were equal.

Stability of R and RA in serum and plasma

Serum and plasma anticoagulated with four different anticoagulants were obtained in duplicate from three different subjects. After standing at room temperature in the dark for 1 h, the samples were centrifuged and the serum or plasma removed. All samples were extracted using method A. The results are given in Table III. Serum and plasma anticoagulated with heparin demonstrated equivalent values of R, RA, and R/RA ratio (for each patient, the mean value from the serum samples was defined as 100%). Recovery of R was slightly less when EDTA was used as the anticoagulant, and was much less when potassium oxalate or sodium citrate were used as anticoagulants. Recoveries of RA were not affected by choice of anticoagulant.

TABLE III

RECOVERY OF R AND RA FROM SERUM AND VARIOUS PLASMA PREPARATIONS

Duplicate samples of serum and four types of plasma were obtained from three subjects. RA was added (1000 ng/ml), and peak heights of R and RA were measured. For each patient, the values of R, RA, and R/RA ratio obtained with serum samples were defined as 100%.

Treatment	R height	RA height	R/RA ratio
Serum	100.0%	100.0%	100.0%
Plasma (heparin)	98.7 ± 1.9	97.9 ± 0.3	96.6 ± 1.9
Plasma (EDTA)	92.8 ± 6.7	98.9 ± 1.9	92.1 ± 8.3
Plasma (oxalate)	82.4 ± 7.6*	97.0 ± 0.8	80.0 ± 7.4**
Plasma (citrate)	76.7 ± 8.0**	100.3 ± 2.7	76.8 ± 7.3**

*$p < 0.05$.
**$p < 0.01$.

Stability of R and RA in butanol—ethyl acetate

Because of the concern about the effect of the extraction solvent on R and RA stability [17, 18], duplicate plasma samples were extracted and injected

immediately, and injected again 2.5 h later, after having been left at room temperature in the dark. Duplicate samples were so analyzed on four different days. Relative to the sample injected immediately after extraction (defined as 100%), those injected after 2.5 h produced R heights of 99.3 ± 0.9%, RA heights of 101.3 ± 1.4% and R/RA ratios of 98.1 ± 0.9%. These differences were not significant.

Precision

Each day, 30—35 plasma samples were analyzed for R concentrations. Naturally the R concentrations varied but all specimens analyzed should have had the same RA peak height. On seven consecutive days, the RA peak heights had C.V. values of 2.8%, 2.0%, 1.8%, 1.9%, 1.5%, 4.7%, and 2.6%, with an average same-day C.V. of 2.5%. When multiple injections were made on the same day of the same plasma sample, R peak heights had similar C.V. values. Over seven consecutive days of analysis, a pooled sample of heparinized plasma was analyzed in duplicate each day. These seven daily mean values were: 460, 420, 468, 414, 421, 481 and 472 ng/ml. These seven measurements had a mean of 448 ± 29 ng/ml, with a between-day C.V. of 6.4%.

Sensitivity

Fig. 1A and B illustrate chromatograms of one subject's plasma, with and without the addition of RA (1000 ng/ml). The calculated concentration of R

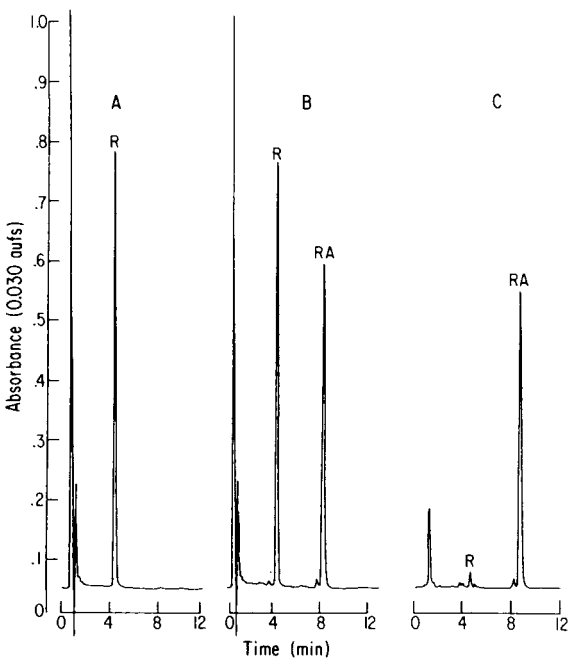

Fig. 1. Chromatograms from human plasma and aqueous samples extracted using method A, with detector sensitivity set at 0.030 a.u.f.s. (A) Human heparinized plasma with normal retinol peak (R, calculated to be 805 ng/ml); (B) the same human plasma, with retinol acetate (RA) added as the internal standard (approximately 1000 ng/ml); (C) water spiked with retinol (25 ng/ml) and retinol acetate (approximately 900 ng/ml).

was 805 ng/ml. Fig. 1C demonstrates that a standard aqueous solution containing R (25 ng/ml), carried through the extraction process, is easily quantitated. At this low detector sensitivity (0.030 aufs), solutions with R concentrations as low as 10 ng/ml are quantifiable (peak height > 3× baseline noise).

DISCUSSION

Since we must measure over 2000 samples each year we felt it would be highly advantageous to develop an extraction process which avoided a solvent evaporation step. We first attempted to use the extraction method proposed by Goodman et al. [17]. However, we noticed that this method, which precipitated serum and plasma protein with 5% perchloric acid (0.83% final concentration), produced inconsistent results. We found that R, and to some extent RA, are rapidly destroyed when exposed to 0.83% perchloric acid. Perchloric acid causes less destruction of R and RA when they are in a serum or plasma matrix, but since the precision of R and RA peak heights is suboptimal with this method, it was not used. While the original authors have used this method to quantify plasma retinol levels [20], their methods paper did not measure R recovery [17]. However, they reported that recovery of 13-*cis*-retinoic acid from plasma using this method was quite variable (83.1—94.7%), possibly reflecting similar problems with the effects of perchloric acid upon this retinoid.

Another question which our work has answered is the issue of whether serum R levels and plasma R levels are equal. This appears to depend on which anticoagulant is used. It was previously shown that recovery of R from plasma anticoagulated with EDTA was slightly less than from serum; oxalate caused an even greater loss [18]. We have confirmed this observation, and in addition demonstrated that anticoagulation with citrate should be avoided as well. Recovery of R and RA from heparinized plasma is equal to that from serum. It is possible that EDTA, oxalate, and citrate all reduce R recoveries by causing acid-induced deterioration or oxidation, similar to the destruction seen following exposure to perchloric acid. In any case, it would seem best to confine blood samples to either serum or heparinized plasma, avoiding other anticoagulants.

Concerning the stability of R and RA in the extraction solvent, we demonstrated no loss in peak heights of either compound after incubation for up to 2.5 h at room temperature in the dark in butanol—ethyl acetate. This is consistent with the results of McLean et al. [18], who found that R was stable for up to 2 h at room temperature when extracted into butanol—acetonitrile. This stability allows the lab technician to perform extractions and operate the HPLC system simultaneously, without worrying that extracted samples have to be injected onto the HPLC system immediately.

The optimal HPLC technique includes the presence of an internal standard (preferably commercially available), and the generation of a standard curve each day, made from spiked samples subjected to the extraction process. As several other groups have done [9, 14, 18], we chose RA as our internal standard, since it is commercially available, has a retention time slightly longer

than R, has similar UV absorption characteristics, and is not present in human blood. Our standard curve was prepared each day from aqueous standard solutions which contained known amounts of R and RA, and which were subsequently extracted. This has the advantage that the R height measured is the net R height, since there is no intrinsic R content of water. We demonstrated that recovery of both compounds was constant and complete from water and from plasma samples from three different subjects. Thus, the efficiency of recovery does not vary from patient to patient. While excellent (96%) recovery of R and RA has been demonstrated previously [18], other workers have experienced recoveries of R and RA as low as 70% [14]. Also, other groups have not demonstrated that recovery was equal in plasma from different patients.

It is important that any assay method has sufficient sensitivity and precision. We were easily able to quantify concentrations of R as low as 10 ng/ml with the UV detector set at 0.030 aufs. Sensitivity could be increased by either increasing the volume of organic matrix injected (we currently inject only 50 μl), or increasing the sensitivity of the UV detector (our detector is able to operate as low as 0.001 aufs, affording an approximately 30× increase in sensitivity). However, since clinically apparent R concentrations are in the range of 400—1400 ng/ml [18] it was not necessary to "push" the sensitivity of the assay further. Even at the level of 10 ng/ml, this sensitivity was better than that reported with other assays [6, 16—18]. Our same-day precision (C.V. average of 2.5%), and between-day precision (C.V. = 6.4%) were similar to those reported by other groups [6, 9, 16, 18].

One final factor of cost concerns the stability of HPLC columns under the required chromatographic conditions. We used a guard pre-column to protect the analytical column. By changing the guard column every 500 injections, the pre-column was not overloaded, pressures were constant, and the analytical column remained protected. We have made over 1100 injections on our first analytical column, and have thus far not detected any change in retention time, or any decrease in the number of theoretical plates.

In conclusion, this method should be of use to investigators who must measure serum or plasma retinol concentrations in large numbers of patients. The ease, speed, sensitivity, accuracy, and precision of the method, its established constant recoveries from different plasma samples, and its standard curve made with aqueous standard solutions utilizing a commercially available internal standard, are all features which other researchers may find useful.

ACKNOWLEDGEMENTS

I wish to thank Debbie Lester for her expert technical assistance. This research was supported in part by PHS grant number CA32934 awarded by the National Cancer Institute, DHHS.

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Journal of Chromatography, 311 (1984) 249—255
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2262

RAPID MEASUREMENT OF OESTRADIOL AND OESTRIOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER AUTOMATIC PRETREATMENT

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(First received March 27th, 1984; revised manuscript received July 16th, 1984)

SUMMARY

A fluorometric liquid chromatographic method was developed for measurements of unconjugated oestradiol and oestriol in the serum of pregnant women. The serum samples are injected directly into the apparatus and pass to a pretreatment column, where oestrogens are adsorbed while hydrophilic components such as proteins and carbohydrates are not. The oestrogens then pass into a separation column containing a new type of polymer gel. The mobile phase consists of an acetonitrile—water mixture, and separation is achieved by a reversed-phase mechanism. The eluent is monitored for fluorescence. Data on the reproducibility and recovery by this method and the correlation of values with those obtained by radioimmunoassay are reported. Results on the increases of oestradiol and oestriol in the serum during pregnancy are also reported.

INTRODUCTION

The importance of determining serum oestrogens as indicator of foetal distress is well recognized [1—3]. Many methods have been used to measure serum oestrogens, and now high-performance liquid chromatography (HPLC) is widely used for their analysis [4—13]. However, a troublesome and time-consuming pretreatment procedure is necessary before HPLC. Therefore, we have developed a liquid chromatographic procedure that requires no such pretreatment procedure.

The serum sample is first introduced into a pretreatment column where

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deproteinization occurs and oestrogens are concentrated. Then, by turning a stop cock, the oestrogens pass through an HPLC column where they are separated. This rapid procedure for serum oestrogen determination is simple and gives excellent recovery.

MATERIALS AND METHODS

Apparatus

A flow diagram of our HPLC system is shown in Fig. 1. The system is composed essentially of a pretreatment part and an HPLC part. Two liquid chromatographic Model LC 3A pumps (Shimadzu, Kyoto, Japan) are used (pump 1 and pump 2). A Model 7125 Rheodyne injector and Model 7000 Rheodyne six-port valve, both from Kemko, Osaka, Japan, are used. The fluorescence detector, Model SK-4, equipped with a liquid chromatographic flow cell is from Sekisui (Osaka, Japan).

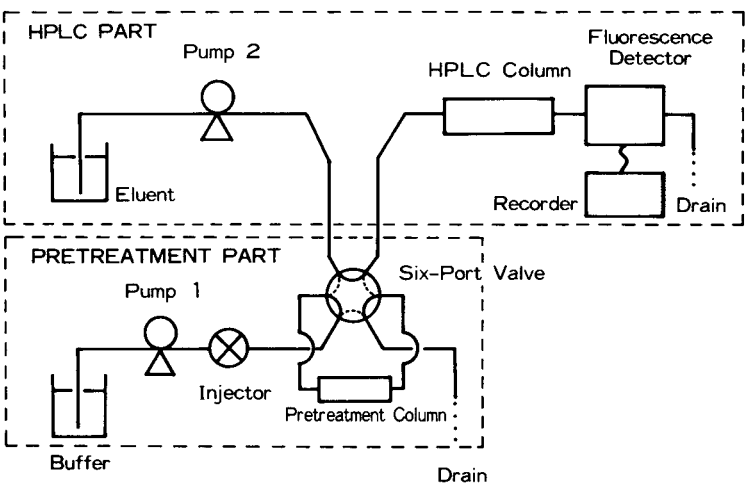


Fig. 1. Flow diagram of the HPLC system (for details, see text).

HPLC and pretreatment columns

Copolymer beads were synthesized by a standard suspension polymerization method. The aqueous portion of the polymerization mixture was prepared in a reaction flask (5 l) by adding 60 g of polyvinyl alcohol as dispersing agent to 2.5 l of distilled water. The monomer phase consisted of 250 g of tetraethyleneglycol diacrylate, 50 g of tetramethylolmethane triacrylate (both from Sin Nakamura, Wakayama, Japan), 300 g of toluene, and 3.5 g of benzoyl peroxide.

The aqueous phase was warmed to 50°C, and then the monomer—diluent—initiator solution was added with stirring and the temperature was maintained at 80°C for 10 h. When polymerization was complete, the resin beads were filtered off and washed. About 25 g of spherical porous beads about 10—15 μm in diameter were obtained by sieving. These porous beads were packed into a stainless-steel column (250 mm × 6 mm I.D.; HPLC column). Similar beads were packed into the pretreatment column (20 mm × 4 mm I.D.).

Reagents and standards

Acetonitrile and methanol, both reagent grade, were from Nakarai (Kyoto, Japan). Oestriol (E_3), oestradiol (E_2) and other oestrogenic steroids were also obtained from Nakarai.

Standard solutions of E_2 and E_3 at 0.5 μg/ml in methanol—water (1:1, v/v), and a mixture of E_2 and E_3 in the same solvent were prepared and stored at 4°C until used.

Pretreatment buffer

Phosphate buffer, 0.05 mol/l, adjusted to pH 8.5, was used.

Eluent

Acetonitrile—water (65:35, v/v) was used as eluent.

Serum samples

Samples (5 ml) of whole blood were taken from about 50 pregnant women. The samples in glass test tubes were left to stand at room temperature long enough (about 30 min) for clot retraction to occur. Then they were centrifuged (1000 *g*, 10 min) and the resulting supernatant serum was transferred to another glass tube and stored at −10°C until use.

Radioimmunoassay (RIA)

E_2 and E_3 in serum samples were measured by RIA at Teikokuzoki Clinical Laboratory (Tokyo, Japan).

Chromatographic procedure

Pretreatment. A sample of 150 μl of serum was injected into the apparatus through the injector. The flow-rate of the pretreatment buffer was maintained at 0.6 ml/min. In the pretreatment column, oestrogens and other hydrophobic components were adsorbed on the porous beads, whereas serum components such as proteins and carbohydrates were not. At 6 min after injection of the sample, when all the hydrophilic components had flowed out of the column, the six-port valve was turned and the eluent was allowed to flow (0.8 ml/min) into the pretreatment column. The oestrogens were eluted and passed into the HPLC column.

Separation and detection. A reversed-phase system was used for the separation. The flow-rate was 0.8 ml/min, and the column temperature was 25°C. The fluorescence detector was set at an excitation wavelength of 220 nm and emitted light was measured at 320 nm.

RESULTS

Chromatograms

The chromatogram of a standard mixture of E_2 and E_3 is shown in Fig. 2A. The right chromatogram (Fig. 2B) is of a serum sample from a normal pregnant woman (35 weeks of pregnancy), for which we calculated the concentrations of E_2 and E_3 to be 32.7 and 17.2 ng/ml, respectively.

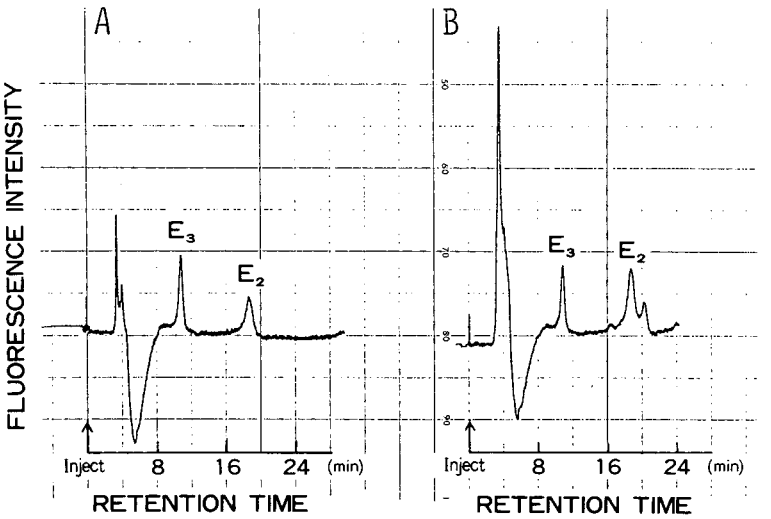


Fig. 2. (A) Chromatogram of a mixture of E_2 and E_3 (20 ng of each). (B) Chromatogram of serum E_2 and E_3 of a pregnant woman.

Interference

We injected several naturally occurring oestrogenic steroids into the chromatograph to determine their potential interference in this procedure. The glucuronides and sulphates of E_2 and E_3 were not adsorbed on the pretreatment column, and so could not be measured by this technique.

Linearity

A mixture of E_2 and E_3 was added to serum from male subjects that had previously been shown to contain no E_2 or E_3. Standard samples of serum

TABLE I

PERCENTAGE RECOVERIES OF E_2 and E_3, DETERMINED BY ADDING DIFFERENT AMOUNTS OF OESTROGENS TO THREE SERUM SAMPLES WHICH CONTAINED DIFFERENT AMOUNTS OF ENDOGENOUS OESTROGENS

E_2				E_3			
Amount added (ng/ml)	Amount expected (ng/ml)	Amount determined (ng/ml)	Recovery* (%)	Amount added (ng/ml)	Amount expected (ng/ml)	Amount determined (ng/ml)	Recovery** (%)
0	—	17.0	—	0	—	4.0	—
5	22.0	20.8	95	5	9.0	9.2	102
10	27.0	27.7	102	10	14.0	16.9	121
20	37.0	38.9	105	20	24.0	28.2	117
0	—	25.4	—	0	—	11.2	—
5	30.4	31.2	103	5	16.2	15.6	96
10	35.4	34.7	98	10	21.2	20.5	97
20	45.4	45.7	101	20	31.2	31.8	102
0	—	31.3	—	0	—	20.2	—
5	36.3	35.9	99	5	25.2	24.6	98
10	41.3	39.3	95	10	30.2	29.0	96
20	51.3	51.6	101	20	40.2	40.8	101

*Mean ± S.D. = 99.9 ± 3.4%.
**Mean ± S.D. = 103 ± 9.0%.

containing E_2 and E_3 at concentrations of 5, 10, 20, 40 ng/ml were prepared and chromatographed. The peak heights of E_2 and E_3 were linearly proportional to their concentrations in the range 0–40 ng/ml. The standard curves obtained are $Y = 0.5539X - 0.2609$ ($r = 0.9996$) for E_2, and $Y = 1.086X - 0.2391$ ($r = 0.9998$) for E_3, where Y is the peak height (mm) and X is the oestrogen concentration (ng/ml sample).

Recovery

Mixtures of E_2 and E_3 were added to sera from pregnant women, and the peak heights of oestrogens in these sera were converted into oestrogens concentrations with the aid of the standard curves. The recoveries thus obtained are shown in Table I.

Reproducibility

Intra-assay reproducibility was examined by measuring the oestrogen concentration in the same sample (serum of a woman in week 38 of pregnancy) six times in one day. The coefficient of variation (C.V., %) was calculated from the mean and standard deviation of the values. Table II shows the results.

TABLE II

INTRA-ASSAY VARIABILITY OF OESTROGEN VALUES

Six determinations were made on serum of a woman in week 38 of pregnancy.

Oestrogen	Average (ng/ml)	S.D.	C.V. (%)
E_2	40.2	1.62	4.0
E_3	16.6	0.59	3.5

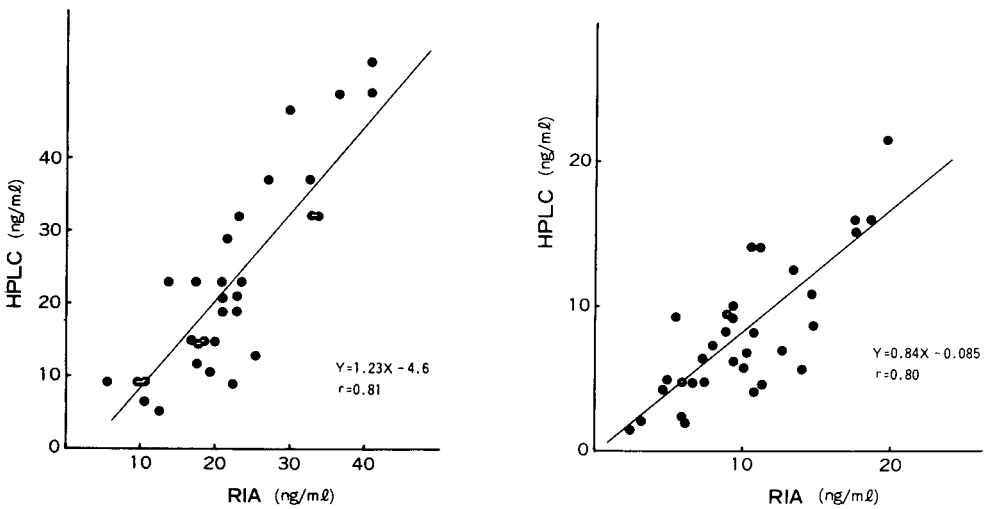


Fig. 3. Correlation between E_2 values determined by HPLC and RIA.

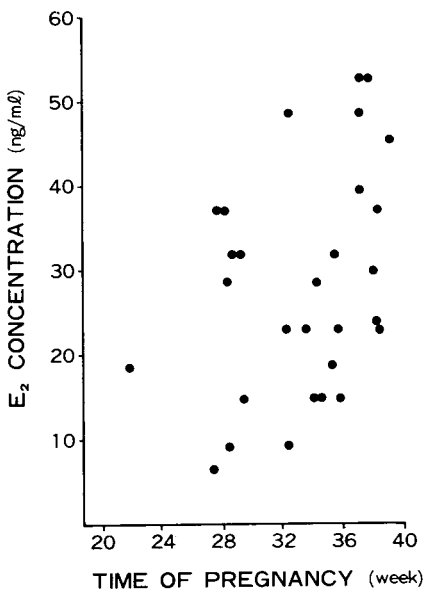
Fig. 4. Correlation between E_3 values determined by HPLC and RIA.

Correlation between values obtained by HPLC and RIA

Samples were divided into two portions and assayed by HPLC and RIA. The values for E_2 and E_3 obtained by HPLC correlated well with those obtained by RIA (r = 0.81 and r = 0.80, respectively), as shown in Figs. 3 and 4, respectively.

Changes in concentrations during pregnancy

Figs. 5 and 6 show plots of E_2 and E_3 concentrations against the duration of pregnancy. Both E_2 and E_3 showed increases in median values and in the distribution of values, which were skewed toward higher values with progress of pregnancy. These tendencies are similar to those observed previously by RIA [1, 14].



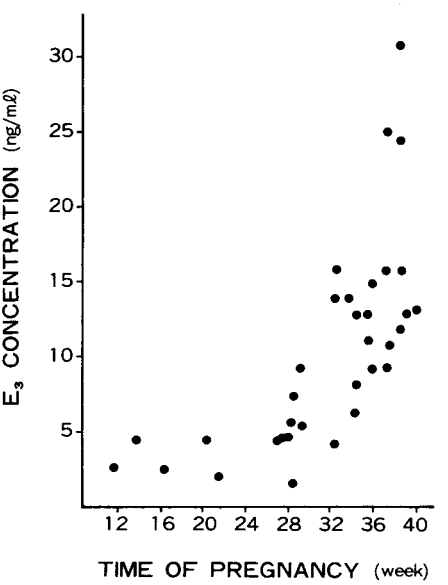


Fig. 5. Serum E_2 concentrations as a function of the duration of pregnancy.

Fig. 6. Serum E_3 concentrations as a function of the duration of pregnancy.

DISCUSSION

The present procedure is highly specific in that it is virtually unaffected by compounds such as oestrogen derivatives and other steroids that might interfere with the accurate determination of serum unconjugated oestrogens by RIA. Many liquid chromatographic methods for the measurement of serum oestrogens have been reported, but these all involve a time-consuming pretreatment consisting of solvent extraction, evaporation, and reconstitution, and thus are not suitable for routine use. Our procedure does not involve a complicated pretreatment and so the total time required for one measurement is only about 25 min. With this procedure it is possible to measure 1.0 ng/ml E_2 or E_3 using a sample of 100 μl of serum. The recovery, reproducibility, and correlation with RIA values are also sufficient for clinical purposes.

In summary, the present procedure for determination of serum E_2 and E_3 is simpler and more rapid than previous chromatographic methods, and seems suitable for use in routine measurements of foetoplacental function.

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Journal of Chromatography, 311 (1984) 257—266
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2266

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF GUANIDINO COMPOUNDS OF CLINICAL IMPORTANCE IN HUMAN URINE AND SERUM BY PRE-COLUMN FLUORESCENCE DERIVATIZATION USING BENZOIN

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(First received April 17th, 1984; revised manuscript received June 13th, 1984)

SUMMARY

High-performance liquid chromatographic microanalyses for guanidino compounds in human physiological fluids have been accomplished by means of a pre-column fluorescence derivatization method using benzoin. The guanidino compounds in urine or deproteinized serum after ultrafiltration are converted to the fluorescent derivatives with benzoin in an alkaline medium, and the derivatives are separated simultaneously within 25 min on a reversed-phase column (μBondapak Phenyl) with a linear gradient elution of methanol in aqueous mobile phase (pH 8.5). The method permits the quantitative determination of guanidinosuccinic acid, methylguanidine, taurocyamine and guanidinobutyric acid at concentrations of as low as 8—78 pmol/ml in human urine and serum.

INTRODUCTION

Several guanidino compounds occur in human physiological fluids and tissues. Among the biogenic guanidino compounds, guanidinosuccinic acid and methylguanidine have been considered to be uraemic toxins in uraemic syndrome since these compounds accumulate in the body fluids of uraemic patients [1—3] and give rise to a complex of symptoms similar to uraemia [4, 5]. Recently, taurocyamine also has been suspected as a uraemic toxin since it is increased in uraemic rabbit brain and induces uraemic convulsions [6]. The concentrations of the above three guanidino compounds in normal human serum are generally too low to be quantified by the conventional chromatographic methods including ion-exchange high-performance liquid chromatography (HPLC) [6—11], thin-layer [12, 13], paper [14, 15] and gas [16, 17] chromatography.

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We previously developed a fluorimetric method for the selective determination of guanidino compounds based on their reaction with benzoin in an aqueous methyl cellosolve—potassium hydroxide solution in the presence of β-mercaptoethanol (to stabilize the fluorescent products) and sodium sulphite (to suppress blank fluorescence) [18, 19]. The fluorescent derivatives of the guanidino compounds (excluding guanidine) produced by this benzoin reaction, corresponding to the chemical structures of 2-substituted amino-4,5-diphenylimidazoles [20], were separable by reversed-phase HPLC as reported previously [21], though the reaction gave a small by-product peak identical to the benzoin derivative of guanidine in the chromatogram. Moreover, the sensitivity of that HPLC method enabled the detection of guanidino compounds at the femtomole level. Thus, the pre-column derivatization method with the benzoin reaction may be useful for the HPLC quantification of biogenic guanidino compounds, particularly at very low concentrations in biological samples.

On the other hand, the HPLC method utilizing the benzoin reaction in post-column detection, which was recently described in a separate paper [22], did not allow the reliable quantification of methylguanidine, guanidinosuccinic acid and taurocyamine in normal human sera because the sensitivity of the on-line post-column detection was limited by the dilution of the HPLC column eluate with the reagent solutions. However, the method [22] as well as other post-column derivatization methods (using 9,10-phenanthraquinone [9, 10] or ninhydrin [11] as fluorogenic reagent) permits the quantification of guanidino compounds at the picomole level.

The purpose of the present research was to establish a simple and rapid HPLC method utilizing the benzoin pre-column fluorescence derivatization technique [21] for the microanalysis of those guanidino compounds especially implicated as uraemic toxins in human serum and urine. Phenylguanidine (PG), which is not present in human physiological fluids, was used as an internal standard.

EXPERIMENTAL

Chemicals and solutions

Deionized and distilled water was used. Taurocyamine was kindly supplied by Prof. A. Mori (Institute for Neurobiology, Okayama University Medical School, Okayama, Japan). Tris(hydroxymethyl)aminomethane (Tris) and benzoin (both from Wako, Osaka, Japan) were recrystallised from aqueous methanol (60%, v/v) and absolute methanol, respectively, to remove fluorescent impurities. Other chemicals were of reagent grade. Diaflo membranes (UM 05) used for ultrafiltration were obtained from Amicon (Lexington, MA, U.S.A.). A standard solution of guanidino compounds was prepared in 0.01 *M* hydrochloric acid. The reagent solutions used for the fluorescence derivatization were prepared as described previously [21].

Preparation of human physiological fluids

Serum and urine specimens were obtained from healthy volunteers in our laboratory and from patients with various diseases in hospital (Chidoribashi Hospital, Fukuoka, Japan).

Dialysed serum. The ultrafiltration conditions were examined using dialysed serum. A 10-ml aliquot of normal human serum was dialysed against 6 l of water for 24 h at 4°C using a cellophane tube (Wako).

Serum sample solution. A 300-μl aliquot of serum was mixed with 300 μl of water or a standard solution of guanidino compounds, 200 μl of 2.5 nmol/ml PG and 100 μl of 0.9 *M* hydrochloric acid. The mixture was placed in an Amicon dialysing device (5-ml cell volume) and then ultrafiltered through a UM 05 membrane under nitrogen gas at a pressure of 3 kg/cm^2. A 200-μl aliquot of the filtrate was used for the fluorescence derivatization.

Urine sample solution. A 50-μl aliquot of urine was mixed with 550 μl of water or a standard solution of guanidino compounds, 200 μl of 10 nmol/ml PG and 100 μl of 0.9 *M* hydrochloric acid. A 200-μl aliquot of the resulting mixture was used for the fluorescence derivatization.

Fluorescence derivatization

A 200-μl aliquot of the sample solution was placed in a test tube, to which were added 100 μl each of 4.0 m*M* benzoin solution (in methyl cellosolve) and an aqueous solution containing 0.1 *M* β-mercaptoethanol and 0.2 *M* sodium sulphite, and 200 μl of 2.0 *M* potassium hydroxide with cooling in ice—water. The mixture was heated in a boiling water-bath for 5 min, cooled in ice—water for approx. 1 min, and then 200 μl of an aqueous acidic solution containing 2.0 *M* hydrochloric acid and 0.5 *M* Tris—hydrochloric acid buffer (pH 9.2) were added. A 100-μl aliquot of the final mixture was used for HPLC.

HPLC apparatus and conditions

The HPLC system consisted of a Hitachi 635 high-pressure pump, a Shimadzu SIL-1A syringe-loading sample injector and a Jasco FP-110 HPLC fluorescence spectrophotometer equipped with a xenon lamp. The fluorescence of the eluate was monitored at 425 nm emission against 325 nm excitation. The column was μBondapak Phenyl (particle size 10 μm; 300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.). This column can be used for more than 300 analyses with only a small decrease in the theoretical plate number. For the separation of the benzoin derivatives of guanidino compounds on the column, a linear gradient elution with a methanol concentration between 50% and 80% (v/v) in the aqueous mobile phase containing 10% (v/v) 0.5 *M* Tris—hydrochloric acid buffer (pH 8.5) was carried out during 25 min at a constant flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

The following biogenic guanidino compounds have been demonstrated in human urine and serum [23] (abbreviation in parentheses); guanidine (G), methylguanidine (MG, δ-guanidinovaleric acid (GVA), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), guanidinobutyric acid (GBA), guanidinosuccinic acid (GSA), arginine (Arg), N-α-acetylarginine (AArg), homoarginine (HArg), taurocyamine (TC), argininosuccinic acid (ASA), creatine (Cr) and creatinine (Crn). Of these, TC, GSA, GBA and MG could be separated and quantified fluorimetrically by the present HPLC method.

Fig. 1 shows typical chromatograms obtained with sera from a patient with renal failure and from a normal subject, and with a normal urine. The benzoin derivatives corresponding to the guanidino compounds and PG as an internal standard in the samples are separated within 25 min on the μBondapak Phenyl column with linear gradient elution of methanol in aqueous mobile phase (pH 8.5) under the HPLC conditions described in the experimental section. All the peaks produced by the derivatization with benzoin (except for the peaks eluted around the time of the void volume) can be readily identified on the basis of their retention times in comparison with the standard compounds and also by co-chromatography of the standards and the sample with a different eluent, i.e. using a lower methanol concentration (40—70%), than that used for the recommended procedure. This elution provided better separation of the peaks but their elution was delayed; the complete separation needed 45 min.

The benzoin reaction does not give fluorescent derivatives of biological substances having no guanidino moiety in the molecule such as amines, amino acids, sugars, lipids, keto acids, steroids, vitamins and nucleosides, as demonstrated previously [18, 19]. In addition, some biological compounds tested (e.g. tyrosine, tryptophan, vitamin A, adenosine, guanosine and thymidine) which have weak native fluorescence do not interfere with either the detection or separation of the peaks of the biogenic guanidino compounds in

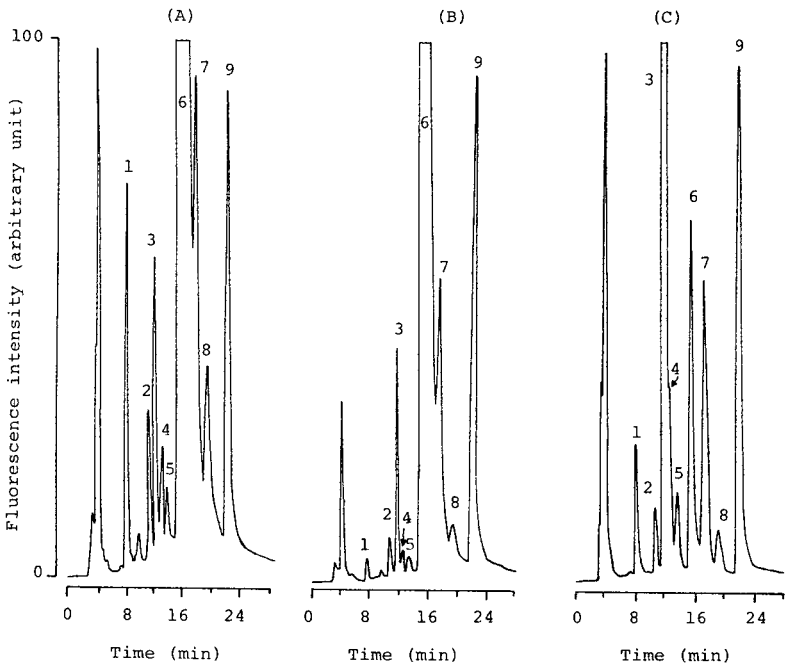

Fig. 1. Chromatograms of the benzoin derivatives of guanidino compounds in serum from a patient with renal failure (A) and from a normal subject (B), and in a normal urine (C). The samples were treated as described in the experimental section. Peaks: 1, GSA; 2, TC; 3, GAA, Cr and Crn; 4, GPA and AArg; 5, GBA; 6, Arg, ASA, GVA and HArg; 7, G and by-products produced from the other guanidino compounds during the derivatization [21]; 8, MG; 9, PG (internal standard).

the chromatograms, because they are co-eluted around the time (3.0—5.0 min) of the void volume.

The chromatographic condition recommended is one of the results of the investigations for the complete separation of TC, GSA and MG which are the compounds of most interest in the toxic manifestation of uraemia, while the basic separation conditions for the derivatives of the guanidino standards were previously described [21]. However, the established HPLC method did not permit the separation of some arginyl-containing oligopeptides in serum from the biogenic guanidino compounds. The benzoin derivatives of several oligopeptides tested with one or two arginyl residues [e.g., tuftsin, angiotensins I, II and III, bradykinin, luteinizing hormone releasing hormone (LHRH) and

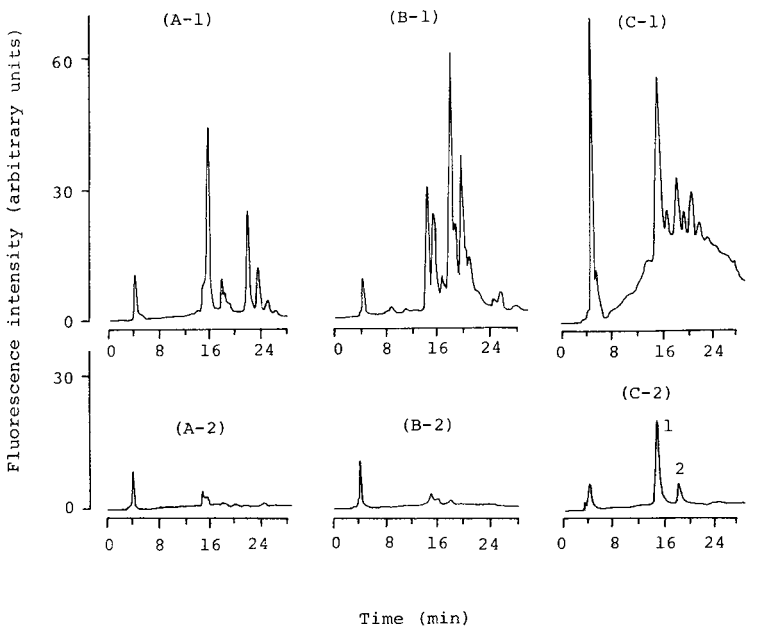

Fig. 2. Effects of ultrafiltration with a UM 05 membrane on removal of peptides and proteins from the peptide solutions (A and B) and a dialysed serum (C). A-1 and B-1 (without ultrafiltration): aliquots (200 μl) of two solutons of peptides (A-1, 2 nmol/ml each of tuftsin and angiotensin III, molecular weight 500—1000; B-1, 2 nmol/ml each of angiotensin II, bradykinin, angiotensin I, LHRH and neurotensin, molecular weight 1000—2000) were directly used for the fluorescence derivatization. A-2 and B-2 (with ultrafiltration): aliquots (900 μl) of the peptide solutions used for A-1 and B-1 were ultrafiltered with a UM 05 membrane, then 200-μl portions of the filtrates were used for the fluorescence derivatization. C-1 (by deproteinization with perchloric acid): to 300 μl of dialysed serum, 250 μl each of the peptide solutions used for A and B and 100 μl of 4 *M* perchloric acid were added. After centrifuging at 800 *g* for 10 min, 200 μl of the supernatant were neutralized with 100 μl of 2 *M* potassium carbonate. A 200-μl portion of the resulting supernatant was used for the fluorescence derivatization. C-2 (by deproteinization with ultrafiltration): to 300 μl of the same dialysed serum as that for C-1, 250 μl each of the peptide solutions used for A and B and 100 μl of 0.4 *M* hydrochloric acid were added. The mixture was then ultrafiltered with a UM 05 membrane. A 200-μl portion of the filtrate was used for the fluorescence derivatization. All the chromatograms were obtained with the same sensitivity range of the detector. Peaks: 1, arginine; 2, by-product; other peaks were from peptides and/or proteins.

TABLE I

RECOVERIES OF GUANIDINO COMPOUNDS ON ULTRAFILTRATION USING A DIAFLO UM 05 MEMBRANE

Conditions*	Percentage recovery** [mean (C.V.)]					Recovery ratio*** [mean (C.V.)]			
	GSA	TC	GBA	MG	PG	GSA	TC	GBA	MG
Three-fold dilution, 0.1 *M* HCl	74 (10)	65 (13)	80 (8)	75 (7)	73 (6)	1.02 (7)	0.90 (7)	1.10 (5)	1.03 (2)
Two-fold dilution, 0.1 *M* HCl	76 (9)	68 (13)	67 (8)	65 (11)	60 (6)	1.25 (4)	1.12 (8)	1.11 (4)	1.08 (6)
Three-fold dilution, H_2O	7 (26)	69 (19)	56 (20)	75 (13)	30 (31)	0.23 (26)	2.37 (16)	1.92 (10)	2.61 (16)
Two-fold dilution, H_2O	5 (38)	67 (9)	58 (9)	52 (18)	36 (11)	0.15 (27)	1.87 (6)	1.61 (6)	1.45 (19)

*Three-fold dilution: to 300 μl of the dialysed serum, 300 μl each of a standard solution of the guanidino compounds (PG, 0.25 nmol/ml; others, 1.0 nmol/ml each) and 300 μl of water or 0.3 *M* hydrochloric acid were added. The mixture was then ultrafiltered with a UM 05 membrane. Two-fold dilution: to 500 μl of the dialysed serum, 300 μl of the standard solution of the guanidino compounds and 200 μl of water or 0.45 *M* hydrochloric acid were added. The mixture was then ultrafiltered with a UM 05 membrane.

**The values were calculated from the results of five analyses using a new UM 05 membrane each time.

***The recoveries of the guanidino compounds on each ultrafiltration were divided by that of PG (internal standard).

neurotensin] are eluted at retention times between 14 and 28 min with two or more fluorescent peaks due to cleavage of peptide bonds during the fluorescence derivatization, as shown in chromatograms A-1 and B-1 of Fig. 2.

An ultrafiltration technique with a UM 05 membrane, which excludes substances larger than approx. 500 daltons, was thus employed to remove such interfering peptides present in samples. Fig. 2 shows the efficacy of the ultrafiltration on the removal of the peptides and serum proteins. The oligopeptides tested (molecular weight 500—2000) in 0.1 *M* hydrochloric acid were not present in the ultrafiltrate (Fig. 2, A-2 and B-2). When the dialysed serum was ultrafiltered, no peak (other than those of arginine and a by-product of arginine due to the fluorescent derivative of guanidine) appeared in the chromatogram (Fig. 2, C-2), even though oligopeptides such as tuftsin, angiotensins I, II and III, bradykinin, LHRH and neurotensin were added to the dialysed serum before the ultrafiltration. The observation of the arginine peak in this chromatogram means that the endogenous arginine still remains in the serum even after the dialysis since the arginine content is generally very high in human serum.

On the other hand, in the chromatogram (Fig. 2, C-1) obtained with the same dialysed serum but which was deproteinized with perchloric acid, there are still several peaks corresponding to the peptides and an enormous broad peak probably due to small proteins not removed by the deproteinization. Thus, deproteinization with an acidic precipitating reagent such as perchloric acid is not suitable for clean-up of serum samples.

Table I shows the recoveries of the guanidino compounds added to the dialysed serum and taken through the ultrafiltration step. Because of the basic nature of the guanidino compounds, acidification of the serum sample gave fairly good recoveries (60—85%) with coefficients of variation (C.V.) of 6—13% for the guanidino compounds tested, though low recoveries for the guanidino compounds especially for GSA and PG were observed when the same sample was diluted two- or three-fold with water. Additionally, the coefficients of variation of their recoveries, depending on the lot number of the membrane, can be reduced by comparing the recovery of each guanidino compound to that of the internal standard, as shown in Table I. Consequently, in the procedure recommended, the serum samples spiked with the internal standard were diluted three-fold with hydrochloric acid (final concentration 0.1 *M*) and then ultrafiltered.

The ultrafiltration procedure was not necessary for normal urine because the determined values of the guanidino compounds in the urine treated or untreated by ultrafiltration were identical within the error of the assay. However, some other urines, particularly from patients with diseases such as proteinuria, may require ultrafiltration. We therefore checked the protein content of each urine sample by a simple test using protein-test paper (Wako) before proceeding with the HPLC.

Calibration curves for both serum and urine, which were constructed by plotting the ratios of net peak heights of the spiked guanidino compounds against the peak height of the internal standard, were linear in the relationship between the ratios and the amounts of the guanidino compounds added to urine or serum. The correlation coefficients (r) of all the curves were greater than 0.997 and no change of the slopes in the graphs was observed depending

on the urine or serum used. In the curves, the ratios of GSA, TC, GBA and MG against the internal standard (PG: 0.5 nmol per 0.3 ml of serum and 2.0 nmol per 50 μl of urine) were 0.24, 0.50, 0.54 and 0.27, respectively, for the concentration of 0.6 nmol per 0.3 ml of serum, and 0.19, 0.40, 0.42 and 0.20, respectively, for the concentration of 2.0 nmol per 50 μl of urine.

The above results indicate that the present internal standard method permits the quantitative determination of the biogenic guanidino compounds in serum and urine.

Concentrations of the guanidino compounds in human serum and urine were analysed by the present method (Tables II and III). The concentrations of GSA and MG in normal serum are very low compared to those in normal urine. However, the levels in sera from patients with uraemia or chronic renal failure undergoing haemodialysis are dramatically increased, as has also been demonstrated in other reports [1—3, 22]. On the other hand, there are no significant differences in the TC and GBA concentrations in the serum between uraemia and other diseases, though both compounds are slightly increased in sera from these patients. The concentration values of the individual guanidino compounds in serum and urine are in good agreement with the data obtained by the post-column fluorescence derivatization methods using 9,10-phenanthra-

TABLE II

CONCENTRATIONS OF GUANIDINO COMPOUNDS IN SERA FROM HEALTHY ADULTS AND PATIENTS

Sex	Guanidino compound (nmol/ml)			
	GSA	TC	GBA	MG
Healthy adults				
F	0.27	0.45	0.11	ND*
F	0.36	0.16	0.04	0.28
M	0.20	0.58	0.05	ND
M	0.10	0.33	0.05	0.13
F	0.34	0.38	0.04	0.12
M	0.49	0.33	0.08	0.22
M	0.23	0.40	0.06	ND
M	0.38	0.43	0.06	0.24
M	0.25	0.42	0.04	0.08
M	0.46	0.43	0.06	ND
Patients**				
M (a)	10.83	2.56	0.32	6.66
M (b)	7.75	1.34	0.46	1.85
F (b)	9.10	0.84	0.43	6.63
M (c)	0.76	3.31	0.43	0.20
F (d)	1.53	0.48	0.30	0.28
M (e)	1.53	0.61	0.52	0.76
F (e)	1.61	1.31	0.36	0.34

*ND, not detected.

**Disease: (a) uraemia; (b) chronic renal failure; (c) gastritis with haemorrhage; (d) stomach cancer; (e) arteriosclerosis.

TABLE III

CONCENTRATIONS OF GUANIDINO COMPOUNDS IN URINE FROM HEALTHY ADULTS

Sex	Urine volume in a day (ml)	Guanidino compounds (μmol/day)			
		GSA	TC	GBA	MG
M	800	40.7	0.4	4.0	19.0
M	880	34.7	9.2	11.5	14.6
M	860	33.6	ND*	3.8	16.6
M	1230	52.5	1.2	4.9	29.9
M	1160	21.3	12.7	12.1	29.3
M	1800	33.1	ND	4.5	23.3
F	720	37.6	0.4	7.1	6.4
F	1030	59.7	7.7	8.1	17.9
F	920	35.5	7.3	15.5	13.7
F	780	32.3	1.2	3.9	10.0

*ND, not detected.

quinone [9, 10], ninhydrin [11] or benzoin [22] with HPLC. But the post-column derivatization methods do not allow the concentrations of GSA, MG and TC in normal sera to be determined. Their concentrations (100—400 pmol/ml) can be determined by the post-column detection methods provided the serum samples are concentrated.

As demonstrated in the Introduction, the present pre-column derivatization method is about 50 times more sensitive than the post-column derivatization method [22] utilizing the benzoin reaction in both methods, even in the analysis of guanidino compounds in normal sera. The sensitivity of determination by post-column reaction may be affected by various factors (other than the column efficiency) such as dilution with the reagent solutions, diffusion in the reactor system and/or the limiting conditions of the derivatization on-line to the chromatograph.

The lower limits of detection for the guanidino compounds determined by the proposed method are 16, 8, 8 and 12 pmol/ml in serum, and 78, 29, 30 and 60 pmol/ml in urine for GSA, TC, GBA and MG, respectively. These values correspond to amounts of approx. 50—100 fmol in an injection volume suitable for this HPLC method, giving a signal-to-noise ratio of 2.

The coefficients of variation in repeatability assays (n = 10) of the guanidino compounds [the compound and its concentration (nmol/ml) in parentheses] in serum are 5.4% (GSA, 0.49), 6.4% (TC, 0.33), 6.1% (GBA, 0.08) and 3.3% (MG, 0.22), and for urine are 4.2% (GSA, 39.4), 4.7% (TC, 10.5), 3.6% (GBA, 13.1) and 3.1% (MG, 16.6).

The present HPLC method with the benzoin pre-column derivatization technique necessitates a clean-up procedure for serum or protein-rich samples but gives a satisfactory sensitivity in the quantitative analysis of the biogenic guanidino compounds especially with suspicion of uraemic toxin; the sensitivity allows the use of less than 300 μl of serum or urine. This method is also rapid and simple to perform and can therefore be applied for routine use.

ACKNOWLEDGEMENT

The authors thank Dr. T. Hirata, Chidoribashi Hospital, for the supply of serum samples from patients.

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Journal of Chromatography, 311 (1984) 267—276
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2279

ANALYSIS OF 1- AND 3-METHYLHISTIDINES, AROMATIC AND BASIC AMINO ACIDS IN RAT AND HUMAN URINE

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(Received May 22nd, 1984)

SUMMARY

A procedure based on automated amino acid analysis has been developed to simultaenously quantify 1-methylhistidine (1-MH), 3-methylhistidine (3-MH), tyrosine, phenylalanine, tryptophan, lysine, histidine and arginine levels in human and rat urines. Deproteinized urine samples containing amino acids in the range 1—10 nmol were analyzed using single-column methodology with ninhydrin detection. Standard curves produced correlation coefficients $\geqslant 0.99$ with duplicate analyses agreeing to within ± 1.9%. Quantitative recovery was ensured by using L-α-amino-β-guanidinopropionic acid as an internal standard. Elution was accomplished in less than 90 min at pH 5.7 with sodium citrate buffers at 45°C and 65°C. Since 3-MH in the rat is acetylated at the α-amino group, rat, but not human, urine ultrafiltrates required acid hydrolysis prior to analysis. The utility of the technique of analysis of 1-MH and 3-MH in human urine was demonstrated for an adult male on a meat-free diet for 21 days; urinary excretion rates for 3-MH and 1-MH were determined to be 3.06 ± 0.10 and 0.72 ± 0.07 μmol/kg body mass/day, respectively. The technique was also used to measure the effect of disuse atrophy of rat skeletal muscle which induced a 40—60% increase in 3-MH. The procedure is also highly suited for measurement of urinary aromatic and/or basic amino acids.

INTRODUCTION

Changes in urinary levels of specific amino acids can provide information about metabolism in the intact organism. Wellner and Meister [1] have recently reviewed alterations of amino acid metabolism in man. In 1954, Tallan et al.

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0378-4347/84/$03.00

[2] identified 3-methylhistidine (3-MH) as a normal component of human and animal urine. Subsequent experiments demonstrated that residues of 3-MH occupy specific sites in the primary sequences of actin and myosin [3, 4]. Since 3-MH is not reutilized and is quantitatively excreted, urinary 3-MH levels can provide an index of muscle protein turnover [5—7]. Much less is known about the metabolism of 1-methylhistidine (1-MH) although it occurs in the dipeptide, anserine (β-alanyl-L-1-methylhistidine) [8].

One of the reasons that there is a paucity of information on 1-MH is the lack of a convenient and sensitive method for simultaneously determining both 1-MH and 3-MH. These methylated amino acids are often difficult to resolve from other basic amino acids and ammonia. 3-MH has been determined by automated amino acid analysis, by gas chromatography—mass spectrometry (GC—MS), or by high-performance liquid chromatography (HPLC) [9—11]. Small variations in temperature, pH, ionic strength, resin and column dimensions may affect the resolution of these amino acids.

Additionally, sample preparation plays a critical role in the quantitation of amino acids in physiological fluids. Such fluids usually contain soluble proteins which should be removed prior to analysis. A further consideration is that the α-amino groups of 1-MH and 3-MH in the rat are blocked and require acid hydrolysis prior to separation and quantitation with an amine-specific reagent such as ninhydrin.

This paper describes rapid procedures for the preparation and automated amino acid analysis of protein-free urine specimens. We have optimized our conditions for the resolution of 1-MH and 3-MH from other basic amino acids and ammonia. The protocol also allows for the resolution of tyrosine, phenylalanine and tryptophan in less than 40 min.

EXPERIMENTAL

Reagents

Chemicals were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Standards for amino acid analyses were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.) or Beckman (Palo Alto, CA, U.S.A.). Ninhydrin was obtained from Pierce (Rockford, IL, U.S.A.).

Animals

Male Sprague—Dawley rats were purchased from Charles River Breeding Lab. (Boston, MA, U.S.A.) and maintained in metabolism cages on a Purina Rat Chow diet with a 12 h light/12 h dark cycle.

Urine collection

Rat urine was collected under mineral oil, free of fecal contamination, utilizing Hoeltge stainless-steel separatory funnels and was stored at −20°C prior to analysis. Human urine was collected from a 91-kg normal 38-year-old male (RCF) on a meat-free diet for 21 days. At each urine collection the volume was measured and a 10-ml aliquot was saved in a glass vial containing five drops of toluene. Samples were either frozen immediately or stored at 4°C for up to 48 h. The equivalent of a 24-h urine collection was prepared by

taking an aliquot of each individual sample as a percentage of total daily urine volume. Such a procedure permits analysis of the metabolite content at each collection point as well as the total metabolites present in a 24-h collection. Daily urine collection was started at 12.00 midnight.

Preparation of urine ultrafiltrates

Urine (1 ml) was added to a 75 × 12 mm polystyrene tube containing 38 mg of citric acid (1 ml of 0.2 *M* citric acid; lyophilized) such that the final concentration was 0.2 *M* and the pH range was 2—3. Of a 25 m*M* stock of L-α-amino-β-guanidinopropionic acid (AGP) 10 μl were added to each urine sample as an internal standard. Following mixing, the urine sample was transferred to an Amicon (Lexington, MA, U.S.A.) MPS ultrafiltration apparatus. Ultrafiltrates were obtained by centrifugal ultrafiltration (1500—1700 *g* for 15 min) through a 14-mm YMB membrane having a molecular mass cut-off of 10,000 daltons. Aliquots of each sample were analyzed directly on a Dionex D-300 amino acid analyzer (Sunnyvale, CA, U.S.A.) or were hydrolyzed prior to analysis. Control experiments demonstrated the complete retention of ^{14}C-labeled human albumin and that ultrafiltration had no effect on the recovery of any amino acid determined in this study.

Acid hydrolysis of urines

Rat urines require hydrolysis prior to analysis because the α-amino groups of methylhistidines are acetylated or blocked in peptide linkage and will not react with ninhydrin. For hydrolysis, 250-μl aliquots of the ultrafiltrates were transferred to 2-ml glass ampules. An equal volume of concentrated hydrochloric acid was added and the ampules were sealed under vacuum. After hydrolysis at 110°C for 24 h the samples were dried in a desiccator overnight under house vacuum and for an additional two days with a vacuum pump. Samples were dissolved with 250 μl of loading buffer pH 2.2 [composition 0.2 *M* Na^+; 0.5% (v/v) thiodiglycol; 0.1% (w/v) phenol] and were stored frozen in 400-μl polypropylene tubes (Sarstedt, Princeton, NJ, U.S.A.). Prior to analysis, thawed samples were centrifuged 5 min in a Beckman microfuge.

Amino acid analysis

This procedure, which should be routinely applicable to other automated amino acid analysis systems, represents modifications of a protocol developed for Durrum Instrumentation (May, 1977) and provided by Dionex Corporation. Samples (20—40 μl) were analyzed using single-column methodology with sodium citrate eluents and a ninhydrin detection system. The column (17 cm × 4 mm) was packed with Dionex DC-5A cation-exchange resin (6 μm diameter). The three sodium citrate eluents (buffers A, B, and C) at pH 3.25, 5.68, and 5.72 contained 0.5% (v/v) thiodiglycol, 0.1% (w/v) phenol and Na^+ at 0.2 *M*, 0.35 *M* and 1.0 *M*, respectively. Samples were injected onto the column (previously equilibrated with buffer A) and eluted at a flow-rate of 20 ml/h with buffer B for 61 min at 45°C followed by buffer C for 25 min at 65°C. Ninhydrin reagent was pumped at 10 ml/h with the products monitored at 570 and 440 nm. Between each run the column was washed for 5 min with a 0.1 *M* sodium hydroxide solution containing 0.1 *M* sodium chloride and 1 m*M* EDTA.

It is essential that the column be re-equilibrated for 10 min with buffer A prior to loading the next sample.

Sample quantitation

Samples and standards were run in the range of 1—10 nmol of each amino acid. Individual components were quantitated by measurements of peak heights relative to those of standards. AGP served as an internal standard. Standard curves of 1-MH, 3-MH, and AGP produced correlation coefficients $\geqslant 0.99$. Duplicate analyses routinely agreed to within ± 1.9% with a range of 0—5.3%. Control experiments for the ultrafiltration step have been described above.

Statistics

1-MH and 3-MH levels for the rat and human data are calculated as the mean ± S.E.M. Inter-group significance of rat data (see Fig. 5) was evaluated with a two-tailed Student *t*-test for unpaired samples.

RESULTS

In preliminary experiments the effects of ionic strength, pH and temperature on methylhistidine separation by automated ion-exchange chromatography were investigated. The buffer conditions described in Experimental were found to be optimal. In agreement with the work of others [12], we observed that control of column temperature was important for the resolution of methylhistidines. As shown in Fig. 1, the rate of elution of 3-MH increases more with increasing temperature than does that of histidine or 1-MH. In practice, slight adjustments in temperature and/or pH can be made to achieve optimal resolution. A sample at pH 2—3 is loaded onto a column equilibrated with buffer A at pH 3.25. This allows the separation of 1-MH, 3-MH, tyrosine, phenylalanine, tryptophan, lysine, histidine and arginine (Fig. 1). Total analysis time was shortened by increasing the temperature and ionic strength after 1-MH elution. We have also determined that hydroxylysine elutes 4 min before tryptophan, ornithine 1.5 min before lysine, and the dipeptides, anserine and carnosine, 2.5 and 6 min after lysine, respectively. The method is, therefore, suitable for analysis of methylhistidines, basic and aromatic amino acids.

The methylhistidine levels in ultrafiltrates of human urine can be determined directly. However, the majority of the 3-MH present in rat urine is acetylated at the α-amino group and must be hydrolyzed prior to reaction with ninhydrin. To circumvent this problem, protein-free urine samples are usually hydrolyzed in 2—6 *M* hydrochloric acid at 100—110°C for 1—22 h [5, 12—15]. Because of the variable conditions reported in the literature, the effect of hydrolysis time on the recovery of 3-MH from rat urine was investigated. As shown in Fig. 2 the minimal time required for the hydrolysis of the acetyl groups with 6 *M* hydrochloric acid was 2 h with recovery being quantitative through 48 h. It should be noted that we have assumed 100% recovery following 24 h of acid hydrolysis. This is justified because the hydrolysis loss is less than the 2% error associated with replicate analyses. We routinely hydrolyze rat urine samples for 24 h. This ensures determination of both 1-MH and 3-MH because acetylated 3-MH is quantitatively recovered as 3-MH and the dipeptide, anserine, is hy-

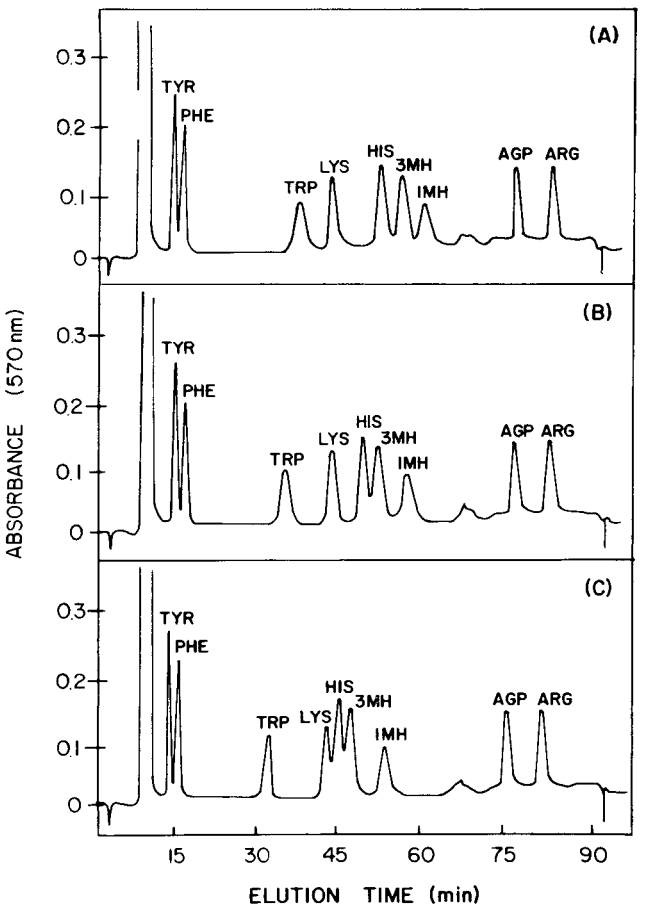

Fig. 1. Effect of temperature on the resolution of 1-MH, 3-MH, basic and aromatic amino acids. Elution conditions are described in Experimental. Panels show the separation of a standard mixture containing 5 nmol of each amino acid. Column temperature for the first 61 min of each analysis was: (A) 45°C; (B) 50°C; (C) 55°C. Peaks: Tyr = tyrosine; Phe = phenylalanine; Trp = tryptophan; Lys = lysine; His = histidine; 3-MH = 3-methylhistidine; 1-MH = 1-methylhistidine; AGP = internal standard; Arg = arginine.

drolyzed to β-alanine and 1-MH. Fig. 3 shows typical analyses of unhydrolyzed human urine and hydrolyzed rat urine. Table I summarizes the effects of acid hydrolysis on the quantitation of 1-MH and 3-MH in human and rat urine. Acid hydrolysis was shown to have no effect on the recovery of 1-MH and 3-MH in human urines confirming that trace amounts of these amino acids occur in the acetylated form or in small peptides [6]. In contrast, 80—90% of 1-MH and 3-MH in rat urine was found to be acetylated or in peptide linkage.

We applied this method to the quantitation of 1-MH and 3-MH in 24-h samples of human urine obtained from a normal human male on a diet free of meat, but containing protein adequate to maintain nitrogen balance. Variations in fluid intake and exercise pattern caused the daily urine output to vary over a four-fold range. The data in Fig. 4 show an inverse relationship between methylhistidine concentration and urine volume. Total daily methylhistidine

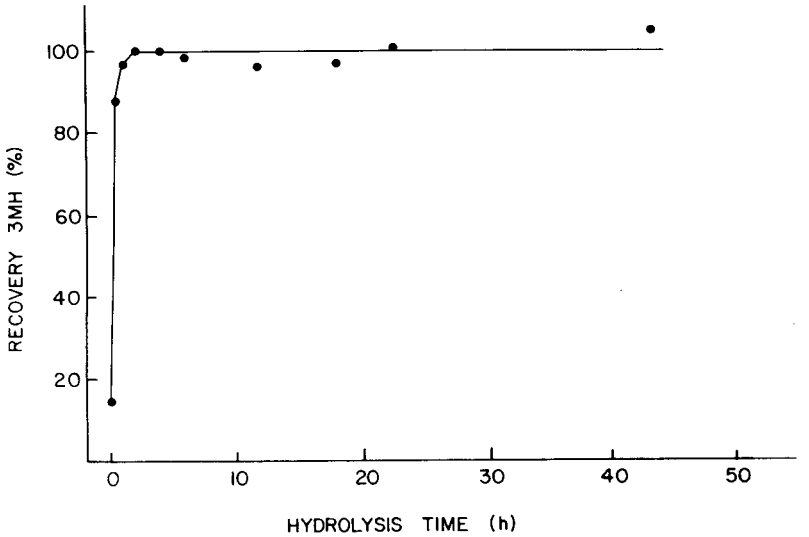

Fig. 2. Effect of hydrolysis time on recovery of 3-MH from rat urine. Ultrafiltrates were hydrolyzed in 6 *M* hydrochloric acid at 110°C in vacuo. Samples were analyzed in duplicate at 0, 0.5, 1, 2, 4, 6, 12, 18, 24 and 48 h.

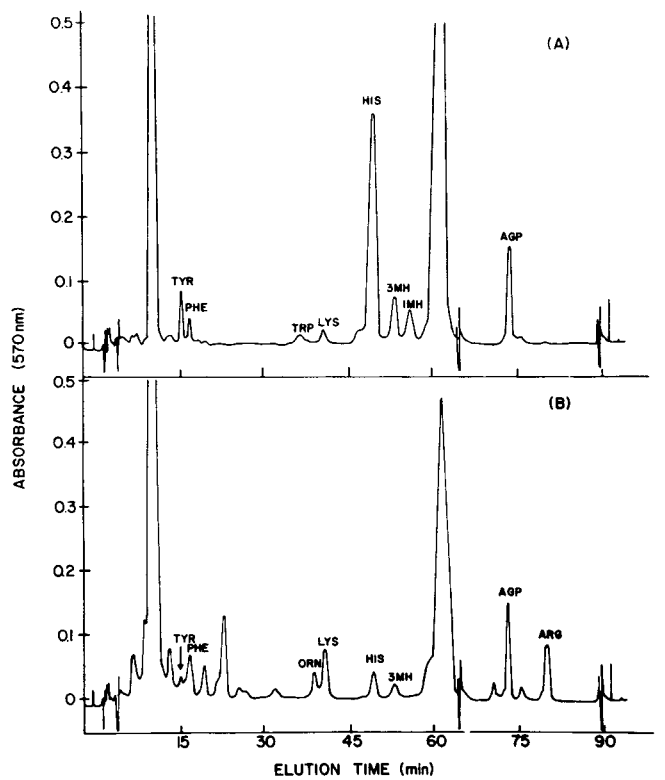

Fig. 3. Typical amino acid analysis of (A) human and (B) rat urine. Ultrafiltrates of urine were prepared and analyzed as described in Experimental. Rat urine but not human urine was hydrolyzed for 24 h prior to analysis. For peak identification see Fig. 1; Orn = ornithine.

TABLE I

EFFECT OF HYDROLYSIS ON DETERMINATION OF 1-MH AND 3-MH

Urine	*n*	3-MH (nmol per 20 μl ± S.E.M.		1-MH (nmol per 20 μl ± S.E.M.	
		No hydrolysis	24-h Hydrolysis	No hydrolysis	24-h Hydrolysis
Human	9	5.00 ± 0.42	5.11 ± 0.45	1.21 ± 0.21	1.07 ± 0.23
Rat	4	0.78 ± 0.13	5.02 ± 0.31	1.36 ± 0.34	9.42 ± 0.81

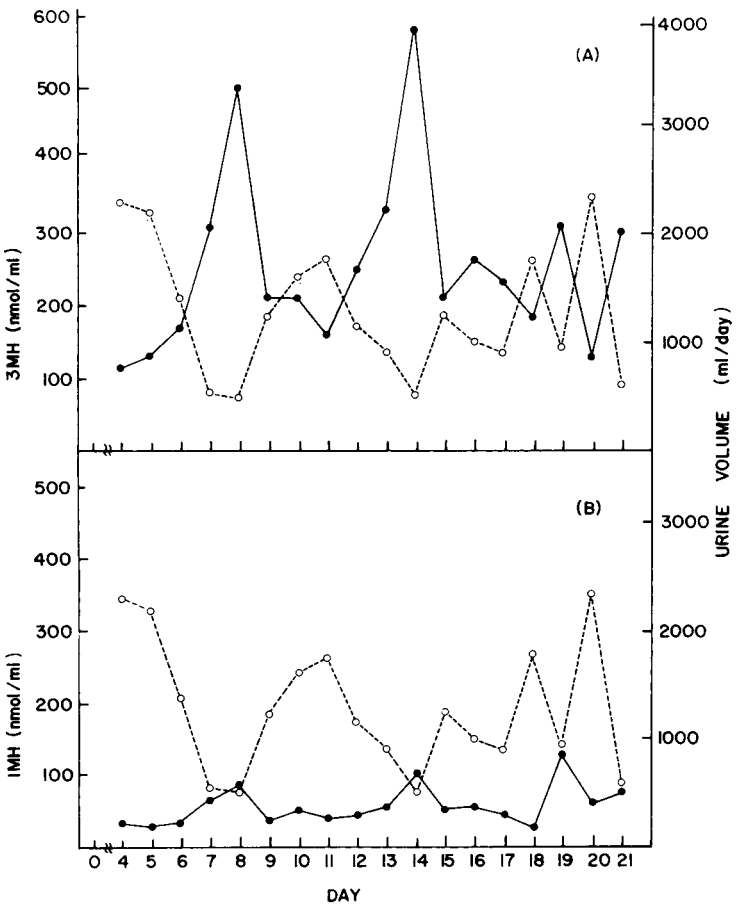

Fig. 4. Daily variations in urine volume (○) and concentration (•) of (A) 3-MH and (B) 1-MH. Urine from an adult human male on a meat-free diet was analyzed as shown in Figs. 1 and 3.

excretion was obtained by multiplying urine volume (ml/day) × MH concentration (nmol/ml). For this 91-kg individual 3-MH and 1-MH excretion averaged 278 ± 9.1 and 66 ± 6.2 μmol/day, respectively. Alternatively, the results for 3-MH and 1-MH can be expressed per kg body mass as 3.06 ± 0.10 and 0.72 ± 0.07 μmol/kg/day, respectively.

The procedure was also utilized for the experiments shown in Fig. 5; the data depict alterations in 3-MH excretion resulting from disuse atrophy of rat hindlimb muscles. Disuse was induced by a whole body suspension technique

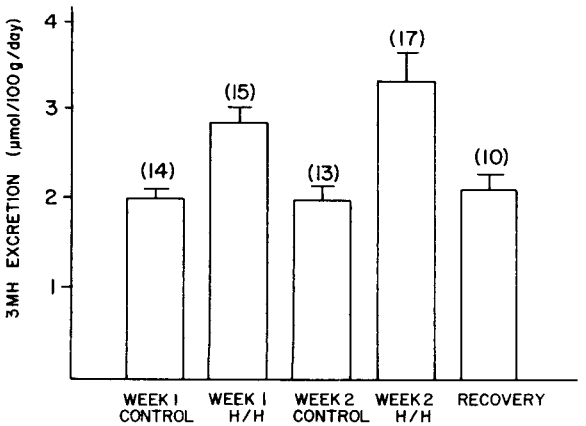

Fig. 5. Effect of hindlimb muscle disuse and recovery on the average daily urinary excretion of 3-MH in the rat. Hindlimb disuse was induced by a hypokinetic/hypodynamic (H/H) suspension as previously described by Musacchia et al. [16]. Some rats were removed from the suspension apparatus after one week and maintained in metabolism cages to study recovery. Ultrafiltrates of urines were hydrolyzed and analyzed as described in Figs. 1—3. Number of rats are indicated in parentheses. The error bars indicate 1 S.E.M.

previously demonstrated to produce hindlimb muscle atrophy through both hypokinesia (reduced limb movement) and hypodynamia (decreased mechanical loading) [16]. Control animals, housed in metabolism cages for up to two weeks, maintained a uniform rate of 3-MH excretion which approximated 2 μmol per 100 g per day. Hindlimb disuse for one and two weeks significantly ($P<0.001$) elevated urinary 3-MH excretion by 40—60%, consistent with the increased protein turnover associated with muscle atrophy. One week of recovery, following removal of rats from the suspension apparatus and return to metabolism cages, was associated with a decrease in 3-MH excretion to values not significantly different from those observed in controls. These results demonstrate that the present technique can be utilized to assess metabolic alterations at the tissue level.

DISCUSSION

This paper describes efforts to develop a rapid, quantitative procedure for the simultaneous analysis of 1-MH and 3-MH in rat and human urine. These metabolites are derived from the catabolism of methylated peptides and proteins [17]. Although there is some controversy [18, 19] it appears that urinary levels of 3-MH can reflect muscle protein turnover when mammals are maintained on a diet lacking in 3-MH for at least four days [5—7].

A variety of methods have been employed for the quantitation of 3-MH in physiological specimens [9—11]. A protein-free sample is usually prepared prior to the actual analysis. Blanchard [20] has reviewed some of the common techniques for sample deproteinization. We have utilized a centrifugal ultrafiltration deproteinization procedure for the preparation of urine samples for methylhistidine analysis. In a single step, each 1-ml sample of urine is adjusted to pH 2—3, mixed with an internal standard and made protein-free. A dozen

human urine samples can be prepared for automated amino acid analysis in 0.5 h.

The procedure also allows for the quantitation of phenylalanine, tyrosine, tryptophan, lysine, histidine, arginine, ornithine, hydroxylysine, anserine and carnosine. Since alterations in the urinary levels of several of these amino acids have been correlated with various disease states this procedure could be helpful in diagnosis and monitoring [1].

Measurements of 3-MH in rat urine are complicated by the increasing amount of acetylation which occurs during development [5]. We found that a minimum of 2 h of hydrolysis in 6 *M* hydrochloric acid at 110°C was required, although recovery was quantitative for all time points up to 48 h. Ammonia does not interfere with the determination of 1-MH by our method although it does in other procedures [17, 21].

Presently, the automated determination of methylhistidines and other amino acids in physiological specimens is accomplished by GC—MS, HPLC or ion-exchange chromatography. GC—MS techniques require sample derivatization prior to analysis and relatively expensive, specialized equipment [10, 22]. Most automated ion-exchange chromatographic procedures for 3-MH determination require 4—6 h per analysis and only provide information on one of the methylhistidines [9, 12, 17, 23]. HPLC methods are often rapid with high sensitivity. Samples can be quantitated by ultraviolet or fluorescent detection following pre-column or post-column derivatization.

However, none of the published methods allow for the simultaneous determination of 1-MH and 3-MH from rat or human urine [11, 24, 25]. Additionally, the resolution of acetylated 1-MH and 3-MH has not been demonstrated. Our procedure, based on high-sensitivity single-column amino acid analysis coupled with deproteinization by centrifugal ultrafiltration, allows for the rapid quantification of 1-MH, 3-MH, basic and aromatic amino acids in human urine. The data (Fig. 4) agree with other reported values [21, 24, 26, 27]. As noted previously, determination of methylhistidine levels in rat urine usually requires hydrolysis. The daily excretion of 3-MH reported here is similar to urine concentrations determined by ion-exchange chromatography based on 4-h analyses [13, 14]. Our procedure may be useful for assessing alterations in muscle metabolism as a consequence of cancer, other muscle wasting diseases and therapy utilizing total parenteral nutrition.

ACKNOWLEDGEMENTS

This research was supported in part by grants from the University of Louisville (RCF), the KY Affiliate of the American Heart Association (RCF), NIH: GM29434 (RCF) and NASA: NSG 2325 (XJM and JMS). The technical assistance of Mr. Benjamin Van Osdol with the amino acid analyses and Mr. Leroy Heron with photography is greatly appreciated. We are also grateful to Ms. Ellen Ford for skillful secretarial assistance and to Drs. Mary Hilton, Pamela Feldhoff, and Richard Ward for their review of this manuscript.

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Journal of Chromatography, 311 (1984) 277—289
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2265

LIQUID—LIQUID EXTRACTION OF MEMBRANES FROM CALF BRAIN USING CONVENTIONAL AND CENTRIFUGAL COUNTER-CURRENT DISTRIBUTION TECHNIQUES

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(First received April 17th, 1984; revised manuscript received June 26th, 1984)

SUMMARY

Neural membranes isolated from calf brain have been partitioned in aqueous two-phase systems containing dextran and polyethyleneglycol. When the partition was repeated several times, using counter-current distribution technique, the distribution of the membranes between the upper phase and the interface changed in a non-ideal manner and in favour of the interface. By using a centrifugal counter-current distribution device the time for the experiment could be reduced by a factor of 7—8 and the distribution was similar to what could be expected for ideally behaving membranes. The time-dependent change of the membranes is discussed in terms of aggregation and lateral membrane perturbations. Despite this effect a certain fractionation has been achieved as deduced from analysis of cholesterol content, opiate receptor activity and acetylcholinesterase activity along the counter-current distribution row of fractions. Compared to the starting material these activities were enriched some two-fold in certain fractions.

INTRODUCTION

In order to understand the structure and function of a particular membrane it is important to isolate that individual membrane in a pure and native state. This is particularly important for membranes of the highly organized brain tissue. Homogenization of such tissue inevitably gives an extremely complex mixture of membranes. It is therefore desirable that several fractionation methods, working according to different separation principles, are used in sequence. The most common method used so far is some type of centrifugation. Another method of great potential value is partition of the material between the aqueous liquid phases (and interface between them) of aqueous dextran—polyethyleneglycol two-phase systems. This method complements

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centrifugation in that it separates material according to differences in surface properties rather than size and density. The properties and preparation of aqueous two-phase systems have been summarized elsewhere [1—3].

Of the great variety of membranes studied in this way the thylakoid membranes in chloroplasts constitute a considerable part [2, 3], but cholinergic membranes from electroplax of *Torpedo californica* [4—6] and *Torpedo marmorata* [7] have also been purified and examined. Typical for the partition of membranes is that they not only distribute between the two phases but they can also accumulate at the interface between the phases [1, 2]. The fractionation of heterogeneous material can be drastically influenced by addition of various salts [2, 8], charged polymers [7, 9, 10], or polymer-bound groups which bind to the membranes [4, 6]. Especially promising is the technique where specific high-affinity ligands have been bound to polyethyleneglycol [4—6, 11, 12]. To improve the purification obtained in a single partition step, counter-current distribution (CCD) is often used. This is carried out by successively shifting the position of the upper phases of a row of two-phase systems, where the sample has been introduced in the first system of the row. The phase volumes are chosen to maintain the interface, and the material concentrated at it, stationary.

Using the CCD technique it should be possible to resolve membrane preparations that differ in their chemical composition and biochemical activities into various fractions. Even if systems with a rather high degree of specificity are designed, multi-step partition may be necessary to resolve membranes with small variations in composition, e.g. two kinds of receptors bound to otherwise identical membrane fragments. In such cases CCD would offer two interesting features: the quantitative composition of a membrane mixture can be established and at the same time several fractions of increased purity may be isolated. A possible drawback is, however, that the membrane structure may be influenced by the two-phase system, causing, for example, loss of biological activity, formation of large membrane aggregates, or gradual change of the partition between the phases. The fluidity of membranes and the well known fact that polyethyleneglycol causes capping of membrane proteins and induces fusion of cells make it necessary to investigate the stability of membrane structures in aqueous two-phase systems.

In the present work the heterogeneity of a crude preparation of synaptic membranes from brain has been studied by using the CCD technique. The influence of the two-phase systems on the status of the membranes has been analysed by using CCD with different numbers of transfers and by comparing two types of CCD apparatus: the conventional thin-layer CCD device, constructed by Albertsson [13], and a new construction based on centrifugation which allows the CCD process to be run in a considerably shorter time [14]. Short (nine steps) CCD has also been performed by manual transfer of the upper phases along a row of test-tubes.

MATERIALS AND METHODS

Materials

Polyethyleneglycol (PEG), M_r = 3500—4500, was purchased from Union

Carbide (New York, NY, U.S.A.). Dextran T500 (M_r = 500,000) was from Pharmacia (Uppsala, Sweden). Hexaethonium—polyethyleneglycol (HE—PEG) and bis(triethylaminoethyl)resorcinol-NH-azelate polyethyleneglycol (BTR—PEG) were prepared as described by Johansson et al. [6]. The biochemicals and substrates used for enzyme analysis were from Sigma (St. Louis, MO, U.S.A.), except for the set for cholesterol determination, which was obtained from Boehringer (Mannheim, F.R.G.). Tritiated etorphine was purchased from Amersham Radiochemical Centre. Dextrophan and levorphanol were kind gifts from Dr. M. Kanje, Department of Zoophysiology, University of Lund (Lund, Sweden). All salt and buffer substances were of analytical grade.

Membrane preparation

Synaptic membranes from calf brain cortex were prepared according to the method of Hajós [15] with slight modifications. The synaptosomes were lysed in ice-cold distilled water for 1 h and collected by centrifugation at 45,000 g_{max} for 1 h. The resulting pellet was suspended in water and passed twice through a Yeda press before it was used for partition or CCD.

Two-phase systems

The two-phase systems were made from 40% (w/w) PEG and 20% (w/w) dextran stock solutions. The concentration of polymers in the systems were 5.3% (w/w) dextran and 5.3% (w/w) PEG. All systems contained 5 m*M* potassium phosphate buffer, pH 7.4, and various concentrations of potassium chloride. Detailed descriptions of the preparation of aqueous two-phase systems can be found elsewhere [1, 16].

Counter-current distribution

The two types of CCD apparatus used were the thin-layer machine constructed by Albertsson [1, 13] and the centrifugal machine invented by Åkerlund [14]. Both were built by the Chemical Center Workshop at the University of Lund. The volumes of the two phases in each chamber, settling (or centrifugation) time and shaking time are given in the figure legends. Each set of discs contained 60 chambers (see Fig. 1). The samples were included in the systems of chamber 0-2 (26 transfers) or 0-3 (55 transfers). After the run the systems were transformed to one phase by addition of 0.9 ml of ice-cold water. The obtained fractions (diluted 30—50 times) were analysed with respect to apparent absorbance at 400 nm, caused by light scattering, using a Hitachi 100-60 spectrophotometer with 1-cm cuvettes. Pooled fractions were concentrated by centrifugation 60 min at 45,000 *g* and resuspended in a small volume of 50 m*M* Tris—HCl buffer, pH 6.4 (1—2 ml). These fractions were analysed for acetylcholinesterase [17], cholesterol [18], phosphate [19], and stereospecific opiate binding [20]. Protein was determined according to the method of Bradford [21] after treatment of the membranes for 3 h with 0.5 *M* phosphoric acid at 50°C.

Manual CCD was performed by using 8-g systems in test-tubes as described elsewhere [16]. After each transfer (performed with a Pasteur pipette), leaving all material at the interface with the lower phase, the tubes were mixed by

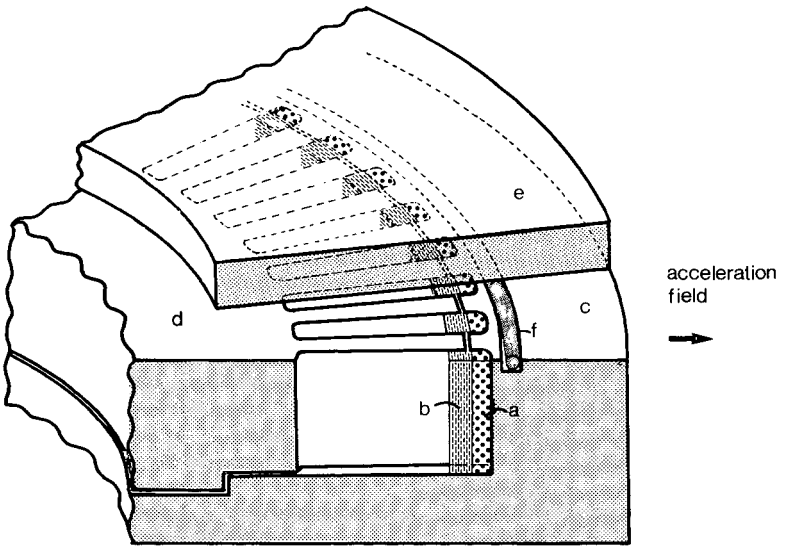


Fig. 1. Section of the separation unit for the centrifugal CCD apparatus. It is composed of four units: c = the outer ring with cavities for the lower phase; d = the inner ring with cavities for the upper phase; e = the lid ring; f = an O-ring for sealing. The position of the two-phase system during centrifugation (b = upper phase; a = lower phase) is shown in the sectioned chamber.

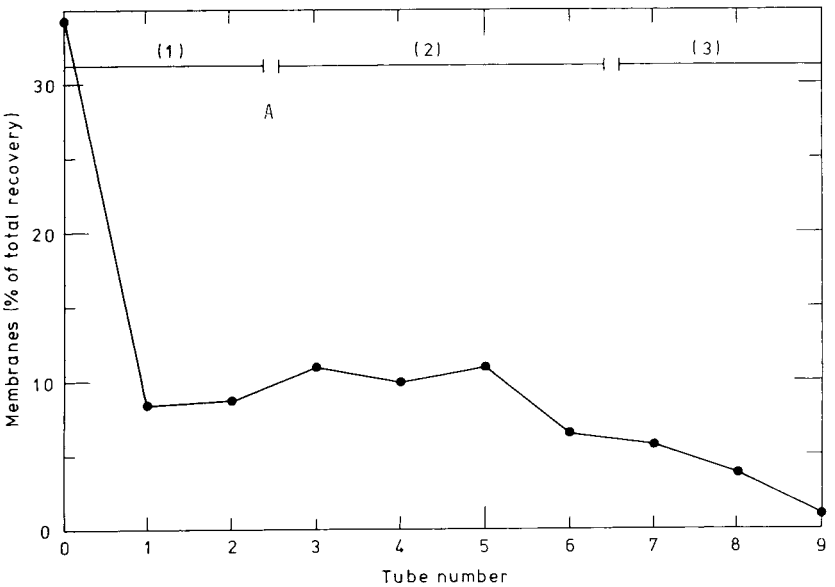
twenty inversions and centrifuged for 5 min at 800 *g*. After nine transfers 4 ml of water were added to break the two-phase systems.

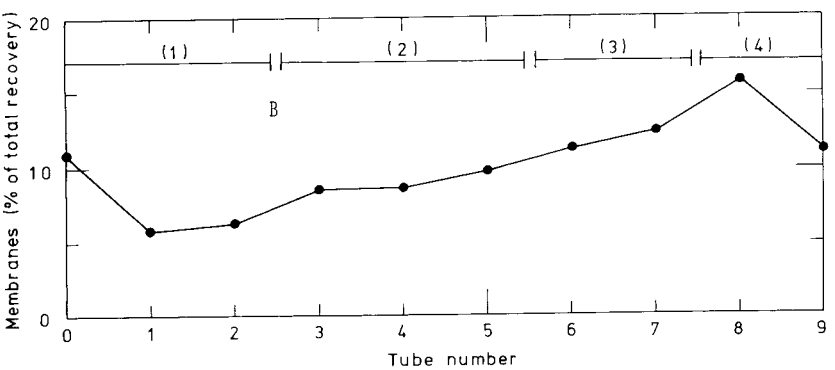
RESULTS

Conventional CCD

Manual CCD with nine transfers (Fig. 2A and B) showed that synaptic membranes partly moved along with the upper phase when the system did not contain any ligand—PEG but only the standard salts, 10 m*M* potassium chloride and 5 m*M* potassium phosphate buffer, pH 7.4. In this experiment (Fig. 2A) the membranes were not pre-extracted and a large fraction remained at the start position, tube No. 0. The membranes in this tube had a marked yellow tint in contrast to the moving material. When HE—PEG was included in the system (Fig. 2B), the material was spread out and more membranes were found in the tubes to the far right. Hexaethonium is a ligand for nicotinic cholinergic receptors [6].

The distribution of membranes in a conventional CCD apparatus is shown in Fig. 2C—E. With 55 transfers and no ligand—PEG the material was found in the left part of the CCD train (Fig. 2C) with a clear tendency to split into two peaks. A corresponding CCD with a HE—PEG-containing system (Fig. 2D) gave rise to a heterogeneous peak which had travelled more to the right. By using HE—PEG but reducing the number of transfers to 26 the peak had travelled relatively more to the right (R_f = 0.57) than with 55 transfers (R_f = 0.41). R_f is defined as the peak position divided by the number of transfers. This relative mobility should be independent of the number of equilibration steps if an ideally behaving substance is partitioned [22].





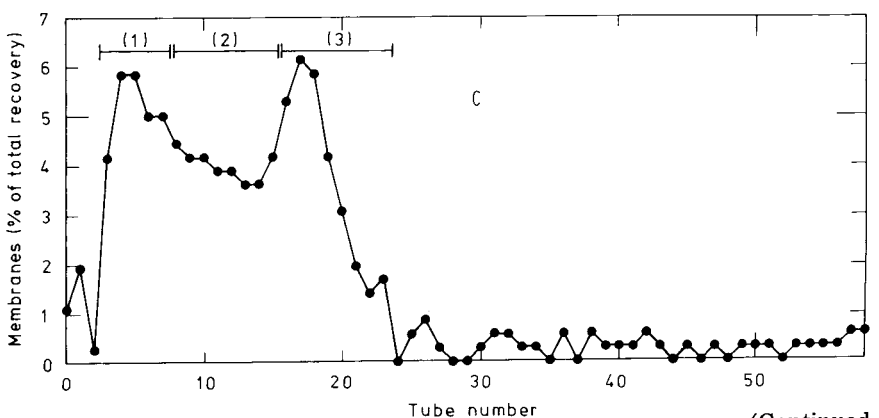


Fig. 2.

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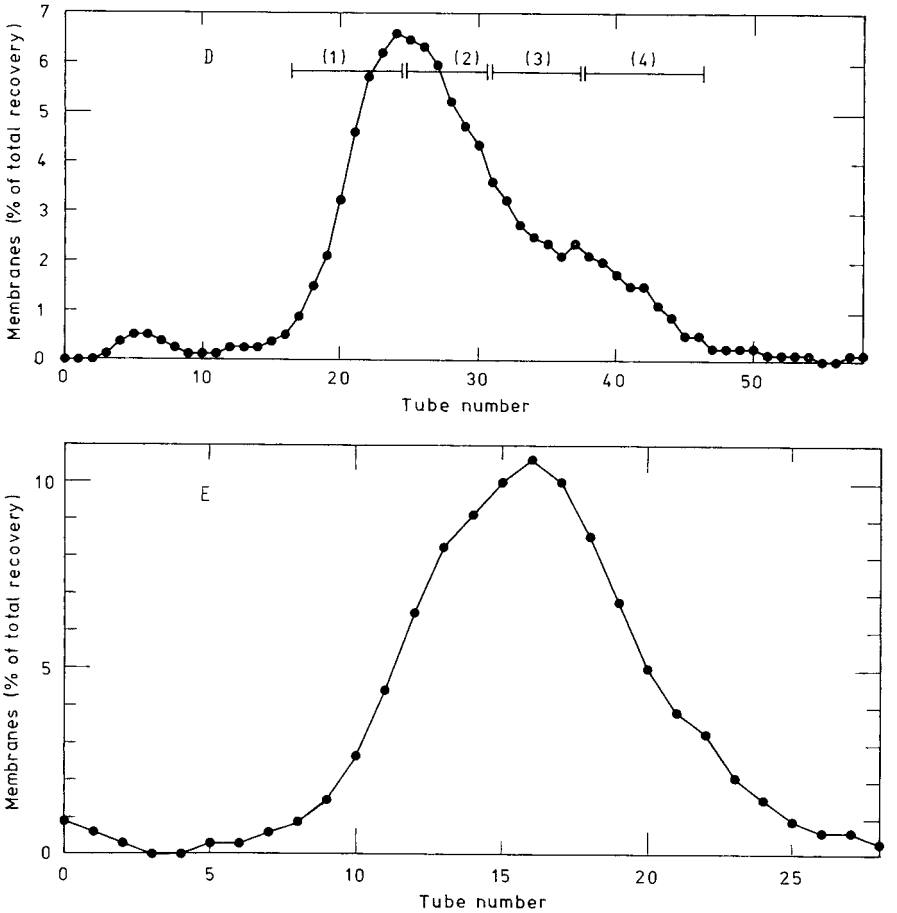

Fig. 2. Conventional CCD, carried out either manually, A and B, or with use of a thin-layer CCD apparatus, C—E. The two-phase systems contained 5.30% (w/w) dextran, 5.30% (w/w) PEG (including ligand—PEG), 5 m*M* potassium phosphate buffer, pH 7.4, and 10 m*M* potassium chloride. Temperature 3°C. The membranes, except in A, were pre-extracted in a system with the same composition as above but with only 5 m*M* potassium chloride, and the upper phase was used together with a pure lower phase as sample system after adjustment of the concentration of potassium chloride. (A) No ligand—PEG, nine transfers; (B) HE—PEG (1% of total PEG), nine transfers; (C) no ligand—PEG, 55 transfers; (D) HE—PEG (1% of total PEG), 55 transfers; and (E) HE—PEG (1% of total PEG), 26 transfers. Mixing time 30 sec and separation time 5 min (manual CCD), or 15 min (thin-layer CCD); 5.6 or 0.7 ml of upper phase (of which 0.35 or 0.19 ml was stationary) and 4.2 or 0.6 ml of lower phase were used for manual and thin-layer CCD, respectively. Pooled fractions are indicated with bars. The ordinate shows percentage in each tube of totally recovered membranes.

Because of the slow settling of the two phases, each transfer cycle required 15 min, corresponding to four transfers per hour. When the number of transfers was increased, the mobility of the main peak decreased (Fig. 2 and Table I).

Centrifugal CCD

By applying an acceleration field, the settling time for the phases can be speeded up. The functional unit (Fig. 1), like in the case of the conventional

TABLE I

THE RELATIVE MOBILITY (R_F) OF THE MAIN PEAKS AFTER COUNTER-CURRENT DISTRIBUTION OF SYNAPTIC MEMBRANES USING VARIOUS TIMES, NUMBER OF TRANSFERS, AND PEG-BOUND LIGANDS

The data have been calculated from the experiments in Figs. 2 and 3.

Composition of the two-phase system	Type of CCD	Time (min)	Number of transfers	Peak position (tube number)	R_F
Without ligand	Manual	110	9	4	0.44
	Centrifugal	110	55	24	0.42
				6	0.08
	Thin-layer	842	55	17	0.28
				5	0.06
With HE—PEG (1% of total PEG)	Manual	110	9	8	0.89
	Centrifugal	110	55	46	0.81
	Thin-layer	413	26	16	0.57
	Thin-layer	842	55	24	0.41
With BTR—PEG	Centrifugal	110	55	18	0.30
	Centrifugal	220	110	21	0.18

thin-layer CCD apparatus, comprises two discs of Plexiglass. The centrifugal machine, however, allows the disc system to rotate at relatively high speed. The mixed two-phase systems in the discs will therefore be centrifuged which results in a reduced settling time. The cycling time was 2 min, allowing 30 transfers per hour. Compared with the conventional apparatus the centrifugal CCD is seven to eight times faster. The apparatus has been described in detail elsewhere [14].

The results obtained with the synaptic membrane preparation are shown in Fig. 3, and the relative mobility of the main peak is shown in Table I. Comparison of the results obtained with the two machines shows clearly that the mobility changes with time and that the centrifugal CCD technique makes it possible to run CCD with a large number of transfers ($\simeq$55) without large changes in the partition behaviour. With an even higher number of transfers (Fig. 3D) the retarding phenomenon was also seen with this apparatus. The longer run was performed by continuing the transfers to cover almost two full turns of the circular mobile unit carrying the upper phases. The last 55 transfers seem to have added very little to the mobility of the peak, which means that the material was partitioned exclusively at the interface and bottom phase after 50—60 transfers.

Use of ligand—PEG

The partition of the membranes was found to depend on both the concentration of polymers and the salt composition. The system used here, without any ligand attached to PEG, was chosen so that a large part of the membranes was at the interface or in the lower phase. Adjustment of the partition could be done, from 85% of the membranes in the upper phase to less than 10%, by changing the concentration of potassium chloride from 0 to 20 m*M* (Fig. 4).

The introduction of PEG-bound cholinergic ligands, enriched in the upper phase, changed the partition of the membranes in favour of this phase. This caused a change in the CCD pattern, where part of the material was distributed further to the right (Figs. 2 and 3).

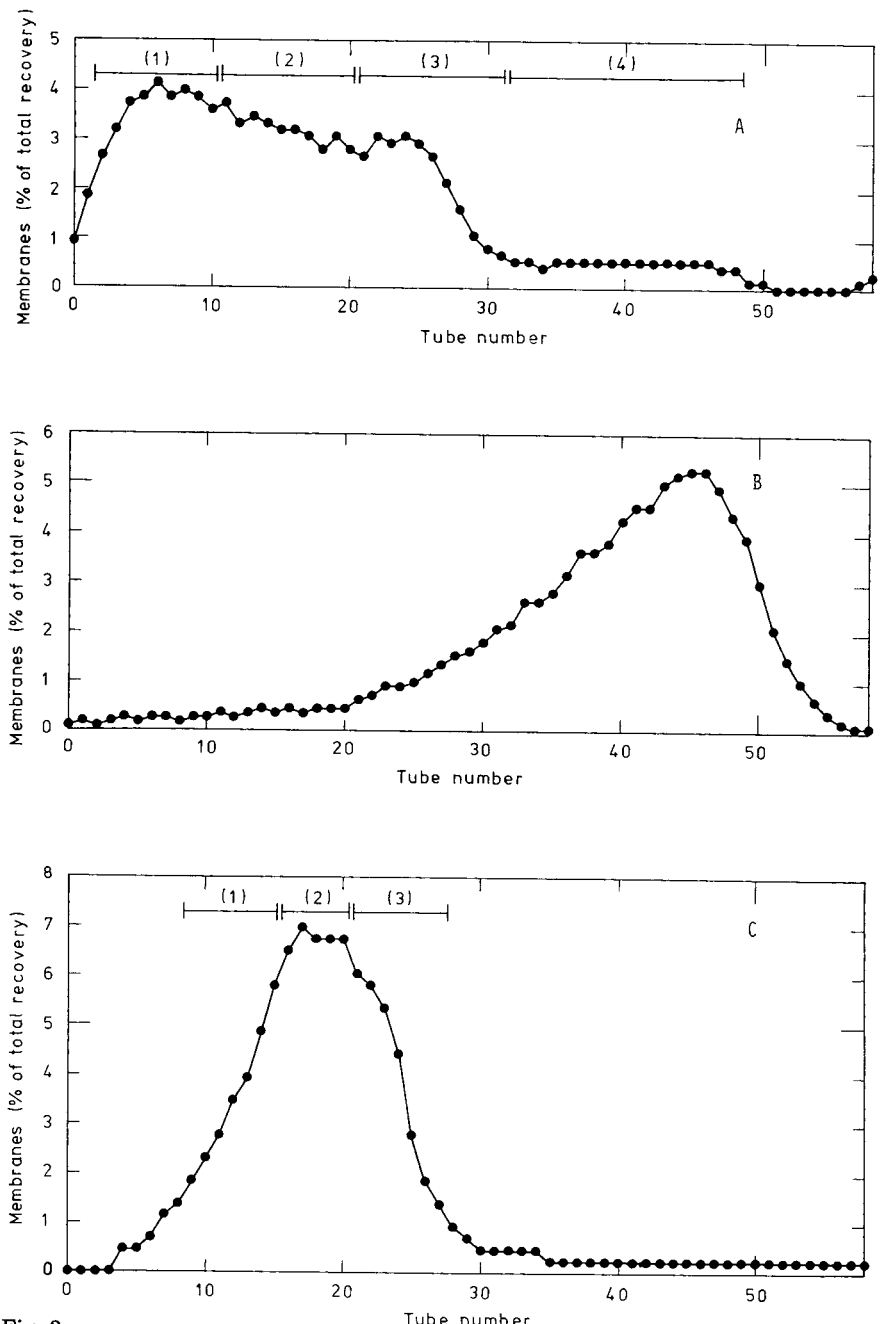

Fig. 3.

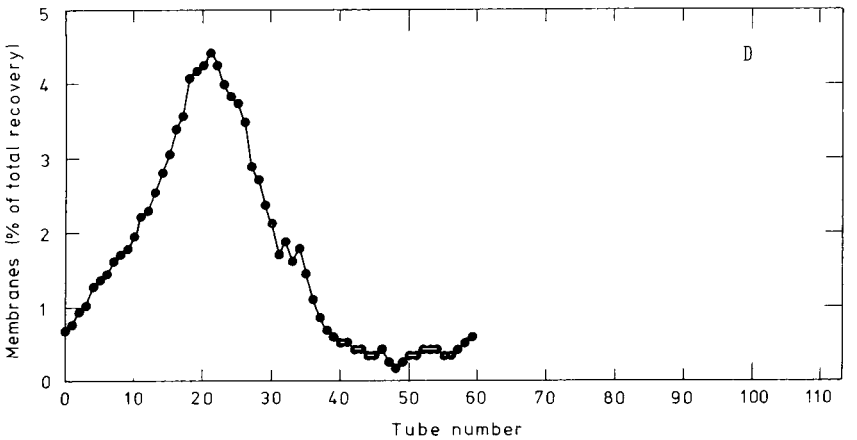

Fig. 3. Centrifugal CCD with the same two-phase system as in Fig. 2. (A) No ligand—PEG, 55 transfers; (B) HE—PEG (1% of total PEG), 55 transfers; (C) BTR—PEG (3% of total PEG), 55 transfers; and (D) BTR—PEG (3% of total PEG), 110 transfers by using two turns with the 60-chamber unit. Mixing time 30 sec and separation time 72 sec; 0.97 ml of upper phase (0.18 ml stationary) and 0.78 ml lower phase.

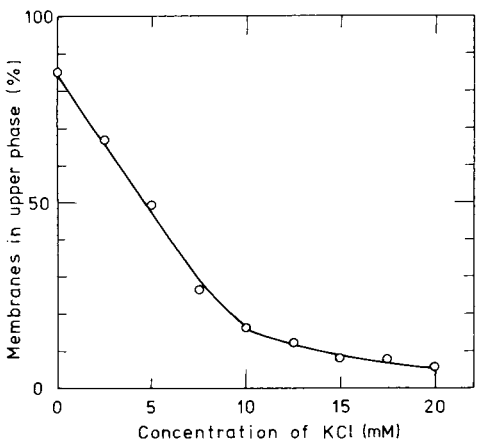

Fig. 4. Effect of potassium chloride on the partition of synaptic membranes (measured as protein content) in the same system as in Fig. 2, except for the concentration of potassium chloride. The membranes corresponded to 0.18 mg of protein per ml of phase system.

Single partition

The partition of membranes within the system was measured as a function of time (Fig. 5). Differences in partition behaviour were found when the system was continuously and gently mixed compared with systems mixed only before sampling. Over a longer period of time 7—20% of the material in the upper phase was decreased by 7—16% units. The decrease in partition, without ligand—PEG, was excluded from this phase. The opposite was true for a system containing HE—PEG where mixing caused decreasing affinity of the material for the upper phase.

TABLE II

PROPERTIES OF THE POOLED FRACTIONS FROM THE CCD EXPERIMENTS IN FIGS. 2 AND 3

The fraction numbers given for the pooled fractions are marked in the corresponding figures.

Figure No.	Ligand—PEG	Fraction	Light scattering (percentage of total)	Protein (percentage of total)	Membrane constituents and activities relative to protein (percentage of total/percentage of total protein)			
					Acetyl-cholin-esterase	Stereo-specific opiate binding	Cholesterol	Phosphate
2A	—	1	51	58	0.5	0.4	—*	—
		2	38	34	1.6	2.0	—	—
		3	11	8	2.1	0.8	—	—
2B	HE—PEG	1	23	21	0.3	0.06	—	—
		2	27	26	0.7	0.9	—	—
		3	23	24	1.3	1.5	—	—
		4	27	29	1.6	1.3	—	—
2C	—	1	26	21	1.0	0.8	—	1.2
		2	32	36	0.9	0.9	—	1.2
		3	22	39	1.1	1.3	—	0.8
2D	HE—PEG	1	33	32	0.8	0.6	0.8	0.9
		2	35	40	0.9	1.2	0.9	0.9
		3	20	20	1.2	1.0	1.4	1.2
		4	12	9	1.4	1.6	1.2	1.3
3A	—	1	33	33	0.7	0.5	—	0.9
		2	37	38	0.9	1.2	—	1.0
		3	26	27	1.4	1.3	—	1.1
		4	4	2	1.8	1.1	—	1.7
3C	BTR—PEG	1	30	18	0.9	0.07	0.6	—
		2	37	40	0.9	1.1	1.1	—
		3	30	39	1.2	1.4	1.2	—

*— = not determined.

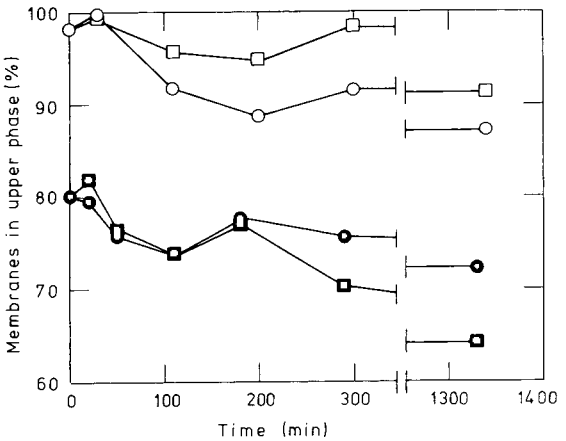

Fig. 5. Incubation of synaptic membranes in two-phase systems. Composition of systems as in Fig. 2 but with 5 m*M* potassium chloride. The membranes were pre-extracted in a system of the same composition using only material in the upper phase. The incubation was carried out at 3°C using resting (□, ▪) and continuously mixed (○, ●) systems, either without ligand—PEG (filled symbols) or with HE—PEG, 1% of total PEG (open symbols). The concentration of membranes was measured as the light scattering at 400 nm. Total concentration of membranes corresponded to 0.25 mg of protein per ml.

DISCUSSION

Preparations of synaptic membranes obtained by centrifugation are not homogeneous. Both the basic composition and the size of the membrane fragments from the synaptic regions may vary considerably. Also outer membranes from non-synaptic regions, as well as membranes from other cells (glial cells) and cell organelles, are present to various degrees. The countercurrent distribution is an attractive way to purify synaptic membranes further. By using specific ligands bound to PEG it had earlier been shown that a high selectivity can be achieved in the extraction of synaptic membranes from electroplax [6]. The ligand, concentrated in the upper PEG-rich phase, causes an increased affinity of membranes with ligand-binding sites for this phase. In the case of brain tissue the receptor densities are much lower, and the preparations of synaptic membranes are much more complex.

The results of CCD of the crude synaptic membrane preparation from calf brain (Table II) show that even if the material could not be split up into discrete fractions (or peaks), the composition along the CCD train was not constant but changed gradually. Membranes enriched in certain activities may therefore be isolated by liquid—liquid extraction using these systems. A general trend was that opiate binding, acetylcholinesterase (marker for synaptic membranes), phosphate (mainly from phospholipids) and cholesterol were all enriched in the material distributed to the right. This shows that membranes from the nerve endings have a higher affinity for the upper phase than the bulk material. While a positive ligand effect was seen when the PEG-bound hexaethonium was used, the CCD experiments did not follow the quantitative rules for this kind of distribution. Another cholinergic ligand, BTR, did not show the expected effect when bound to PEG but decreased the mobility of the material.

The mobility was generally reduced when the time for the experiment was longer. This time-dependent effect influenced in a negative way the possibilities of obtaining a high resolution by using a large number of steps in CCD. Time alone was, however, not sufficient to cause the increased affinity of the membranes for the interface relative to the upper phase, as was shown by incubating the membranes for longer times in the two-phase systems. A possible explanation for the change in partition is that the membrane status changes when fragments of different kinds begin to separate from each other during the CCD run. A partial purification may facilitate membrane aggregation as well as lateral changes within the membranes. An increased affinity for the interface can be expected if some of the membrane components, e.g. certain proteins, are collected in a small region of the membrane fragment (capping). The fragment can be pictured as composed of two parts, one with affinity for the upper phase and one for the lower phase. Such a particle will have a strong affinity for the interface where it can orientate its respective parts towards the two phases. Capping of membrane proteins can be induced by PEG which leads to fusion of cells when these are treated with the polymer [23]. However, the concentration of PEG required for cell fusion [24] is normally much higher than that necessary to obtain two-phase systems with dextran. The changes of the partition may also be due to formation of (small) aggregates of the fragments. It is a general tendency that large particles have higher affinity for the interface than small ones. Aggregation may also reduce the exposed parts of the membranes responsible for favourable interaction with PEG.

The possibility to decrease the time for phase settling by centrifugation, thereby allowing larger numbers of transfers per time unit, works well and no tendency of the membranes to sediment at the relatively low centrifugal force (100 *g*) has been observed.

ACKNOWLEDGEMENTS

We wish to thank Miss Monica Andersson and Miss Sophie Bingsmark for skilful technical assistance. This work has been supported by grants from the NFR — Swedish Natural Science Research Council.

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Journal of Chromatography, 311 (1984) 291—299
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2263

SIMULTANEOUS DETERMINATION OF LIDOCAINE AND ITS METABOLITES IN PLASMA AND MYOCARDIUM

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(First received March 27th, 1984; revised manuscript received July 19th, 1984)

SUMMARY

No validated method exists for measuring lidocaine and its metabolites in myocardial tissue. We modified a previously described high-performance liquid chromatographic assay and applied it to plasma and to homogenized myocardial samples obtained from dogs that had received lidocaine by a double-infusion technique. Recovery of lidocaine, monoethylglycylxylidide and glycylxylidide after homogenization and extraction is reported. Assay variability, sensitivity and linearity over a wide range of sample sizes are also described. The results obtained with high-performance liquid chromatographic analysis are compared to quantitation of ^{14}C-labeled lidocaine plus metabolites measured by an oxidation—scintillation technique. Myocardium to plasma partition coefficients for lidocaine, monoethylglycylxylidide and glycylxylidide were 2.16, 4.27, and 2.91, respectively.

INTRODUCTION

Lidocaine, a widely used local anesthetic and antiarrhythmic agent, is deethylated in the liver to monoethylglycylxylidide (MEGX) and glycylxylidide (GX) [1, 2]. Extensive data already exist regarding plasma concentration of lidocaine and its relationship to clinical effects. These data call attention to lidocaine's narrow toxic-to-therapeutic ratio, which often requires plasma concentration monitoring in patients treated with the drug. Even when one ensures that plasma concentrations of lidocaine fall within the usual therapeutic range toxic reactions still have been reported to occur [3—5]. It is possible that alterations in lidocaine metabolism or metabolite excretion may contribute to variability in the toxic-to-therapeutic ratio. Specifically, MEGX and GX have both been proposed to contribute to the antiarrhythmic and toxic effects of the parent compound [6—9]. Until recently, however, it has not been possible

0378-4347/84/$03.00

to measure these metabolites and, as such, the nature of their contributions has not been rigorously established. The effectiveness of the intravenously administered compound and the rapidity with which lidocaine is de-ethylated after administration, provide incentives for making such determinations.

Although assays of plasma MEGX and GX are now available [10—12] little work has been done to clarify the concentration—response relationships of these compounds and how their effects may relate to myocardial levels. As yet no method has been described for simultaneously measuring lidocaine, MEGX and GX in tissue. Previous efforts have sought only to measure tissue lidocaine concentration [13—16]. The marked variability in myocardium-to-plasma lidocaine concentration ratios reported in these studies suggests the possibility of inadequate assay methodology. A better understanding of tissue uptake of this drug and its metabolites may also help explain variations in lidocaine's actions.

A previous report from our laboratory describes lidocaine and metabolite levels in canine plasma and myocardium [17] using a modification of the plasma assay described by Nation et al. [11]. In the present paper we describe in detail our methodology, which permits accurate simultaneous quantification of lidocaine, MEGX and GX in canine myocardium via homogenization followed by extraction and subsequent high-performance liquid chromatographic (HPLC) analysis.

EXPERIMENTAL

Surgical procedure and drug infusion

Four mongrel dogs of either sex weighing 10—15 kg were anesthetized with sodium pentobarbital, 30 mg/kg intravenously. An additional 60-mg dose was given just prior to sacrifice. The dogs were intubated and ventilated at 20 cycles per min with a tidal volume of 13 ml room air per kg body weight plus dead space using a Harvard Apparatus Model 607 respirator. The right femoral artery and vein were exposed and catheterized. Blood samples for lidocaine plasma concentration determinations were obtained via the arterial catheter just before sacrifice and centrifuged at 1000 *g* for 15 min. Plasma was subsequently pipetted into screw cap tubes and stored at −10° C.

Using a Harvard Apparatus Model 906 infusion pump, lidocaine was administered intravenously by a two-stage procedure at 0.4 mg/kg/min for the first 5 min, and at 0.08 mg/kg/min for the remainder of the 90-min study. ^{14}C-Radiolabeled lidocaine (>97% pure, labeled at the carbonyl carbon, New England Nuclear) was infused along with the cold drug at a ratio of 40 μCi per 150 mg cold lidocaine.

A left thoracotomy was performed and the heart exposed 15 min prior to the study's conclusion. Sacifice of the dog was carried out at 90 min by excision of the heart, which in turn was blotted and stored at −10° C until time for assay.

Preparation of tissue samples

Triplicate transmural samples of frozen left ventricular (LV) tissue from each

dog (n = 4) were isolated, weighed (0.9–1.4 g) on a Mettler H51 balance and placed into ice cold siliconized test tubes each containing 2 ml of distilled deionized water.

Homogenization was carried out using a Brinkman Instruments ST10 polytron. Of each homogenate 0.5 ml was dispensed into a 15-ml siliconized tube and covered with a PTFE-lined cap (American Scientific Products) for extraction.

Drug-free tissue samples for standard curve generation were treated in the same fashion as the above except that the distilled deionized water used was supplemented with known amounts of lidocaine, MEGX and GX.

HPLC system

Reagents. All reagents were of HPLC grade unless otherwise specified. Lidocaine, ethylmethylglycylxylidide (the internal standard), MEGX and GX were supplied by Astra Pharmaceuticals.

Extraction. The extraction procedure used here is similar to those described previously for extraction of lidocaine, MEGX and GX from plasma [11, 17]. To each of the 0.5-ml tissue homogenates or to 0.5-ml samples of plasma 100 μl of 10 μg/ml internal standard, 100 μl of 1 mol/l NaOH and 4 ml of ethyl acetate were added. Extraction into the organic phase was completed by vortex mixing for 1 min followed by centrifugation at 1000 *g* for 5 min. Back-extraction from the organic phase was accomplished, after careful removal of the ethyl acetate layer to a separate tube, by vortex mixing with 100 μl of 0.005 mol/l sulfuric acid. Portions of 20 μl from the acid–water pellet were injected onto the HPLC column using a micro syringe. All glassware was siliconized with Aquasil (Pierce).

Apparatus. Analyses were carried out with an HPLC system employing a Spectromonitor III variable-wavelength ultraviolet detector (Laboratory Data Control) set at 200 nm and recorded on a Linear Products recorder. The mobile phase consisted of 0.04 mol/l sodium phosphate buffer pH 3.0–acetonitrile–triethylamine (Aldrich, 99% pure) (87:12:1). Separation was accomplished with a Zorbax ODS column from Dupont (5–6 μm column particle size, 25 cm × 4.6 mm I.D.). A constametric pump (Laboratory Data Control) maintained a flow-rate of 1.5 ml/min.

Data analysis. Linear regressions (standard curves) were constructed by plotting the peak height ratios (drug-to-internal standard peak height ratio obtained from the HPLC chromatograms) against known (standard) concentrations of drug contained in tissue homogenate or plasma samples. For tissue homogenates, standard curves were constructed for lidocaine over a range of 1.25–10.0 μg/g, and for MEGX and GX over a range of 0.9–5.0 μg/g. Standard curves for plasma samples were constructed for lidocaine from 0.5 to 4.0 μg/ml, and for MEGX and GX from 0.25 to 2.0 μg/ml. Lidocaine, MEGX and GX levels in experimental myocardium and plasma were determined using each sample's HPLC-derived peak height ratio and the appropriate standard curve.

Oxidation–scintillation analysis

Samples to be analyzed were dispensed into individual combustocones and

left to dry overnight. A Packard Instruments oxidizer was used to combust the samples into $^{14}CO_2$, which was subsequently trapped by 7 ml of Carbosorb per sample. Permafluor (12 ml) was added to each sample; radioactive content was then measured using a Beckman LS 100C spectrometer. All instruments and solutions used, with the exception of the scintillation counter, were Packard products.

Assay evaluation procedures

Drug recovery after homogenization. Four 1-g samples of experimental myocardium containing ^{14}C-labeled lidocaine and metabolites were homogenized, dispensed in triplicate volumes of 0.5 ml, and subjected to oxidation–scintillation analysis. These results were compared to those obtained by direct oxidation–scintillation analysis of non-homogenized tissue obtained from contiguous sites of myocardium.

Extraction efficiency. Extractions from homogenized myocardial tissue were carried out for lidocaine (at 2.7, 4.5 and 6.3 μg/g), ethylmethylglycylxylidide (at 6.0 μg/g), MEGX and GX (at 0.9, 1.8 and 2.7 μg/g). Extraction recoveries were determined by comparing the peak heights of these samples to the peak heights generated by direct injection onto the column of non-extracted drug of the same concentrations.

Coefficient of variation. The coefficient of variation (C.V.) of the assay was determined by dividing the standard deviation obtained from chromatogram ethylmethylglycylxylide peak heights (n = 14 for myocardium and n = 12 for plasma) by each set's mean value.

Sensitivity of the assay. Samples containing variable amounts of lidocaine, MEGX and GX, 2 ml of distilled deionized water, and 1 g of LV tissue were homogenized and extracted. The peak heights generated by injection of 20 μl of back-extracts onto the column were evaluated for their signal-to-noise ratios.

Tissue size and assay linearity. To determine the accuracy of our method for analyzing tissue samples of varying sizes, pieces of LV tissue (from dog No. 2) ranging from 0.165 to 1.98 g (n = 16) were assayed for uniformity of drug concentration.

Comparison of HPLC and oxidation data. Of the back-extraction phase, prepared as described in *HPLC system, Extraction*, 40 μl were subjected to oxidation–scintillation analysis to measure the content of ^{14}C-labeled drug. By determining the hot-to-cold drug ratio of the infusate, as well as the extraction efficiency and molecular-weight differences amongst the three compounds of interest, the drug concentrations from HPLC samples were converted to dpm/g and compared to drug concentrations obtained from oxidation–scintillation analyses.

RESULTS

At a flow-rate of 1.5 ml/min the chromatographic peaks resolved clearly with retention times of 4, 5, 6 and 8 min for GX, MEGX, ethylmethylglycylxylidide and lidocaine, respectively (Fig. 1). Recovery of ^{14}C-labeled drug from the homogenization step was 91%. The C.V. derived from internal standard peak heights was 12.0% for myocardial samples and 6.1% for plasma samples. Linear

regression coefficients for all standard curves were > 0.999. We chose a signal-to-noise ratio of 3:1 as the minimum clearly measurable limit for peak heights. Using this definition and a standard 20-μl injection of back-extract, the assay sensitivity for each drug was found to be 1.5 ng per 20 μl injection of back-extract. This corresponds to 0.045 μg/g of myocardium.

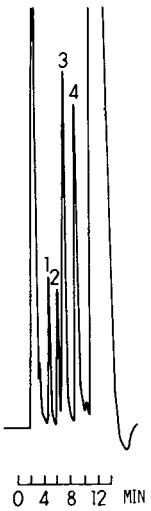

Fig. 1. Typical chromatogram of myocardial sample with peaks at 4, 5, 6 and 8 min for GX (1), MEGX (2), ethylmethylglycylxylidide (3) and lidocaine (4). a.u.f.s. setting = 0.05.

Extraction efficiencies for MEGX and GX at concentrations of 0.9, 1.8, and 2.7 μg/g were $\geqslant$ 88% and $\geqslant$ 55%, respectively (Table I). Extraction efficiency for lidocaine at concentrations of 2.7, 4.5 and 6.3 μg/g was $\geqslant$ 95%. Each of these drugs had better extraction efficiency at the lower concentrations. Extraction was found to be 95% efficient for the one concentration of ethylmethylglycylxylidide used in this study.

Drug concentrations from LV tissue for each dog (n = 4) are shown in

TABLE I

EXTRACTION EFFICIENCIES FOR LIDOCAINE, MEGX AND GX AT NOTED CONCENTRATIONS

Compound	Concentration (μg/g)	Extraction efficiency (%)
Lidocaine	2.7	98
	4.5	99
	6.3	95
MEGX	0.9	98
	1.8	90
	2.7	88
GX	0.9	67
	1.8	57
	2.7	55

Table II. The average lidocaine concentration was 3.37 μg/g, which is a little more than the combined value of its metabolites. Although the mean values of MEGX (1.51 μg/g) and GX (1.40 μg/g) are similar, the ratio MEGX:GX shows considerable variability, ranging from 1.74 for dog 2 to 0.56 for dog 4. Myocardial concentrations at the time of sacrifice appear to correlate with the respective plasma concentrations, including those values noted above for MEGX and GX in dogs 2 and 4.

TABLE II

LV TISSUE AND 90-min PLASMA CONCENTRATIONS ALONG WITH TISSUE-TO-PLASMA PARTITION RATIOS

Compound	Concentration (mean ± S.D.)		Ratio
	Tissue (μg/g)	Plasma (μg/ml)	
Dog No. 1			
Lidocaine	3.40 ± 0.21	1.30 ± 0.01	2.62
MEGX	1.62 ± 0.16	0.33 ± 0.01	4.91
GX	1.18 ± 0.21	0.36 ± 0.01	3.28
Dog No. 2			
Lidocaine	3.64 ± 0.19	1.64 ± 0.01	2.22
MEGX	1.69 ± 0.08	0.43 ± 0.01	3.93
GX	0.97 ± 0.07	0.30 ± 0.01	3.23
Dog No. 3			
Lidocaine	3.91 ± 0.50	1.67 ± 0.02	2.34
MEGX	1.74 ± 0.12	0.40 ± 0.01	4.35
GX	1.63 ± 0.15	0.62 ± 0.01	2.63
Dog No. 4			
Lidocaine	2.51 ± 0.15	1.61 ± 0.01	1.56
MEGX	1.01 ± 0.05	0.26 ± 0.01	3.88
GX	1.81 ± 0.05	0.73 ± 0.01	2.48
Mean			
Lidocaine	3.37 ± 0.60	1.56 ± 0.17	2.16 ± 0.45
MEGX	1.51 ± 0.32	0.36 ± 0.08	4.27 ± 0.48
GX	1.40 ± 0.37	0.50 ± 0.21	2.91 ± 0.41

The relationships between tissue and plasma concentrations are also shown in Table II. Both MEGX and GX are found in higher proportion in myocardium relative to plasma than is lidocaine. The partition coefficient of MEGX (4.27) is almost double that of lidocaine (2.16), whereas the partition coefficient for GX (2.91) lies in between these two values. As can be seen from the standard deviations, the partition ratios vary little amongst experiments. Thus, differences in myocardial metabolite concentration are caused predominantly by variation in hepatic metabolism rather than variation in myocardial uptake.

Fig. 2 displays the linearity between lidocaine, MEGX and GX concentration, and myocardial sample size for dog 2. Tissue weights range from 0.165 to 1.980 g. Drug concentrations (n = 16) show little variation over this range of tissue weights. The slopes for MEGX (—0.094) and GX (0.054) are nearly parallel to the X-axis. The lidocaine slope (—0.362) is accentuated by one outlier at the smallest sample size.

To evaluate the concordance between HPLC and oxidation—scintillation methods, portions from experimental tissue sample back-extractions (n = 12) were assayed by each technique. The results using HPLC give total drug values that are 88 ± 23% (mean ± S.D.) of those obtained with the oxidation—scintillation method.

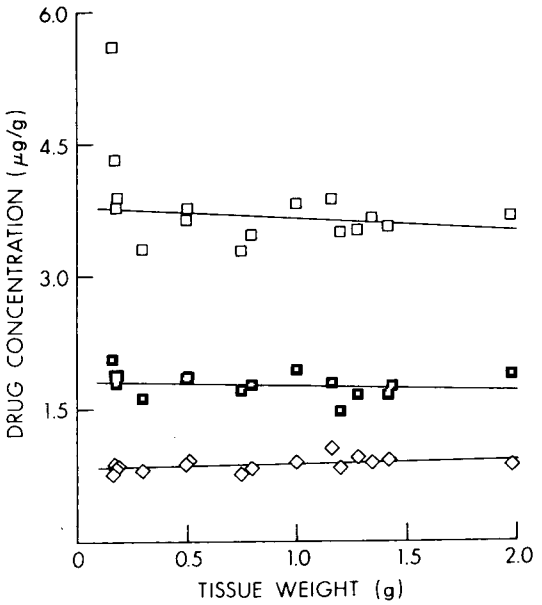

Fig. 2. Myocardial drug concentrations over a range of sample sizes obtained from dog 2. □, Lidocaine; ■, MEGX; ◇, GX.

DISCUSSION

There has been recurrent interest in the measurement of lidocaine and its metabolites in plasma and myocardium. Previous studies using gas—liquid chromatography report a wide range of myocardium-to-plasma partitioning of lidocaine and do not provide data on MEGX and GX [13—16, 18]. Because this range may reflect differences in technique, we set out to describe and validate a method for measuring plasma and tissue concentrations of lidocaine and its de-ethylated metabolites using HPLC.

We evaluated our method for sensitivity (1.5 ng per injection), reproducibility (C.V. of 12.0% for tissue and 6.1% for plasma), homogenization recovery (91%) and extraction efficiency ($\geqslant$ 95% for lidocaine, $\geqslant$ 88% for MEGX, and $\geqslant$ 55% for GX). The sensitivity of the assay can be improved by injecting larger samples of the back-extraction onto the column. In addition, the tissue concentration was shown to be independent of tissue sample size for values ranging between 0.165 and 1.98 g. Myocardium-to-plasma partition coefficients between myocardium and plasma for lidocaine, MEGX and GX were measured to be 2.16, 4.27 and 2.91, respectively, at a sampling time known to be

adequate for equilibrium between plasma and myocardium for these drugs [17].

Other reports of lidocaine myocardium-to-plasma partition ratio vary considerably. Ahmad and Medzihradsky [13] determined lidocaine tissue-to-plasma partitioning in dogs to be 0.45. This method requires large pieces of tissue for homogenization, as well as deproteination and evaporation steps for extraction. Using a simpler extraction procedure, Benowitz and co-workers [14, 15] found lidocaine to partition into myocardium with a ratio of 0.96 relative to plasma in monkeys. Although noted later to be sensitive and reproducible [19], this method does not report extraction efficiencies for tissue lidocaine, and as with the methodology of Ahmad and Medziradsky [13], requires large samples of tissue for homogenization. Naito et al. [18] presented a technique which enabled examination of smaller pieces of tissue (0.3 g) by pulverizing the sample prior to homogenization. No drug plasma levels were reported however, and the assay's sensitivity was 0.5 μg/g. Holt et al. [16] were able to determine lidocaine in pieces of myocardium as small as 0.1 g by digesting samples for 15 h with a bacterial proteinase. The partition ratio was determined to be 4.76 at a sensitivity of 1.0 μg/g. Variations in time of sampling are unlikely to account for much of these discrepancies, since lidocaine equilibrates rapidly between blood and myocardium [17].

In addition to the failure of the different methodologies noted above to yield consistent values for tissue-to-plasma partition ratios, none of these methods measured myocardial content of lidocaine metabolites. Much of the interest in quantifying these metabolites stems from reports of their toxic and pharmacologic activities, in particular their antiarrhythmic potential. Smith and co-workers [8, 20] noted that the peak antiarrhythmic action of lidocaine occurred after peak blood levels were attained following oral administration of the drug to dogs. This observation, it was suggested, may have been due to contributions of metabolites to the parent compound's activity. Similarly, Boyes et al. [5] found that plasma levels of lidocaine required for antiarrhythmic protection following oral administration were less than those needed after intravenous dosing of the drug. In this regard, Smith and Duce [8] showed MEGX to have one-third to one-half the ventricular antiarrhythmic potency of lidocaine in mice and dogs. Also, Burney et al. [7] demonstrated that MEGX was 83% as effective as lidocaine in protecting against ouabain-induced arrhythmias in guinea pig atrial tissue. By administering MEGX and GX for protection against chloroform-induced arrhythmias in mice, Strong et al. [9] determined potencies for the metabolites to be 99% and 26%, respectively, compared to that of lidocaine. Most recently, Broughton et al. [21] found that MEGX and GX each decrease the upstroke velocity of early premature beats and delay the recovery of action potential upstroke velocity following repolarization in guinea pig papillary muscle, confirming that both MEGX and GX have blocking effects on the sodium channel.

These metabolites have been shown to contribute a variety of toxic effects as well. They provoke convulsions [8], impair mental concentration [9] and may cause dizziness [5]. In addition, they have been reported to elicit emesis [8], frontal headaches and possibly hallucinations [9].

Extraction and subsequent HPLC analysis of lidocaine, MEGX and GX

removes the possibility, which may exist with procedures measuring only lidocaine, of non-resolved metabolites contributing to the lidocaine peak on the chromatogram. The extraction efficiencies obtained in our study for these three compounds are similar to those determined using the same procedure in plasma [11]. The lower extraction efficiencies registered at higher concentrations of drug (Table I) may be due to a decreasing acid-to-drug ratio in the extraction phase. This phenomenon may require further investigation if the analysis of higher drug concentrations is desirable. The accuracy with which drug content is measured over a range of tissue sizes adds credence and flexibility to this technique and may allow extension to animal models other than the dog.

ACKNOWLEDGEMENTS

We wish to thank Astra Pharmaceuticals for their generous supply of lidocaine and metabolites. We also wish to thank Mrs. T. LeMay for her secretarial expertise. Funding for this project was provided in part by U.S. Public Health Service Grants HL-17670 and 19216, National Institute of Arthritis, Diabetes, and Kidney Diseases, AM07386 NIADDK, and by a research grant from Burroughs Wellcome Co.

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Journal of Chromatography, 311 (1984) 301—309
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2272

IMPROVED METHOD FOR THE DETERMINATION OF ASPIRIN AND ITS METABOLITES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATIONS TO HUMAN AND ANIMAL STUDIES*

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(First received September 19th, 1983; revised manuscript received July 25th, 1984)

SUMMARY

An improved method has been developed for the determination of acetylsalicylic acid, salicylic acid, gentisic acid, and salicyluric acid in plasma and urine of rabbits and man. Samples are extracted with dichloromethane containing mephenytoin as an internal standard, the solvent is evaporated under reduced pressure, the residue reconstituted and analyzed by high-performance liquid chromatography. Extraction efficiencies, linearity and assay precision were determined. This method has been applied to human bioavailability studies and the data are presented.

INTRODUCTION

The pharmacokinetics of aspirin (ASA), one of the most widely used analgesics, remains a field of active investigation [1, 2]. In the species studied, ASA is hydrolyzed to salicylic acid (SA), which is further metabolized by conjugation to form salicyluric acid (SU), salicyl phenolic glucuronide and salicyl acyl glucuronide [2]. To a minor extent, SA is hydroxylated to form gentisic acid (GA), some of which is conjugated with glucuronide to form gentisuric acid [3]. Recently, SU has been reported to form a double conjugate with glucuronic acid [4]. The kinetics of ASA metabolite formation in

*This paper was presented in part at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, IL, U.S.A., April, 1983.

man have been extensively studied by Levy and co-workers [5—10] who showed capacity-limited formation of SU and salicyl phenolic glucuronide.

In order to conduct a large pharmacokinetic study of ASA in humans, our laboratory needed a rapid, sensitive, and selective assay for aspirin and its major metabolites in plasma and urine. Colorimetric assays for ASA and its metabolites are neither specific nor sufficiently sensitive [11—13]. The more recent assays using high-performance liquid chromatography (HPLC) are selective, but either fail to quantify ASA or fail to prevent its hydrolysis during sample preparation [14—19]. Other HPLC methods do not quantify metabolites of ASA and frequently do not provide for complete analysis of metabolites in urine [20—25]. While this study was in progress, two additional assays for ASA were published. One method requires a back-extraction step, not required in our procedure, and does not quantify GA [26]. The other method does not quantify urinary conjugates and does not use an internal standard [27].

Consequently, we developed a semi-automated method for analysis of ASA and its major metabolites by HPLC, which has been used successfully for measurement of over 2000 samples in studies of ASA bioavailability in humans, as well as pharmacokinetic investigations in rabbits and rats.

EXPERIMENTAL

Reagents

All reagents and solvents were reagent or HPLC grade and used as received. ASA was a gift from Endo Labs. (Garden City, NY, U.S.A.). SU, β-D-glucuronidase (97,900 I.U./ml, crude from *Helix pomatia*, EC 3.2.1.31), and heparin sodium salt, 140 USP K units/mg were obtained from Sigma (St. Louis, MO, U.S.A.). GA was purchased from Aldrich (Milwaukee, WI, U.S.A.). Mephenytoin (MP) was a gift from Sandoz (East Hanover, NJ, U.S.A.). Sodium fluoride, SA, and HPLC-grade phosphoric acid were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.). HPLC-grade methanol, acetonitrile, and dichloromethane from both Fisher Scientific and Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) were used.

Equipment

A Model 1084B liquid chromatograph equipped with a Model 79875A variable-wavelength detector, a Model 79850B LC terminal, a Model 79841A automatic injector and a Model 79842A autosampler, all from Hewlett-Packard, were used for all analyses. A 25 cm × 4.6 mm I.D. Nucleosil C_{18} column with 5-μm spherical particles was used (Alltech Assoc., Deerfield, IL, U.S.A.).

Determination of ASA, SA, and SU in plasma

Blood was collected in sterile 7-ml tubes containing 30 mg of sodium fluoride per tube (Becton-Dickinson, Rutherford, NJ, U.S.A.). The tubes were inverted gently several times to dissolve the sodium fluoride and immediately placed on ice. The blood samples were centrifuged at 0° C in a Beckman TR-6 centrifuge at 1500 *g* for 10 min. Plasma (1 ml) was pipetted into a 50-ml centrifuge tube containing 150 μl of 3 *M* orthophosphoric acid, 400 mg of

sodium chloride, and 12 ml of dichloromethane, which contained 3 μg/ml MP as the internal standard. The time which elapsed from the collection of blood to the pipetting of plasma was 30 min or less and the samples were kept on ice at all times. The tubes were shaken for at least 10 min at 300 oscillations per min on an Eberbach shaker, centrifuged and the upper layer was removed by aspiration and discarded. From the organic (lower) layer 8 ml were transferred to a 15-ml conical centrifuge tube and the solvent evaporated under reduced pressure of a water aspirator at room temperature using a Buchler Evapomix. Care was taken to remove the tubes immediately upon evaporation of the solvent. The residue was dissolved in 200 μl of mobile phase. The mixture was vortexed for 1 min, placed in a septum-capped vial, and a 10-μl portion injected on column. The mobile phase was pH 2.5 5 m*M* phosphate buffer—methanol—acetonitrile (68:16:16) and the flow-rate was 1.3 ml/min. The ultraviolet (UV) absorbance was measured at 237 nm. The retention times of GA, SU, ASA, SA and MP were 4.5, 5.7, 7.4, 10.0, and 11.6 min, respectively. Sample chromatograms are shown in Fig. 1.

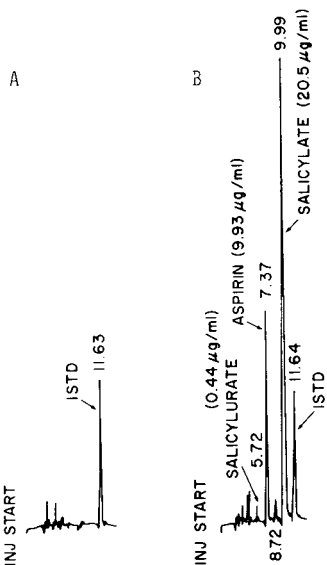

Fig. 1. HPLC chromatograms of an extract of human plasma (A) prior to dosing; (B) 20 min after oral administration of 975 mg of aspirin.

Rabbit plasma was analyzed in a manner similar to human plasma except that ca. 1 ml of blood was collected in a 5-ml polypropylene tube containing 50 μl of sodium fluoride solution (160 mg/ml). A 0.5-ml aliquot of plasma was assayed, and the quantities of salt and phosphoric acid reduced by half.

Determination of SA, SU, and GA in urine

ASA metabolites were analyzed by mixing 1 ml of urine with 1 ml of pH 5.0 0.2 *M* acetate buffer, a drop of chloroform and 20 μl of β-D-glucuronidase, and incubating at 37°C for 20 h in a Dubnoff metabolic shaking incubator. The solution was transferred quantitatively to a 10-ml volumetric flask, diluted

to volume, mixed well, and a 1-ml portion pipetted into a 50-ml conical centrifuge tube containing 12 ml of dichloromethane (9 μg/ml internal standard) and the same amounts of salt and phosphoric acid as above. The samples were shaken, centrifuged and evaporated as for plasma. The residue from evaporation of dichloromethane was reconstituted in 1 ml of mobile phase, of which 10 μl were injected on column and the UV absorbance measured at 237 nm.

When quantification of GA was desired, the residue from evaporation of the organic phase was reconstituted in 0.5 ml of mobile phase of which 50 μl were injected on column. The UV absorbance was measured at 330 nm for 4.8 min to optimize detection of GA acid and then at 237 nm for the remainder of the run. Determinations of free SA and SU in urine were essentially the same except that the addition of β-D-glucuronidase was omitted. Representative chromatograms of extracts of human urine are shown in Fig. 2.

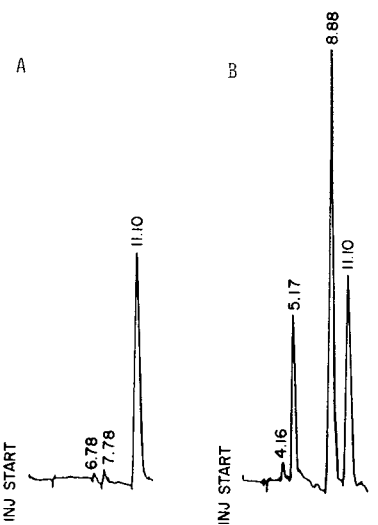

Fig. 2. HPLC chromatograms of an extract of blank human urine (A) prior to dosing with aspirin; (B) after a single oral dose of 975 mg of ASA. GA, SU, SA, and MP (internal standard) appear at retention times of 4.16, 5.17, 8.88, and 11.10 min, respectively.

Standard curves

Standard curves were prepared daily by addition of the analytes to the appropriate fluid (either plasma or urine, human or rabbit) using a 100-μl gas-tight Hamilton syringe. The concentration ranges for standards in plasma were: ASA, 0.2—100 μg/ml; SA, 0.2—100 μg/ml; SU, 0.2—4 μg/ml; and in urine were: GA, 2—20 μg/ml; SA, 20—300 μg/ml; SU, 200—2000 μg/ml. The standards for plasma (ASA, SA, and SU) were prepared in acetonitrile. The standards for urine (SA, SU, and GA) were prepared in water, with sufficient 1 *M* sodium hydroxide added to effect solution. All standards were kept refrigerated, and showed no decomposition over a period of five months. The standard curves were calculated by plotting the ratios of integrated areas of analyte and internal standard against the concentration of analyte. The line

which best fits the data was determined using a linear least-squares program on a Hewlett-Packard 85 computer. The concentrations of analyte in unknowns were then determined by the computer. Standard curves for all analyses were linear over the concentration ranges studied with correlation coefficients $\geqslant$ 0.999.

RESULTS AND DISCUSSION

Stability of ASA under assay conditions

As others have observed, the hydrolysis of ASA is minimized (less than 5%) for samples collected over fluoride, kept on ice, centrifuged at 0°C, and pipetted immediately into the extraction tubes containing solvent [23, 28]. Samples of plasma spiked with ASA and carried through this extraction procedure showed a loss of ASA of 2.2 ± 1.1%. The mobile phase is buffered to pH 2.5 at which point the rate of ASA hydrolysis is reported to be at a minimum [29]. Extracted samples stored in mobile phase for 24 h at room temperature showed a 4% loss of ASA.

Sublimation of SA during solvent evaporation has been reported by others [18, 23, 30]. We investigated the problem by comparing recoveries of SA from extracts of plasma spiked with SA. We observed no loss of SA during solvent evaporation as long as the solvent contained plasma components. However, when SA was added to pure extraction solvent (i.e. dichloromethane that had not been equilibrated with plasma), significant loss of SA occurred during evaporation. At the concentrations tested (equivalent to 40 and 1.6 μg/ml SA in plasma), 3% and 51%, respectively, of SA was lost in the evaporation step. Results in urine were similar. These findings indicate that plasma and urinary components inhibit sublimation of SA. One of us has observed this for other drugs [31]. Since SA is extracted from plasma or urine, including the standards which are spiked into these fluids, its loss through sublimation does not occur at a significant rate under the conditions of this assay.

Recovery and precision

Overall recoveries of ASA, SA, SU, and GA were determined by extracting the substances from plasma or urine and comparing the chromatographic peak areas to those obtained from unextracted standards dissolved in mobile phase. Recoveries (mean ± S.D.) from plasma for ASA, SA, and SU were 92 ± 1%, 85 ± 4%, and 52 ± 8%, respectively, and from urine for SA, SU, and GA were 98 ± 4%, 93 ± 7%, and 55 ± 3%, respectively. To measure the precision of the assay, a volunteer was given 975 mg of ASA by mouth. Blood was collected at 35 and 70 min, divided into five portions and assayed as above. For urine, a sample obtained from 0—8 h after the same dose of ASA was assayed five times. The results of the precision studies in plasma and urine are shown in Tables I and II, respectively.

Sensitivity

The lowest measurable limits of the assay as described here for plasma are: ASA, 0.2 μg/ml; SA, 0.2 μg/ml; SU, 0.2 μg/ml; and for urine are: SA, 0.2 μg/ml; SU, 0.2 μg/ml; GA, 2 μg/ml. Simple modifications can increase the

TABLE I

REPRODUCIBILITY OF FIVE DETERMINATIONS OF ASA, SA, AND SU IN PLASMA OF A HEALTHY VOLUNTEER FOLLOWING ORAL ADMINISTRATION OF 975 mg OF ASA

	Plasma concentration (μg/ml)					
	35 min post dosing			70 min post dosing		
	ASA	SA	SU	ASA	SA	SU
$\bar{X}$ ± S.D.	9.18 ± 0.20	45.5 ± 0.5	1.17 ± 0.06	3.87 ± 0.12	53.8 ± 1.7	1.43 ± 0.09
C.V. (%)	2.2	1.1	5.1	3.1	3.2	6.2

TABLE II

REPRODUCIBILITY OF SIX DETERMINATIONS OF SA, SU, AND GA IN UNHYDROLYZED URINE OF A HEALTHY VOLUNTEER FOLLOWING ORAL ADMINISTRATION OF 975 mg OF ASA

	Urine concentration (μg/ml), 0—8 h post dosing		
	SA	SU	GA
X ± S.D.	85.0 ± 1.1	1480 ± 20	24.5 ± 0.8
C.V. (%)	1.3	1.6	3.1

sensitivity of the assay if this is desired. With plasma samples, we have routinely injected 10 μl; 50-μl injections increase sensitivity five-fold and enhance the detection limit of all analytes. If urine is diluted less than ten-fold, a corresponding increase in sensitivity can be achieved.

Blanks

Human plasma has no interfering substances for the compounds assayed. Human urine has an SU blank as high as 5 μg/ml in some subjects. SU has been reported as a normal constituent of human urine [32]. Since typical SU concentrations are in the range of 1 mg/ml, this is not a serious drawback. In some subjects, an interfering peak eluted within 0.3 min of GA. This peak appears to be the aglycone of an endogenous glucuronide, since it is not present in unhydrolyzed urine. The blank for SA in urine is zero.

Rabbit plasma has no substances which interfere with either ASA or SU. However, the blank for SA amounts to 0—0.2 μg/ml. In rabbit urine, the blank for SA is 6—25 μg/ml and for SU 6—19 μg/ml. The blank for GA is too large to permit its measurement.

Use of the method

The procedure has been used in a 24-patient crossover study of ASA bioavailability. The mean concentration profiles and the pharmacokinetic parameters calculated from this data will be presented elsewhere [33].

Six other volunteers were given 975 mg of ASA by mouth and timed blood samples were removed through an indwelling venous catheter. Figs. 3 and 4 show the mean plasma curves. With ASA, a peak concentration of 11.7 ± 1.7

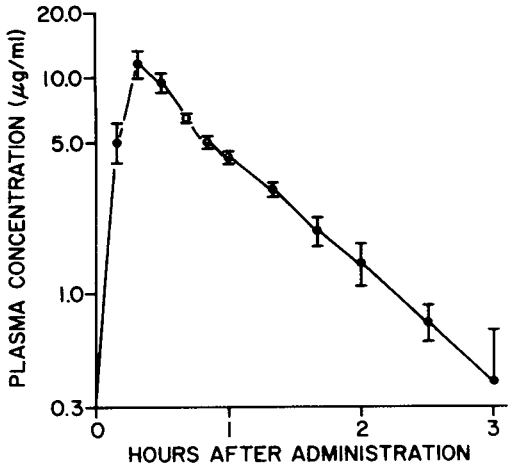

Fig. 3. The concentration of ASA in plasma of humans each given a single oral dose of 975 mg of ASA. Each point represents the mean ± S.E.M. of five different subjects.

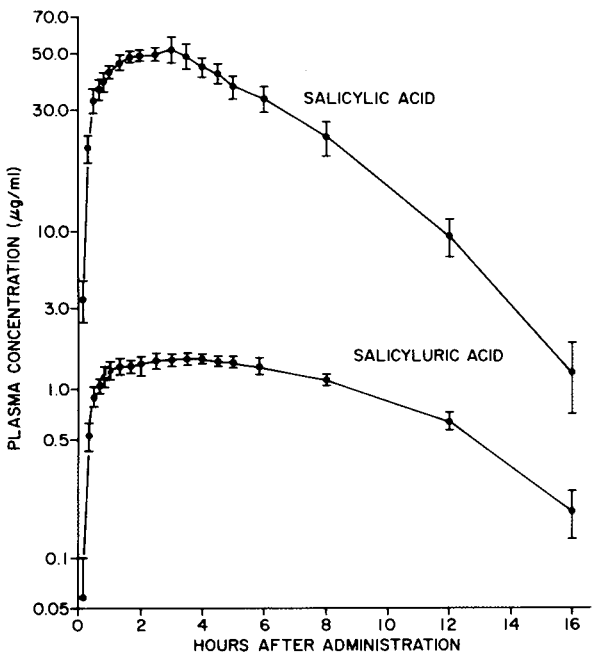

Fig. 4. The concentrations of SA and SU in the plasma of humans each given a single oral dose of 975 mg of ASA. Each point represents the mean ± S.E.M. of five different subjects.

μg/ml was attained 20 min after administration and declined with a half-life of 30.6 min. Half-lives reported for ASA in man following an oral dose of 600—650 mg range from 15 to 20 min [34—36]. Continued absorption of the larger dose of ASA during its exponential decline phase could account for the apparent prolongation of ASA half-life observed in this study. With SA, a peak concentration of 56.7 ± 4.8 μg/ml was attained at 2.37 ± 0.37 h and a terminal half-life of 2.70 h was observed, in good agreement with previously reported

TABLE III

RECOVERY OF URINARY METABOLITES FROM HEALTHY VOLUNTEERS AFTER ORAL ADMINISTRATION OF 975 mg OF ASA

Metabolite	Percent of dose recovered as metabolite						Mean ± S.D.
	KV*	HD	DA	TR	JL	CR	
Free SU	65.6	51.8	69.5	63.2	55.9	61.6	61.3 ± 6.46
Conjugated SU	1.7	2.1	7.2	9.8	16.7	6.8	7.4 ± 5.54
Free SA	17.6	11.7	6.0	7.4	5.0	3.4	8.5 ± 5.27
Conjugated SA	6.6	12.1	6.8	11.3	11.9	8.6	9.6 ± 2.54
GA	1.1	1.2	1.1	1.3	0.9	0.7	1.1 ± 0.22
Total recovery	92.6	78.9	90.6	93.1	90.4	81.1	87.8 ± 6.16

*Subject.

TABLE IV

PHARMACOKINETIC DATA FOR ASA AND SA IN RABBITS FOLLOWING INTRAVENOUS ADMINISTRATION OF 25 mg/kg ASA

Mean ± S.D. (n = 3); k_e = elimination rate constant, $t_{1/2\alpha}$ = distribution half-life, $t_{1/2\beta}$ = elimination half-life, AUC = area under the concentration—time curve, Cl_T = total body clearance.

Parameter	Aspirin	Salicylic acid
k_e (h^{-1})	5.37 ± 0.75	0.305 ± 0.100
$t_{1/2\alpha}$ (h)	0.035*	—
$t_{1/2\beta}$ (h)	0.129*	2.27*
AUC (mg l^{-1} h)	5.88 ± 0.77	135 ± 27
Cl_T (l h^{-1})	15.5 ± 0.3	0.493 ± 0.111

*Harmonic mean.

values [35]. The urinary metabolite and recovery data for these six volunteers were also determined (see Table III). Treatment of urine with β-D-glucuronidase gave significantly higher recoveries (7.4 ± 5.5%) of SU than untreated samples ($p < 0.001$). This indicates that SU, conjugated with glucuronic acid, is present in urine. Chemical evidence for this double conjugate has been observed in rats treated with SU [37] and in humans given ASA [4].

The pharmacokinetics of ASA and its metabolites after intravenous administration of 25 mg/kg ASA to rabbits were determined (Table IV). The data for ASA fitted a two-compartment model with a half-life of 2.1 min and 7.7 min for the α- and β-phases, respectively. The terminal half-life for SA was 2.3 h.

In summary, an improved assay for determination of ASA and its major metabolites in biological fluids was developed. It is rapid, sensitive, and amenable to the analysis of a large number of samples. The automation of the HPLC system allows for the continuous analysis of ASA overnight. Approx.

100 plasma samples can be processed and analyzed within 24 h, thereby eliminating the need to store samples which contributes to the loss of ASA by hydrolysis.

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Journal of Chromatography, 311 (1984) 311—317
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2255

DETERMINATION OF SALBUTAMOL IN HUMAN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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(Received June 7th, 1984)

SUMMARY

A simple and sensitive method for the quantitative determination of salbutamol in human serum using reversed-phase ion-pair high-performance liquid chromatography with electrochemical detection is described. The method involves the combined use of Sep-Pak® cartridges and ion-pair extraction for sample clean-up, and subsequent separation of salbutamol and the internal standard from interfering compounds on a reversed-phase column. An amperometric detector incorporating a glassy carbon electrode was employed for detection. The inter-assay coefficients of variation at plasma concentrations of 2.0, 6.0 and 20.0 ng/ml were 7.3%, 7.2% and 8.5%, respectively (n = 20). The minimum detection limit was 400 pg/ml from a 0.5-ml sample of serum. The method can be readily utilised for clinical pharmacokinetic studies.

INTRODUCTION

Salbutamol, 2-*tert.*-butylamino-1-(4-hydroxymethyl)phenylethanol, is a β_2-adrenoreceptor agonist widely used in the treatment of asthma. Although its pharmacology is well documented [1, 2], pharmacokinetic information on salbutamol is limited largely due to the difficulty in measuring the drug at therapeutic concentrations. Thus far, reported assays for salbutamol have employed liquid scintillation spectrometry [3], gas chromatography—mass spectrometry [4, 5], high-performance liquid chromatography (HPLC) with a rotated disc amperometric detector [6] and more recently, HPLC with fluorescence detection [7, 8]. Some of these methods suffer from the disadvantages of a lack of sensitivity [3], the use of laborious extraction procedures and elaborate equipment [4, 5] and the apparent short lifespan of column and electrode [6]. We have exploited the sensitivity of amperometric detection

0378-4347/84/$03.00

which is capable of measuring catecholamines in the picogram range [9] to quantitatively determine the low therapeutic levels of the electrochemically active salbutamol.

In this report, a sensitive and simple HPLC method coupled with amperometric detection for the determination of salbutamol in human serum is described. This method includes initial sample clean-up with a Sep-Pak® cartridge followed by ion-pair extraction, and subsequent separation on a reversed-phase column with a phosphate buffer—methanol mixture. Selectivity was enhanced by the use of 1-heptanesulfonic acid, an ion-pair reagent in the mobile phase which thereby created a weak cation-exchange column. Pharmacokinetic studies have been carried out on human subjects receiving oral salbutamol using this HPLC—amperometric detection method.

EXPERIMENTAL

Chemicals and standards

Salbutamol sulphate and the internal standard fenoterol bromide were a generous gift from Glaxo Canada (Toronto, Canada) and Boehringer Ingelheim (Burlington, Canada), respectively. Di(2-ethylhexyl) phosphate (DEHP) was a synthetic-grade reagent obtained from Sigma (St. Louis, MO, U.S.A.). Glass-distilled ethyl acetate, Accusolv methanol and chloroform (without ethanol preservative) were purchased from BDH (Toronto, Canada), Anachemia (Mississauga, Canada) and Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), respectively. HPLC-grade sodium 1-heptanesulphonic acid used as ion-pair reagent was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Reagent A was a 70 m*M* sodium phosphate buffer ($NaH_2PO_4 \cdot H_2O$ from Fisher Scientific), pH 6.8, containing 1 m*M* chloride ions, and 2.0 m*M* sodium 1-heptanesulphonic acid. The buffer was filtered through a 0.45-μm membrane before use. Stock 10 mg/ml solutions of fenoterol and salbutamol were prepared in 0.1 *M* hydrochloric acid and stored at 4°C. All working solutions were prepared by dilution with distilled water.

Chromatographic conditions

A high-performance liquid chromatograph, Model 6000A solvent delivery system equipped with a Model U6K injector, both from Waters Assoc. and an Altex Ultrasphere 3-μm ODS column (7.5 cm × 4.6 mm, Beckman, Berkeley, CA, U.S.A.) were employed. The detector system comprised a BioAnalytical System Model LC-4 amperometric detector incorporating a TL-5 thin-layer glassy carbon working electrode and Ag/AgCl reference electrode (BioAnalytical Systems, West Lafayette, IN, U.S.A.) and connected to a Perkin-Elmer Model 024 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.). The detector was typically used at a sensitivity of 10 nA full scale and an applied potential of +0.80 V.

The mobile phase was a mixture of 25% (v/v) methanol in reagent A. Before use, it was degassed in an ultrasonic bath for about 2 h. The flow-rate was 0.5 ml/min and all separations were performed at room temperature.

Sample preparation

To 0.5 ml serum sample diluted with 1.0 ml distilled water 15 μl of internal standard solution (100 ng/ml) were added. This solution was slowly forced through a Sep-Pak cartridge which was previously washed with 10 ml of methanol and 10 ml of water. The cartridge was washed twice with 2 ml of water and the drug and internal standard were then eluted with 2 ml of methanol — the first two drops of eluate were discarded. The methanol was evaporated to dryness under a nitrogen stream at 40°C. Salbutamol and fenoterol in the dry residue were extracted as ion-pair with DEHP by vortexing vigorously for 1 min with 70 μl of reagent A and 300 μl of 0.05% (v/v) solution of DEHP in ethyl acetate. After centrifugation (5000 *g* for 30 sec) the organic phase was transferred to a second microtube (6 × 50 mm) containing 40 μl of reagent A and the vortexing step was repeated. The phases were again separated by centrifugation and the ethyl acetate phase transferred to a third microtube containing 70 μl of 10 m*M* hydrochloric acid into which salbutamol and fenoterol were back-extracted by vortexing for 1 min. Following centrifugation the organic layer was discarded and the acid was briefly washed with 150 μl of chloroform. A 40—60 μl volume of the aqueous phase was injected into the chromatograph after centrifugation.

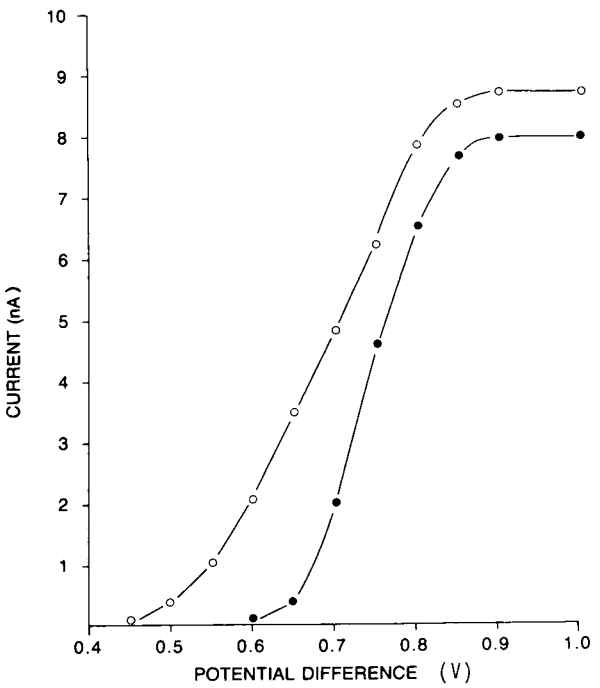

Fig. 1. Hydrodynamic voltammograms for salbutamol (•) and fenoterol (○) on injection of 4.0 ng of salbutamol and 2.0 ng of fenoterol employing the chromatographic conditions described in the text.

RESULTS AND DISCUSSION

Chromatography

Hydrodynamic voltammograms (i.e. profiles of current versus potential) for salbutamol and fenoterol were determined over the range of 0.40—1.00 V in the mobile phase described and are shown in Fig. 1. An applied potential of +0.60 V is required to initiate an electrochemical response for salbutamol whereas a response to fenoterol is initiated at a considerably lower potential. The applied potential was chosen as +0.80 V because sufficient sensitivity could be obtained at this voltage with minimal background noise.

Fig. 2 shows typical chromatograms of extracted blank plasma and plasma containing salbutamol at a concentration of 3.0 ng/ml and at a detector sensitivity of 10 nA full scale. Because the internal standard has almost twice the retention time to that of salbutamol, one chromatographic run requires about 14 min.

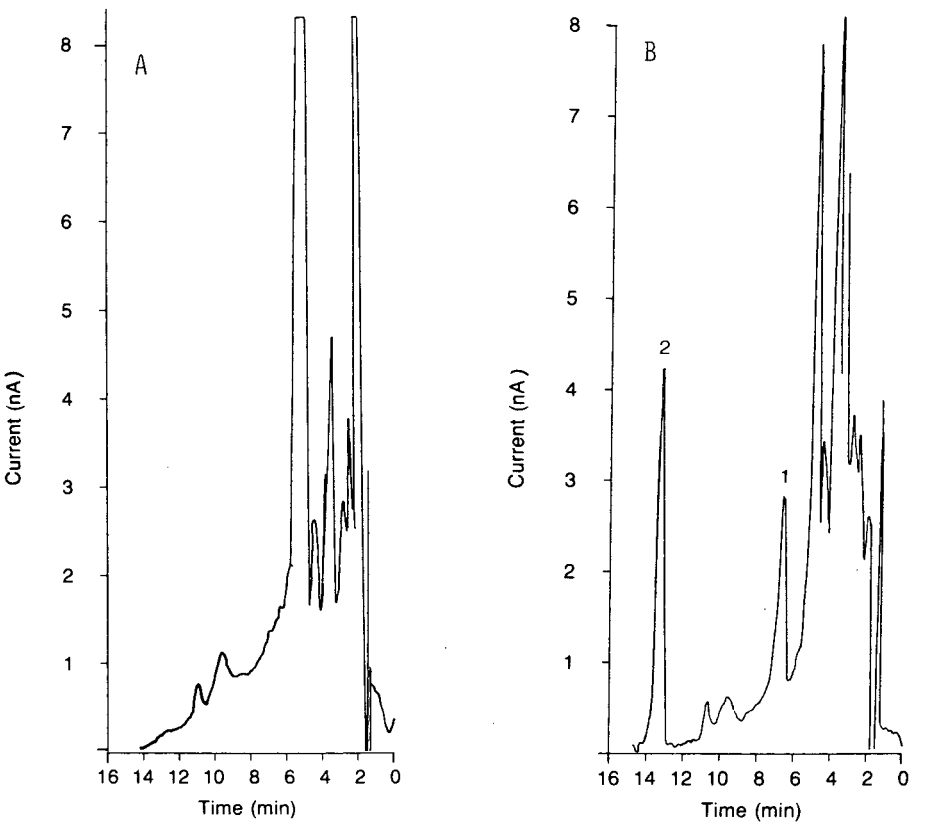

Fig. 2. Chromatograms obtained with (A) blank plasma and (B) plasma containing 3.0 ng/ml salbutamol. Peaks: 1 = salbutamol, 2 = fenoterol (internal standard).

Selectivity

The selectivity of the method was investigated at the retention times of salbutamol and fenoterol. No endogenous interference was found in

chromatograms of samples extracted from 25 pools of plasma. Also, replicate analyses of serum samples by this method containing therapeutic concentrations of theophylline, carbamazepine, phenytoin, phenobarbital, ethosuximide, primidone, valproic acid and gentamycin revealed no interfering peaks. Furthermore, injection of solutions of sympathomimetic agents such as epinephrine, phenylephrine, terbutaline, isoproterenol, metaproterenol, metaraminol, isoxsuprine, dobutamine, buphenine, phentolamine and oxymetazoline did not reveal any peaks with retention times similar to those of salbutamol or fenoterol. However, when heparin is used as an anticoagulant it was found to give an interfering peak with the same retention time as the internal standard. Heparinised plasma is therefore not recommended for the analysis described above.

Recovery

The percentage analytical recovery of salbutamol and fenoterol was measured by comparing the peak heights obtained from the injection of known quantities of the pure compounds with those obtained from the direct injection of extracted plasma samples spiked with three different concentrations of salbutamol. Using this procedure the percentage analytical recovery of salbutamol at various concentrations averaged 79% and that of fenoterol 73% (Table I).

TABLE I

RECOVERY DATA FOR SALBUTAMOL ASSAY (n = 12)

Plasma salbutamol concentration (ng/ml)	Percentage recovery (mean ± S.D.)	Coefficient of variation (%)
20.0	79 ± 4.8	6.1
6.0	79 ± 4.9	6.2
2.0	80 ± 4.3	5.4
Amount of internal standard fenoterol added		
1.5	73 ± 4.4	6.0

Precision and accuracy

The precision and accuracy of the method were assessed by the repeated analysis of a drug-free plasma pool to which salbutamol had been added to provide a series of concentrations ranging from 2.0 to 20.0 ng/ml. Twelve replicate samples at each of the three concentrations were used in the assessment of the within-day variability while between-day variability was assessed for twenty days over a one-month period employing samples which were stored frozen at −20° C; the results are presented in Table II.

Quantitation

The peaks on the chromatogram are identified by their retention time

relative to that of the internal standard. Quantitation was done by comparison of the peak height ratio of salbutamol to fenoterol in the unknown sample to those of control samples containing known quantities of salbutamol, extracted and chromatographed in exactly the same way. Concentration and peak height ratio were verified to be linearly related throughout the concentration range investigated, 1.0—20 ng/ml, yielding a correlation coefficient of 0.9946 and a linear regression equation of $Y = 0.266X - 0.036$.

TABLE II

WITHIN-DAY AND BETWEEN-DAY VARIABILITY OF SALBUTAMOL ASSAY

Salbutamol concentration (ng/ml)	Within-day precision (n = 12)		Between-day precision (n = 20)	
	Mean ± S.D. (ng/ml)	Coefficient of variation (%)	Mean ± S.D. (ng/ml)	Coefficient of variation (%)
2.0	1.97 ± 0.14	7.1	2.06 ± 0.17	8.3
5.0	5.04 ± 0.31	6.2		
6.0			5.84 ± 0.42	7.2
10.0	9.80 ± 0.66	6.7		
20.0			19.9 ± 1.47	7.4

Sensitivity and detection limit

With the above analysis set at a sensitivity of 10 nA full scale and a detector potential of +0.80 V versus Ag/AgCl reference electrode, the detection limit taken as a signal-to-baseline noise ratio of 2 was judged to be 400 pg/ml. This limit can be further lowered by either doubling the detector sensitivity or employing a larger serum volume.

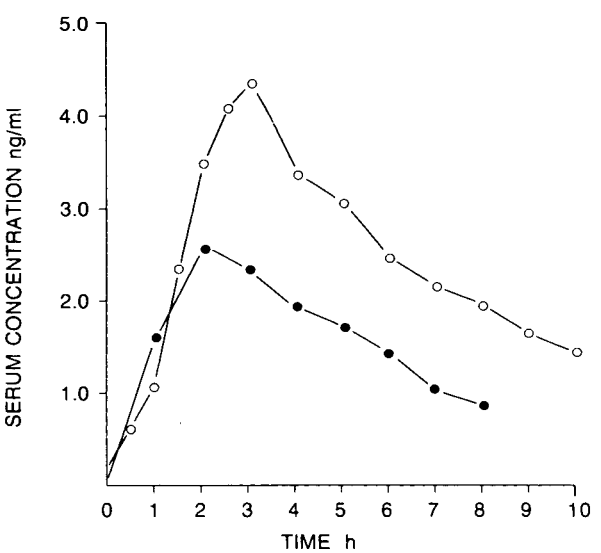

Fig. 3. Serum concentration—time curves following oral administration of 2 mg of salbutamol to two healthy adult volunteers with a body mass of 53 kg (○) and 62 kg (●).

Clinical studies

The HPLC—amperometric detection method described above has been applied to acquire clinical pharmacokinetic data. Fig. 3 shows two serum concentration—time curves in two healthy adults following an oral administration of 2 mg of salbutamol. The peak serum concentration occurred between 2 and 3 h post-dose and the elimination half-lives were between 4 and 5 h. The parameters can be expected to vary between individuals. As fenoterol, also an antiasthmatic agent, is used as the internal standard, the present method cannot be applied to serum from patients receiving simultaneous medication. The assay described for salbutamol can be readily adapted to investigate the fenoterol serum concentration in an asthmatic patient receiving the latter drug. However, we have found that it lacked adequate sensitivity to measure fenoterol concentration following a 2.5-mg oral dose, even with a detection limit of 100 pg/ml. Salbutamol pharmacokinetic studies including concentration—response and bioavailability assessment in healthy and/or asthmatic subjects are currently in progress.

CONCLUSION

We have successfully developed a simple and yet highly sensitive HPLC—amperometric detection assay for the determination of salbutamol in human serum. The method possesses good selectivity and reproducibility and superior sensitivity over all existing salbutamol assays to date [4—8]. The procedure generally requires only 0.5 ml of serum. Acquisition of salbutamol pharmacokinetic and pharmacodynamic information is now feasible.

ACKNOWLEDGEMENT

We gratefully acknowledge the financial support we have received from Glaxo Labs.

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Journal of Chromatography, 311 (1984) 319—328
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2260

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR LABETALOL IN HUMAN PLASMA USING A PRP-1 COLUMN AND FLUOROMETRIC DETECTION

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(First received April 10th, 1984; revised manuscript received June 4th, 1984)

SUMMARY

A high-performance liquid chromatographic assay for the determination of labetalol, a novel antihypertensive agent, in human plasma was developed. Reversed-phase separation of labetalol and the internal standard was accomplished on a 150 × 4.1 mm column commercially packed with a spherical (8—12 μm particle size) macroporous co-polymer (PRP-1). Unlike silica-based columns, the unique properties of PRP-1 permit operation at pH extremes. Based on this advantage, a mobile phase which was sufficiently basic (pH 9.5) to optimize the fluorescent yield of analyte and provide the necessary specificity was selected. Detector response (peak area ratio) was linear from 4 to 500 ng/nl. Following a simple extraction procedure, samples were automatically injected and analyzed using microprocessor-controlled equipment. No interferences were observed in the extracts obtained from drug-free plasma which were processed under the conditions described for unchanged drug. The limit of quantitation using 0.5 ml of plasma was validated to 4 ng/ml. The inter-assay precision (coefficient of variation) was less than 4.6% at all concentrations evaluated from 4 to 300 ng/ml. This method is suitable for the routine quantitation of labetalol or its *RR* isomer (dilevalol) in plasma (0—24 h) following the administration of therapeutically effective doses to man.

INTRODUCTION

Labetalol · HCl (I), 5-{1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]-ethyl}salicylamide monohydrochloride (Fig. 1) is an effective antihypertensive agent with both α- and β-adrenoreceptor blocking activity as well as direct vasodilator actions [1]. Disposition studies with radiolabeled (^{3}H) drug in man have shown that labetalol is well absorbed following oral administration but undergoes extensive first-pass metabolism [2]. Peak plasma concentrations of

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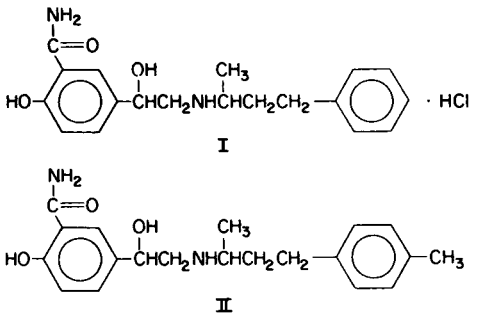

Fig. 1. Structures of labetalol hydrochloride (I) and the internal standard (II).

labetalol after a 200-mg dose were only about 80—100 ng/ml and represented less than 5% of the total plasma radioactivity. For these reasons, analytical methodology which can reproducibly measure nanogram quantities of unchanged labetalol is required to fully characterize the terminal elimination phase in plasma. A selective and sensitive high-performance liquid chromatographic (HPLC) assay for labetalol was therefore developed in our laboratory in order to support a multitude of clinical bioavailability and bioequivalency studies. Several HPLC methods [3—7] for the determination of labetalol in plasma are reported in the literature. Dusci and Hackett [3] described an HPLC method with ultraviolet (UV) detection which was sensitive to 40 ng/ml of labetalol in plasma. This limit of quantitation, however, is unsuitable for detailed pharmacokinetic studies in man following single-dose administrations (per os) of drug. Another more sensitive (10 ng/ml) HPLC assay which employed a UV spectrophotometer (207 nm) was reported by Woodman and Johnson [4]. Although this method requires extensive sample clean-up, significant assay bias (+60%) was observed at the 10 ng/ml concentration in plasma. In a recent publication, Hidalgo and Muir [7] report an HPLC method for labetalol which also used UV detection (216 mm) but with a simplified extraction procedure. Comparable sensitivity was achieved; however, no accuracy or precision estimates at the lower limit of detection (10 ng/ml) were provided.

Meredith et al. [5] and Oosterhuis et al. [6] reported two different procedures for the quantitation of labetalol in plasma which employ HPLC with fluorimetric detection. Both methods share improved lower limits of detection (ca. 1—8 ng/ml) and selectivity. An examination of the method employed by Meredith et al. [5] reveals that detection of drug is accomplished with an acidic mobile phase thus failing to optimize the fluorescence yield of labetalol which is achieved under basic (pH 9—10) conditions [6, 8]. The approach reported by Oosterhuis et al. [6] involved post-column alkalination of the column effluent with buffer in order to enhance the fluorimetric detection of analyte. This, however, was achieved at the expense of an additional pump and associated hardware.

The method detailed herein describes the use of an HPLC column commercially [9] packed with a macroporous co-polymer (PRP-1) for the determination of labetalol in plasma. Unlike silica-based columns, the unique properties of PRP-1 permit operation at pH extremes. Based upon this advantage, a mobile phase was chosen which was sufficiently basic (pH 9.5)

to optimize the fluorimetric detection of labetalol and provide the selectivity necessary for routine determinations of drug at concentrations as low as 4 ng/ml.

EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system composed of a WISP (Model 710B) automatic injector (Waters Assoc., Milford, MA, U.S.A.), and a Waters pump (M6000A) interfaced with a Waters Model 720 system controller. Detection of analyte was achieved by using an Aminco (Urbana, IL, U.S.A.) Fluoro-Monitor equipped with a round 100-μl flow cell after excitation at 370 nm (Corning 7-51 filter) and emission at 415 nm (Wratten gelatin No. 2A filter). A back-pressure coil constructed with 60 cm × 0.23 mm I.D. stainless-steel tubing was connected to the outlet side of the detector to prevent bubble formation in the cell. The amplified signal from the detector was connected to a recorder and an integrating computer (HP 3357 Lab Automation System, Hewlett-Packard, Palo Alto, CA, U.S.A.) in order to generate real-time chromatographic tracings (10 mV) and to integrate peak areas (0—2 V). The attenuator was maintained at a setting of 10.

Reagents and solvents

Labetalol · HCl and the internal standard (II), 5-{2-[4-(4-methylphenyl)-2-butylamino]-1-hydroxyethyl}salicylamide · HCl, were used as received from the Schering Chemical Distribution Center (Bloomfield, NJ, U.S.A.). All other chemicals except acetonitrile, hexane and methanol (Omni Solv, MCB, Cincinnati, OH, U.S.A.) were reagent grade.

Chromatographic conditions

Chromatography was performed on a 150 × 4.1 mm I.D. stainless-steel column commercially (Hamilton, Reno, NV, U.S.A.) packed with PRP-1, a spherical (10 μm particle size) macroporous poly(styrene-divinylbenzene) sorbent.

Reversed-phase separations were accomplished at ambient temperature using a mobile phase consisting of 0.05 *M* $(NH_4)_2CO_3$—NH_4OH, pH 9.5—acetonitrile—tetrahydrofuran (495:125:40, v/v/v). The solvent mixture was prepared daily, filtered (0.45 μm) and degassed under reduced pressure before use. The flow-rate (1.5 ml/min) generated a back-pressure of approx. 124 bars.

Standard solution preparation

An accurately weighed amount (11.1 mg) of labetalol · HCl was dissolved in 3 ml of methanol and then diluted to 100.0 ml with distilled water. This solution contained 100 μg/ml free base form. A solution of the internal standard was similarly prepared with water in a 100-ml volumetric flask. Subsequent dilutions with distilled water were designed so that the desired amount of both drugs could be conveniently delivered in a 100-μl volume with automatic pipettes.

Detector calibration and standard curve

A standard curve was initially generated after repated (n = 6) injections of standard solutions prepared to contain 4, 20, 50, 100, 200, 400 and 500 ng/ml labetalol and a constant concentration (200 ng/ml) of the internal standard. Data from this multipoint calibration curve were subjected to least-squares fit analysis to determine the best straight-line relationship. Computer-reported estimates of labetalol concentration were based upon the average calculated response factors from triplicate injections of a single concentration point near the mid-point of the standard curve. Calibration was performed using samples containing 100 ng/ml labetalol and 200 ng/ml internal standard.

Extraction procedure

An aliquot (0.5 ml) of human plasma was transferred to a 15-ml test tube (125 × 16 mm) fitted with a polytef-lined screw cap. After the addition of 100 ng internal standard which was prepared as an aqueous solution (100 ng per 0.1 ml), each plasma sample was diluted (0.5 ml) with 0.05 *M* Tris buffer (pH 9.0) and extracted with 5.0 ml of ethyl acetate by agitation on an Eberbach (Ann Arbor, MI, U.S.A.) reciprocal shaker for 10 min. Samples were centrifuged (10 min at 1600 *g*) to facilitate separation of the phases. The aqueous portion was frozen in a dry-ice—acetone bath and the organic layer transferred to a clean 15-ml test tube.

Aliquots of hexane (2.0 ml) and 0.05 *M* sulfuric acid (0.5 ml) were added to the ethyl acetate, then shaken and centrifuged as above. The organic layer was discarded and the acidic solution transferred to a disposable polypropylene microcentrifuge tube (1.5 ml). An aliquot (400 µl) of this acidic extract was then automatically injected for analysis by HPLC.

Extraction efficiency

Venous blood was drawn from several human volunteers into heparinized Vacutainers® (Becton-Dickinson, Rutherford, NJ, U.S.A.) and centrifuged at 1600 *g* for 30 min to generate a drug-free plasma pool. The efficiency of extracting drug from human plasma was determined using the following procedure. Labetalol and internal standard were added to aliquots (0.5 ml) of drug-free plasma ($n \geqslant 6$ replicates per group) to achieve labetalol concentrations of 4, 100 and 300 ng/ml with a constant internal standard concentration of 200 ng/ml. Samples were extracted as described above and 400 µl of the acidic layer injected by the WISP.

The recovery of labetalol and internal standard was calculated by comparing the peak area of both compounds from extracted samples with those obtained from the analysis of equivalent amounts of drug injected directly.

Intra-assay accuracy and precision

The precision and accuracy of labetalol quantitation were evaluated in the following manner. Known amounts of drug standards were added to drug-free plasma as described in the previous section and extracted as outlined. In order to calibrate the detector response, extracts from three samples spiked to contain 100 ng/ml labetalol and 200 ng/ml internal standard were injected in triplicate. Quantitation of labetalol concentrations in the remaining samples

was then automatically calculated by the integrating computer using the average (n = 3) internal standard response factor generated at this calibration point.

Inter-assay accuracy and precision

Estimates of inter-assay variability were provided by repeating the accuracy and precision determinations described above for labetalol on two different days. All analytical results within each concentration group were pooled in order to calculate the inter-assay precision (coefficient of variation, C.V.) and accuracy (percentage bias).

Selectivity

Drug-free human plasma specimens were processed and analyzed by HPLC as described above. Chromatograms were examined for the presence of endogenous material which might interfere with the measurement of either labetalol or the internal standard.

Solutions of hydrochlorothiazide, trichloromethiazide and propranolol were injected directly onto the column in order to assess their potential for assay interference.

RESULTS AND DISCUSSION

Chromatography

The retention times for labetalol and the internal standard varied between 4.74 and 5.20 min and 8.53 and 9.6 min, respectively, for three PRP-1 columns which were evaluated for assay suitability. No significant changes in retention time or peak shape were observed for either compound over a three-month period.

Extracts from drug-free human plasma were found to be free of interfering peaks (Fig. 2). Representative chromatograms from plasma spiked with 4 ng/ml

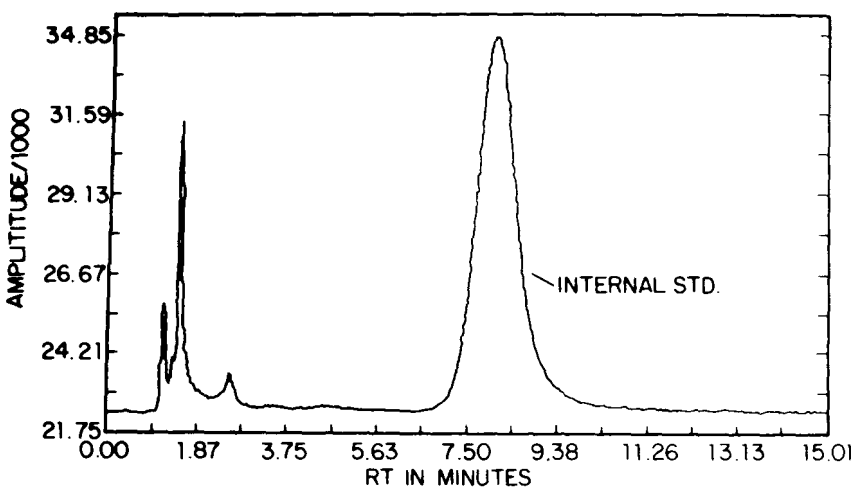

Fig. 2. Computer-reconstructed chromatogram of an extract from drug-free human plasma to which only internal standard (200 ng/ml) was added.

and 100 ng/ml labetalol are shown in Fig. 3. Hydrochlorothiazide, trichloromethiazide and propranolol demonstrated no potential for assay interference.

Detector calibration and standard curves

A standard curve was generated using stock solutions prepared to contain labetalol at concentrations ranging from 4 to 500 ng/ml. The integrated peak area (μV-sec) ratio of labetalol to the internal standard was chosen as the quantitative measure of detector response for each labetalol concentration. Data were then subjected to weighted (1/variance) least-squares fit analysis owing to variance heteroscedasticity in order to determine the best fit straight-line relationship between detector response and labetalol concentration.

Weighted regression of peak area ratio on labetalol concentration yielded a linear ($P > 0.1$) fit of the data with a coefficient of determination (r^2) equal

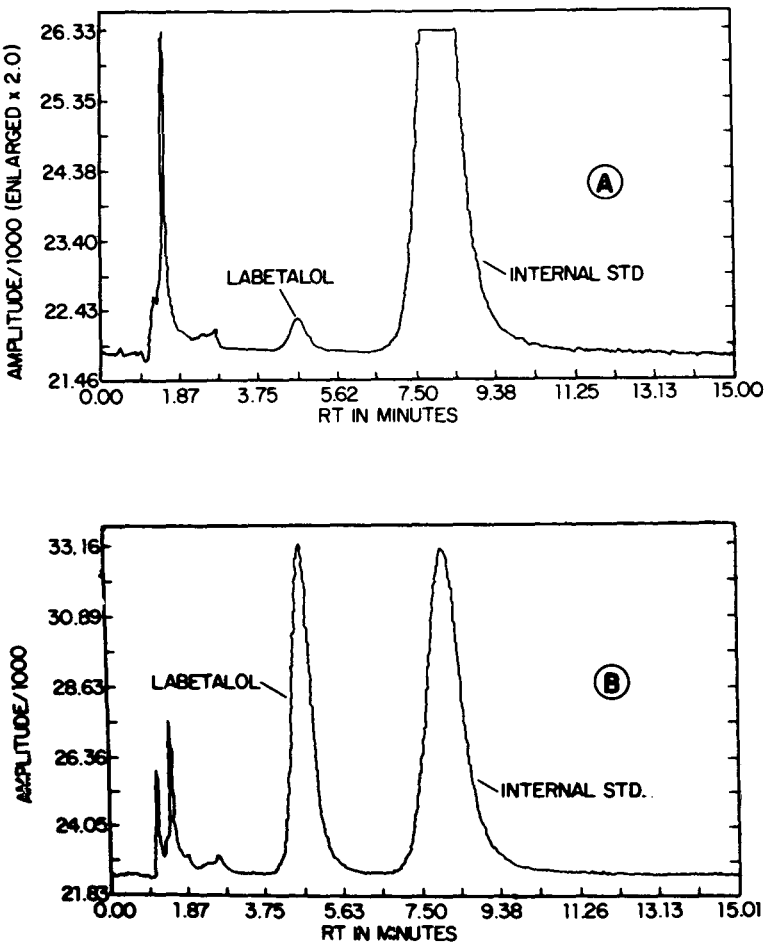

Fig. 3. Computer-reconstructed chromatograms of (A) an extract from plasma spiked to contain 4 ng/ml labetalol and (B) an extract from plasma spiked to contain 100 ng/ml labetalol. Both samples (0.5 ml) were fortified with internal standard to achieve a concentration of 200 ng/ml.

TABLE I

MEAN PERCENTAGE RECOVERY OF LABETALOL AND THE INTERNAL STANDARD FROM HUMAN PLASMA AT VARYING LABETALOL CONCENTRATIONS

n	Labetalol concentration (ng/ml)	Recovery labetalol* (%)	Internal standard concentration (ng/ml)	Recovery internal standard* (%)
8	4	74.8 ± 5.81	200	71.0 ± 5.28
6	100	76.9 ± 5.80	200	70.9 ± 5.04
6	300	74.6 ± 1.73	200	70.4 ± 1.22
		$\bar{X}$ = 75.4 ± 4.81		$\bar{X}$ = 70.8 ± 4.17

*$\bar{X}$ ± S.D.

to 0.998. The *Y*-intercept, which was not significantly different from zero at the 95% confidence interval, was determined to be $8.63 \cdot 10^{-6}$. The slope of the linear response was calculated to be 0.003346 $(ng/ml)^{-1}$.

Extraction efficiency

The average recovery of labetalol from plasma samples to which drug standard had been added at concentrations ranging from 4 to 300 ng/ml was determined to be 75.4 ± 4.81% S.D. (Table I). The internal standard (200 ng/ml) was extracted with a mean efficiency equal to 70.8 ± 4.17% S.D. Statistical analysis by single level ANOVA demonstrated that there were no significant differences ($P > 0.5$) among the mean recoveries for labetalol nor were there significant differences in the recovery of the internal standard as a function of labetalol concentration. These data, therefore, suggest that there is no concentration dependence on extraction efficiency over the range of drug levels in plasma which was investigated.

Intra-assay accuracy and precision

Concentration estimates from plasma spiked to contain 4, 100, and 300 ng/ml labetalol are shown in Table II. The relative accuracy (percentage bias) of

TABLE II

INTRA-ASSAY PRECISION AND ACCURACY OF LABETALOL QUANTITATION FROM HUMAN PLASMA BY HPLC

Theoretical concentration (ng/ml)	*n*	Mean observed concentration (ng/ml)	C.V. (%)	Percentage bias*
4.0	8	3.86	2.97	−3.50
100.0	6	100.11	1.23	0.11
300.0	6	307.44	1.10	2.48
			$\bar{X}$ = 1.77	

*Percentage bias—relative accuracy.

these determinations was found to range from −3.50 to 2.48% with a mean intra-assay precision (C.V.) of ± 1.77%.

Inter-assay accuracy and precision

The inter-assay variability of concentration estimates from plasma spiked with known amounts of drug and processed for labetalol is shown in Table III. These data reflect the variability in labetalol concentration which were determined for each group after extraction and HPLC analysis on two different days. As can be seen in Table III, the inter-assay precision did not exceed ± 4.6% while the relative accuracy of labetalol quantitation from plasma ranged from −3.50 to 0.30% for all concentration groups. These data suggest that the method can reliably quantify labetalol concentrations (4—300 ng/ml) on a day-to-day basis.

TABLE III

INTER-ASSAY PRECISION AND ACCURACY OF LABETALOL QUANTITATION FROM HUMAN PLASMA BY HPLC

Theoretical concentration (ng/ml)	*n*	Mean observed concentration (ng/ml)	C.V. (%)	Percentage bias*
4.0	14	3.86	4.09	−3.50
100.0	12	100.30	1.91	0.30
300.0	12	300.02	4.59	0.01
			$\bar{X}$ = 3.53	

* Percentage bias — relative accuracy.

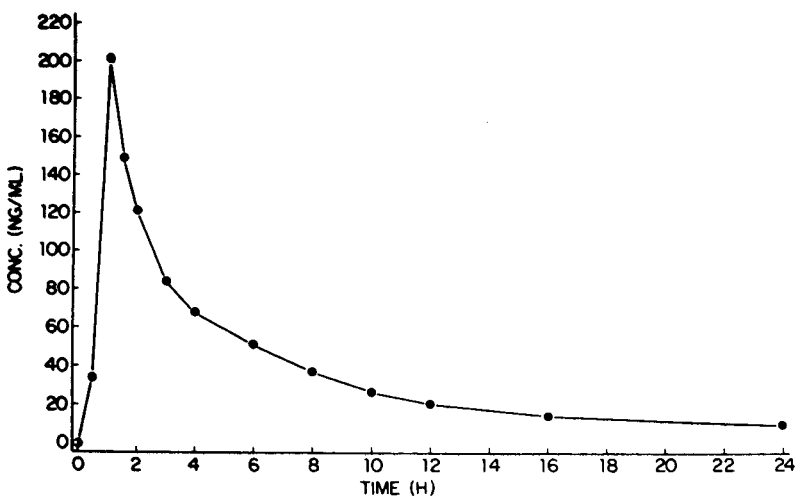

Fig. 4. Plasma concentration—time curve for a subject who received (per os) 300 mg of labetalol.

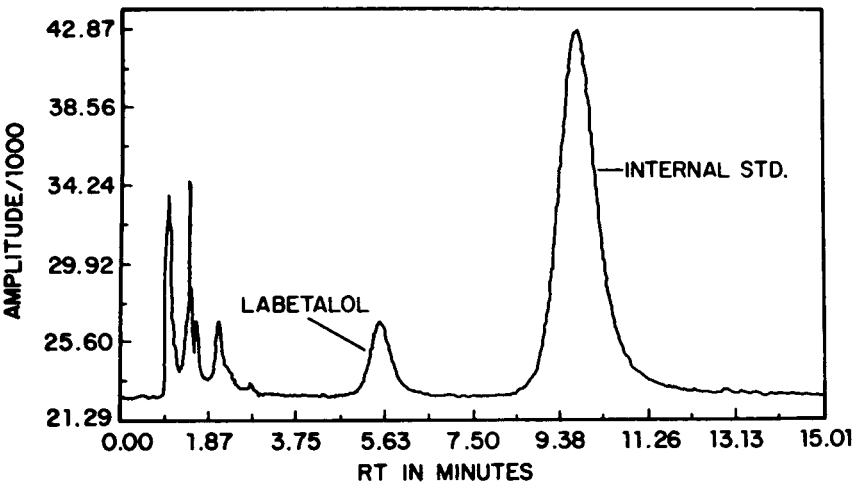

Fig. 5. Computer-reconstructed chromatogram of a 12-h plasma extract which was determined to contain 20.7 ng/ml labetalol.

Assay feasibility

A plasma concentration—time curve is shown in Fig. 4 for a subject who received (per os) 300 mg of labetalol. Duplicate aliquots (0.5 ml) were extracted and analyzed by HPLC as described herein. A chromatogram generated by a 12-h plasma extract (20.7 ng/ml) from this subject is illustrated in Fig. 5. Concentrations of unchanged labetalol remained in excess of the validated lower limit of quantitation (4 ng/ml) throughout the first 24 h of plasma collection. The relative standard deviation of the duplicate analyses ranged from ± 0.04 to ± 5.43%.

CONCLUSIONS

In summary, an HPLC method for the quantitative determination of labetalol in human plasma has been validated for concentrations ranging from at least 4 to 300 ng/ml. This assay takes advantage of a novel separation of labetalol and the internal standard on an analytical column commercially packed with a macroporous co-polymer (PRP-1). Since the support of this column is stable with a moderately basic (pH 9.5) mobile phase, optimized response of labetalol to fluorometric detection can be achieved without post-column alkalination. The increased sensitivity and simplicity of this procedure represents a significant advantage over previously published methods.

Drug agents such as hydrochlorothiazide, trichlorothiazide and propranolol, which may be administered concomitantly with labetalol, demonstrated no potential for interference. This method has been shown to provide quantitatively accurate and precise determinations for labetalol in human plasma and can be routinely employed following the administration of therapeutically effective doses to man. This method is also suitable for quantitating the plasma levels of dilevalol (Sch 19927), the *RR* isomer of labetalol.

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Journal of Chromatography, 311 (1984) 329—337
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2259

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ^{14}C-LABELLED TOLOXATONE AND ITS METABOLITES

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(First received April 10th, 1984; revised manuscript received June 29th, 1984)

SUMMARY

A method for the analytical and micropreparative separation of toloxatone and its urinary metabolites in man is described. Toloxatone was given as an aqueous solution and was labelled with ^{14}C. Following solvent extraction of urine, before and after enzymatic hydrolysis, one-step thin-layer chromatography on silica gel in combination with reversed-phase high-performance liquid chromatography, gave a good micropreparative separation for mass spectrometric analysis. After lyophilization of the high-performance liquid chromatographic fractions, the purity of the metabolites was checked by thin-layer chromatography. Acetic acid was chosen to regulate the pH of the mobile phase (acetonitrile—water) because it can be easily removed by lyophilization when a preparative separation is desired. The retention times as a function of the pH have been evaluated. Formic acid is also proposed for the optimization of the high-performance liquid chromatographic analysis. The quantitative analysis of ^{14}C-labelled toloxatone and its metabolites was carried out, after solvent extraction of 2 ml of urine, using the same high-performance liquid chromatographic method with off-line and flow-through radioactivity detection.

INTRODUCTION

Toloxatone, 5-(hydroxymethyl)-3-(3-methylphenyl)-2-oxazolidinone (Fig. 1) is a reversible inhibitor of monoamine oxidase A [1, 2] which possesses an antidepressant activity [3—5]. Previous studies in man [6] have shown that the drug is extensively metabolized, the majority of the dose being excreted in the urine. Two metabolites have been isolated from urine: 5-(hydroxymethyl)-3-(3-carboxyphenyl)-2-oxazolidinone (metabolite 1) and 5-(hydroxymethyl)-3-(4-hydroxy-3-methylphenyl)-2-oxazolidinone (metabolite 2). Toloxatone and metabolite 2 were also excreted in urine as conjugates with glucuronic acid. The structure of these metabolites was confirmed by comparison with the

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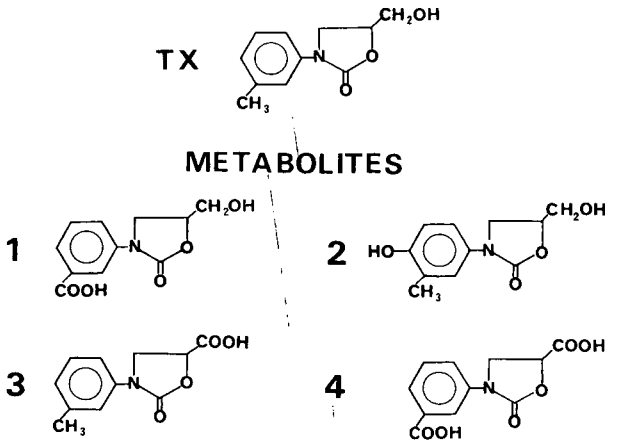

Fig. 1. Chemical structures of toloxatone (TX) and its metabolites.

synthetic compounds. Two other metabolites were isolated by thin-layer chromatography (TLC) but remained unidentified. Further studies showed that, even with successive TLC analyses, the purity of the two unknown metabolites was not suitable for identification by mass spectrometry (MS). Therefore, combined TLC and high-performance liquid chromatography (HPLC) were attempted for the qualitative and quantitative analysis of the metabolites.

MATERIALS AND METHODS

Chemicals and reagents

Toloxatone labelled with ^{14}C in the carbonyl group of the oxazolidinone ring was synthesized by the chemical and radioisotope division of ICN. The radiochemical purity was > 99% after TLC analysis with solvent system 1 (see Table I) and with the system benzene—methanol—acetone (90:10:5, v/v/v). The specific activity was 0.344 μCi/mg.

HPLC-grade water for preparative HPLC analysis was used (Fisons Scientific Apparatus, Loughborough, U.K.). Pro analysi acetic acid, formic acid, orthophosphoric acid (E. Merck, Darmstadt, F.R.G.), acetonitrile HPLC S grade (Rathburn Chemicals, Walkerburn, U.K.), ethyl acetate "RPE-ACS" grade and chloroform RPE (Carlo Erba, Milan, Italy) were used.

Enzymatic hydrolysis was carried out with β-glucuronidase—arylsulphatase (H_1, Sigma, St. Louis, MO, U.S.A.) (2 mg/ml of urine).

HPLC analysis

HPLC was performed with a Micromeritics system (752 gradient programmer, 750 pump, 786 variable-wavelength detector), a WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.), coupled to a Hewlett-Packard 3390A integrator and a Perkin-Elmer 56 recorder. A 25 × 0.46 cm CP-TM-Microspher ODS 3-μm column (Chrompack, Middelburg, Netherlands) was used at a flow-rate of 1 ml/min. The ultraviolet (UV) detector was set at 240 nm. A guard column of 7.5 × 0.21 cm (Chrompack packed guard column, reversed-phase type B) was mounted directly on the head of the analytical column.

UV detection was combined with off-line radioactivity measurements (0.5—1 ml fractions) by liquid scintillation counting (Intertechnique SL 3000, Roche Bioelectronique, Velizy, France) with 10 ml of Unisolve (Koch-Light Labs., Colnbrook, U.K.).

Radioactivity was also determined with a flow-through radioactivity detector (Flo-One HS, Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.) coupled to the UV detector of the HPLC system. Lumapack (Kontron Analytique, Trappes, France) was used as scintillation fluid at a flow-rate of 3 ml/min.

Sample preparation for isolation of metabolites

Toloxatone and its metabolites were isolated from urine (0—12 h fraction) after oral administration of an aqueous solution of [^{14}C]toloxatone (200 mg, specific activity 0.344 μCi/mg) to six healthy adult volunteers. Urine (200 ml) was extracted at pH 1 (1 *M* hydrochloric acid) with ethyl acetate (3 × 500 ml) before and after enzymatic hydrolysis (37°C, pH 5.5, 16 h). The solvent was evaporated at 30°C under reduced pressure (Rotavapor, Büchi, Flawil, Switzerland). The dry residue was dissolved in methanol and applied on silica gel F_{254} plates 20 cm × 20 cm (0.25 mm, Merck). Toloxatone, 150 μgequiv./cm, was spotted in a band of 18 cm using a Linomat III (Camag, Müttenz, Switzerland) and the plates were developed with solvent system 1 (Table I).

TABLE I

SOLVENT SYSTEMS FOR THE TLC SEPARATION

1 Chloroform—methanol—water	65:35:5
2 Toluene—methanol—acetone	50:50:10
3 Toluene—ethanol	50:50
4 Toluene—methanol—acetone	80:20:5
5 Chloroform—acetone	50:50

After detection of the radioactive bands on NS-2T film (Kodak, Rochester, NY, U.S.A.), the silica gel corresponding to the bands was scraped off the plates and toloxatone and its metabolites were recovered from the silica gel with methanol (3 × 5 ml). The fractions isolated by TLC (system 1) were further purified by HPLC; methanol was evaporated at 30°C under reduced pressure, the dry residue was dissolved in water (≃5 μgequiv./μl) and 250 μgequiv. of toloxatone were injected onto the HPLC column. The HPLC fractions containing the radioactivity were collected and the mobile phase was frozen and freeze-dried. The dry residue was dissolved in acetonitrile to a concentration of 1 μgequiv./μl toloxatone for identification by MS. Before MS analysis, the purity of each fraction was verified by TLC analysis (single spot with the solvent systems given in Table I).

Sample preparation for quantitative analysis of labelled drug and metabolites

Analysis of unconjugated compounds. A 2-ml volume of urine (0—12 h fraction) was dispensed into a conical tapered glass tube and the pH was adjusted to 1 with 1 *M* hydrochloric acid. The sample was extracted successively with 5 ml of chloroform and with 3 × 5 ml of ethyl acetate on a mechanical

shaker (15 min). After centrifugation (10 min at 1000 *g*), the organic phase was transferred into a separate tube and evaporated to dryness in a water bath at 40°C under a stream of nitrogen. The two dry residues (chloroform and ethyl acetate) were dissolved in 1 ml of water and 50—100 μl were injected into the HPLC system.

Analysis of conjugated compounds. The same procedure was followed after enzymatic hydrolysis (37°C, pH 5.5, 16 h) to determine the metabolites conjugated with glucuronic acid.

RESULTS AND DISCUSSION

Optimization of the HPLC analysis for isolation of metabolites

It has been shown that reversed-phase HPLC with gradient elution is a rapid and powerful method for the separation of urinary acids [7]. Off-line identification by MS with direct inlet methods is possible with a few micrograms of the compound, but of high purity. For this type of preparative separation, analytical columns can be used with a high efficiency and capacity [8]. However, only volatile salts and acids can be used for the separation, to assure the recovery of the compound from the HPLC fraction [9]. Acetic acid has been used to adjust the pH of the mobile phase in the reversed-phase mode for the preparative separation of polar metabolites [10]. The limitations of its use, however, are worth noting.

The effect of acetic acid on the isocratic mobile phase (water—acetonitrile,

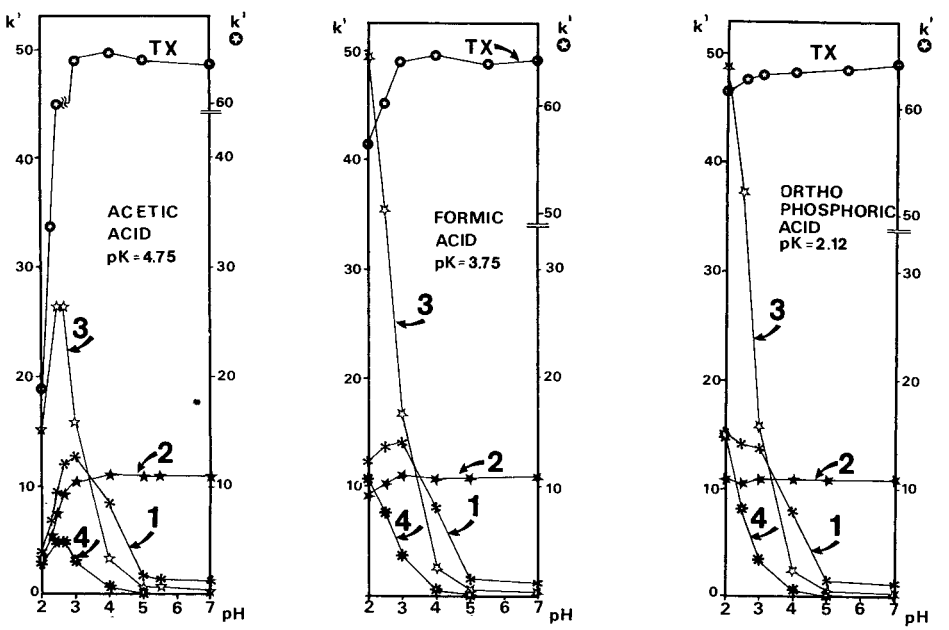

Fig. 2. Effect of pH on the separation (k') of toloxatone (TX) and its metabolites 1, 2, 3 and 4. HPLC analysis was carried out in isocratic conditions (10% acetonitrile); the pH of the water (90%) was adjusted with acetic, formic and orthophosphoric acid. The k' values of toloxatone are given on the right-hand ordinate. $k' = (t_R - t_0)/t_0$.

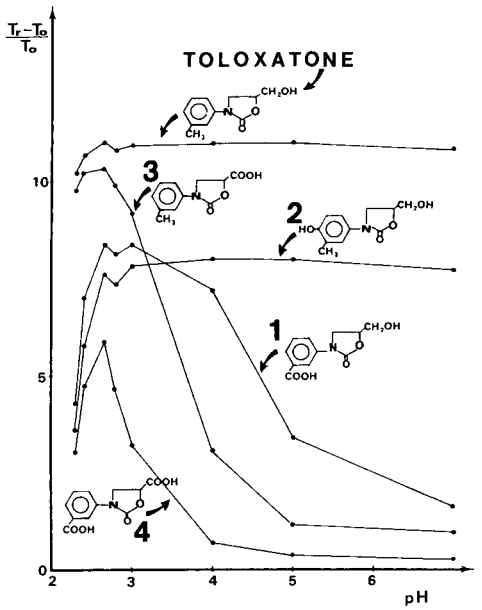

Fig. 3. Effect of pH on the separation of toloxatone and its metabolites 1, 2, 3 and 4. HPLC analysis was carried out with a concave gradient of acetonitrile—water (10:90, v/v, to 40:60, v/v) over 10 min. The pH of the water was adjusted with acetic acid.

90:10, v/v), was investigated. The capacity factor [$k' = (t_R - t_0)/t_0$] of toloxatone and the four metabolites isolated, are shown (Fig. 2) in the pH range 2—7. As expected, no major changes were observed for toloxatone and metabolite 2 between pH 3 and 7, whereas a consistent reduction of k' values was observed for the carboxylic acid derivatives (metabolites 1, 3 and 4). For the acid metabolites, the sigmoid curve predicted by Horváth et al. [11] was not found, since k' values decreased below pH 3. Furthermore, this effect was more pronounced with a lower proportion of acetonitrile in the mobile phase (Figs. 2 and 3).

Since acetic acid is moderately strong (pK 4.75), low pH values (< 3) are reached only with relatively high concentrations of the acid [12]. At this pH most of the acetic acid is in the unionized form, which increases the elution of the compounds analysed and results in a reduction of k' values. This hypothesis was confirmed by the analysis of toloxatone and the four metabolites with formic acid (pK 3.75) and with orthophosphoric acid (pK_1 2.12) (see Fig. 2).

Formic acid, which is also volatile, can be used over a larger pH range than acetic acid, whereas orthophosphoric acid is not recommended because of difficulty in its removal from the sample.

In the present study, optimization of the HPLC analysis was obtained with a concave gradient (No. 9 on the 752 gradient programmer; see Fig. 4) of water (pH 2.7) and acetonitrile (10 to 40% acetonitrile in 10 min). The pH 2.7 (≈1%, v/v, acetic acid in water) corresponded to a good separation of the five compounds (Fig. 3).

Urinary excretion of toloxatone and metabolites

Between 86% and 95% of the dose was excreted by the subjects in the 0—12 h urine fraction after administration of toloxatone. The micropreparative separation of toloxatone and its metabolites was carried out on this fraction. The combination of silica gel TLC and reversed-phase HPLC allowed a good separation of toloxatone and its metabolites whose purity was confirmed by TLC (solvent systems showed in Table I) either with autoradiography or UV detection at 254 nm.

Before enzymatic hydrolysis, 60—65% of the radioactivity administered was extracted with chloroform and ethyl acetate and four major compounds were isolated after TLC (solvent system 1): metabolites 1 (R_F 0.35), 2 (R_F 0.52), 3 (R_F 0.22) and 4 (R_F 0.09).

After hydrolysis with β-glucuronidase—arylsulphatase a fifth band corresponding to toloxatone (R_F 0.62) was isolated after TLC; in this case, the radioactivity extracted from urine corresponded to 75—80% of the dose. After enzymatic hydrolysis, the radioactivity extracted with chloroform and ethyl acetate did not account for the total (86—95%). The difference could be attributed to incomplete enzymatic hydrolysis or to a metabolite which is not conjugated with glucuronic and/or sulphuric acid [6].

Fig. 4 shows typical HPLC chromatograms of samples containing approx. 200 μg of toloxatone equivalents of metabolites 1 and 3. MS analysis of the HPLC radioactive fractions by direct probe insertion into the mass spectrometer, in the electron-impact or chemical-ionization (ammonia) mode, or by gas chromatography—mass spectrometry (GC—MS) after derivatization with trimethylanilinium hydroxide (methylation) or N,O-bis(trimethylsilyl)tri-

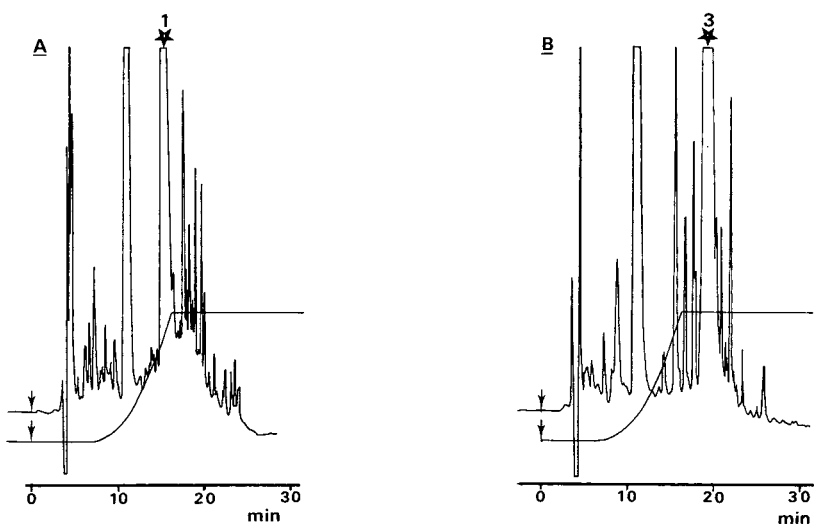

Fig. 4. HPLC chromatograms obtained after TLC separation of urine extracts with ethyl acetate. Urine was hydrolysed with β-glucuronidase—arylsulphatase. The fractions analysed contained ≃ 200 μg of toloxatone equivalent of metabolite 1 (A) and ≃ 200 μg of toloxatone equivalent of metabolite 3 (B). The concave gradient (10 min) was started at the time of injection. The isocratic run (6 min) corresponds to the volume between the gradient programmer and the column at 1 ml/min. Radioactivity peaks in the UV signal are starred (⋆).

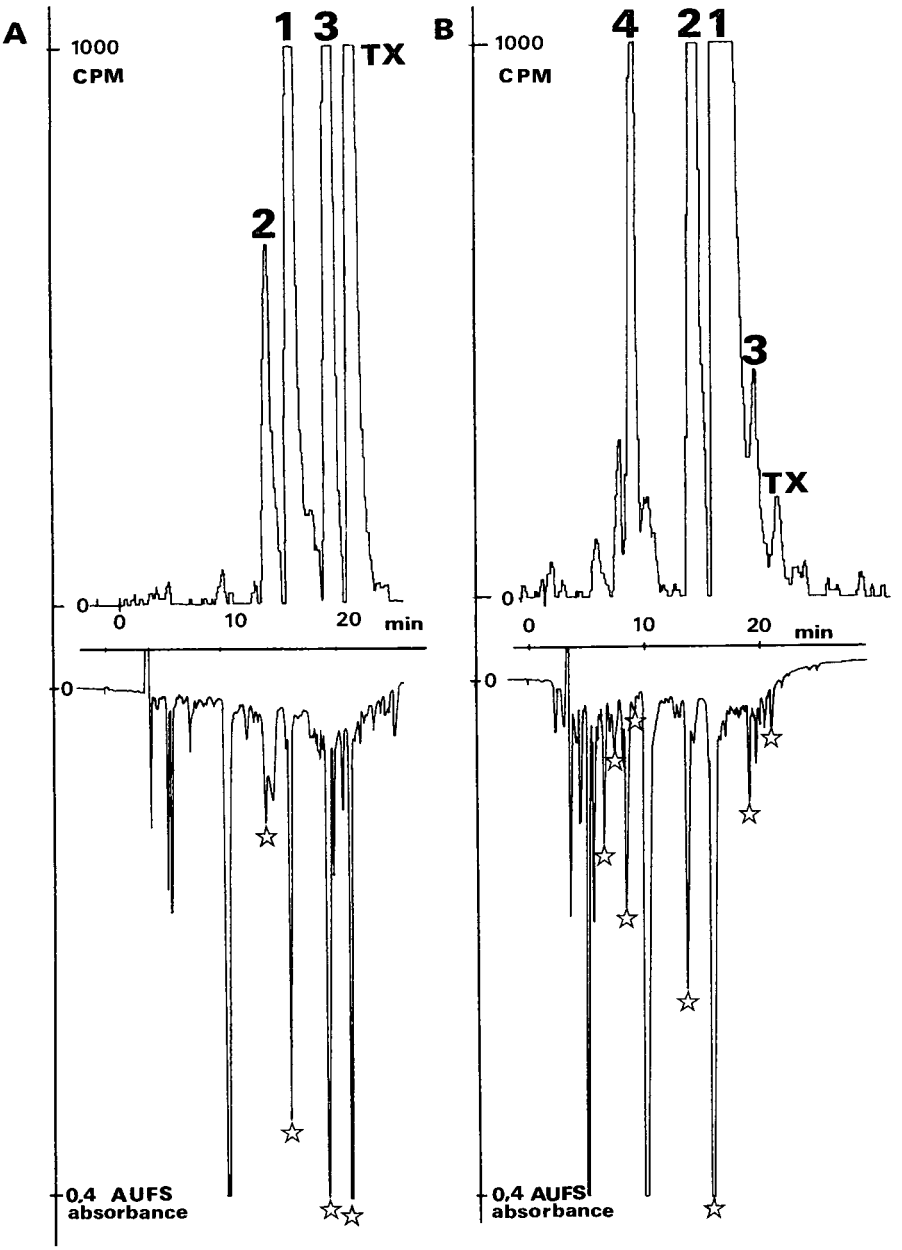

Fig. 5. HPLC chromatograms of urine after hydrolysis with β-glucuronidase—arylsulphatase, extracted with chloroform(A) and with ethyl acetate (B). The peaks of radioactivity, relative to toloxatone (TX) and its metabolites 1, 2, 3 and 4, determined in the UV signal are starred (☆).

fluoroacetamide (silylation), allowed the identification of the metabolites shown in Fig. 1.

The structures were also confirmed by high-resolution MS analysis. MS analysis of the carboxylic acid metabolites 1, 3 and 4 was not possible by

direct introduction into the ion source when HPLC separation of the metabolites was carried out with distilled water. The presence of unidentified impurities prevented the desorption of these compounds, whereas, after methylation or silylation, the same samples were easily analysed by GC—MS. This problem was avoided using commercial HPLC-grade water.

Quantitative analysis of ^{14}C-labelled toloxatone and its metabolites

Optimization of the HPLC analysis allowed the quantitation of toloxatone and its metabolites after the extraction of 2-ml urine samples; 50—100 μl of the aqueous solution of the urine extracts (see sample preparation) were injected into the HPLC system. Both the chloroform and the ethyl acetate extracts were chromatographed and the radioactivity was determined by liquid scintillation counting in each HPLC fraction (0.5—1 ml). Toloxatone and metabolite 3 were extracted principally with chloroform, whereas metabolites 1, 2 and 4 were well extracted with ethyl acetate (Fig. 5).

After the HPLC analysis of each 0—12 h urine fraction the recovery of the total radioactivity injected on the HPLC column was > 98%. The quantitative analysis was obtained without internal standard. The radioactivity counted for toloxatone and its metabolites, after HPLC analysis of the chloroform and the ethyl acetate extracts, was converted to percentage of the dose excreted for each subject; the results are shown in Fig. 6.

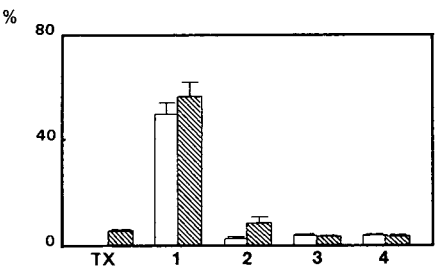

Fig. 6. Urinary excretion profile of ^{14}C-labelled toloxatone (TX) and its metabolites 1, 2, 3 and 4 in 0—12 h post dose urine. Mean ± S.D. values in six subjects, before (open bars) and after (hatched bars) hydrolysis with β-glucuronidase—arylsulphatase, are shown.

Toloxatone and metabolite 2 were excreted in urine mostly as conjugates, whereas metabolites 1, 3 and 4 were principally excreted in the free form. The determination of the radioactivity was repeated with a flow-through radioactivity detector (Flo-One HS). The values obtained with both techniques were similar but direct quantitation with the flow-through detector resulted in a considerable reduction of the overall time of analysis. In addition, a smaller volume of scintillation fluid was used.

In conclusion, this work describes a simple and efficient technique for the isolation of urinary metabolites of ^{14}C-labelled toloxatone in man. The combination of one-step silica gel TLC with reversed-phase HPLC purification is proposed as a general approach for the isolation of polar metabolites in urine, particularly for carboxylic acid derivatives.

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Journal of Chromatography, 311 (1984) 339—346
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2267

DETERMINATION OF A NEW ORALLY ACTIVE CEPHALOSPORIN IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED COLUMN SWITCHING

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(First received April 20th, 1984; revised manuscript received June 26th, 1984)

SUMMARY

A sensitive method for the determination of a new cephalosporin in human serum and urine is described. The sensitivity of the procedure is derived from a high-performance liquid chromatographic separation which utilizes the different selectivities of two columns. Partial separation of the agent from deproteinized serum or diluted urine is achieved by an anion-exchange column. To concentrate the large volume of the eluent fraction containing the compound from the anion-exchange column, a reversed-phase short column is placed between the anion-exchange column and a reversed-phase analytical column. The separation is completed by switching the eluent fraction containing the compound from the second column to the analytical column. The compound is detected by ultraviolet absorption at 295 nm. Quantitation is possible down to 0.05 μg/ml using 300 μl of serum and down to 0.5 μg/ml using 50 μl of urine. The coefficients of variation of the method are 6.8% and 0.6% in serum when spiked at the 0.05 μg/ml and 1.0 μg/ml level, respectively. One assay can be completed in 16 min. Serum levels and urinary excretion data obtained with this method are given for three healthy volunteers who had received a 100-mg oral dose of the compound.

INTRODUCTION

(6*R*,7*R*)-7-[(*Z*)-2-(2-Amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo-(4,2,0)-oct-2-ene-2-carboxylic acid* (hereinafter abbreviated as I) is a new orally active semisynthetic cephalosporin (Fig. 1). The compound differs in structure from commercially available materials which are cephalexin-type analogues. The antibacterial activity of I

*Industrial abbreviation FK-027.;

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against the most commonly isolated gram-negative bacteria is higher than that of other orally active β-lactam antibiotics such as cephalexin, cefaclor and amoxicillin [1].

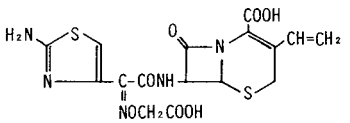

Fig. 1. Chemical structure of compound I.

In recent years, many high-performance liquid chromatographic (HPLC) methods have been reported for the determination of different cephalosporins in biological fluids. The reversed-phase mode using ultraviolet (UV) detection is most commonly used, with precipitation of serum proteins prior to injection [2—10]. These procedures were tried for the determination of I in serum, but the sensitivity was limited to 0.5 μg/ml which was insufficient to study the pharmacokinetics of the compound after oral administration of the usual clinical dose in man. Compound I, a highly polar and water-soluble compound, cannot be extracted from biological fluids by techniques such as conventional two-phase liquid—liquid extraction which works with cefazolin, cephalothin, cefamandole, cefoxitin, cefuroxime, cefotaxime and cefoperazone [11], or by ion-pair extraction with quaternary ammonium salts which works with cephalothin [12]. In addition, I lacks a primary amino function for reacting with fluorogenic reagents such as *o*-phthaldialdehyde and fluorescamine [13, 14].

This paper describes a sensitive method for the determination of I in human serum and urine. The method is based on HPLC column switching using an anion-exchange column and a reversed-phase column.

EXPERIMENTAL

Reagents and materials

Compound I was prepared by Fujisawa Pharmaceutical (Osaka, Japan). Methanol and acetonitrile of UV grade were used. Water used for all solutions and mobile phases was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). All other solvents and reagents were of analytical-reagent grade.

Standard solutions of I were prepared by dissolving the agent in 1/15 *M* phosphate buffer (pH 7.0) and diluting to appropriate concentrations.

Apparatus

All analyses were performed using a liquid chromatograph equipped with two solvent delivery pumps (Model 6000A; Waters Assoc., Milford, MA, U.S.A.), an automatic liquid sampler (WISP710B; Waters Assoc.), a variable wavelength UV detector (at 295 nm, UVIDEC 100-III; Japan Spectroscopic, Tokyo, Japan), and an integrator (Data Module; Waters Assoc.) or a 10-mV recorder. The three columns used were an anion-exchange column 1 cm × 4 mm I.D. (TSK-IEX540 DEAE, 5 μm; Toyo Soda, Tokyo, Japan) and two

reversed-phase columns 1 cm × 4 mm I.D. and 15 cm × 4.6 mm I.D. (TSK-LS410 ODS, 5 μm; Toyo Soda). Three-port and six-port switching valves were placed in line, and operated by a digital programmer (Toyo Soda). Fig. 2 shows the arrangement of the apparatus.

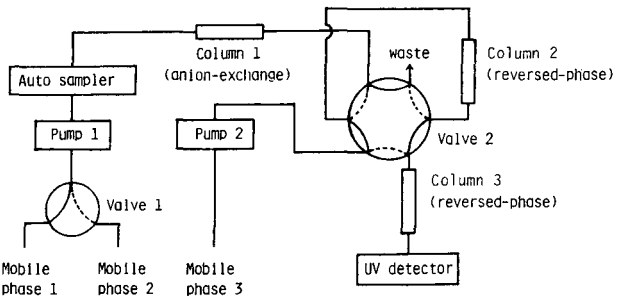

Fig. 2. Flow diagram of automated column switching for analysing I in human serum and urine. Valves 1 and 2 are three-port and six-port switching valves, respectively, and are operated by a digital programmer which is not shown in order to simplify the drawing. The solid and dotted lines in the valves show the off and on modes, respectively.

Mobile phase

Mobile phase 1 consisted of 0.03 *M* ammonium dihydrogen phosphate—phosphoric acid solution (pH 3.5). The solution was prepared from 0.03 *M* ammonium dihydrogen phosphate adjusted to pH 3.5 with dilute phosphoric acid. Mobile phase 2 consisted of 0.5 *M* sodium chloride. Mobile phase 3 consisted of 73% 0.03 *M* ammonium dihydrogen phosphate—phosphoric acid solution (pH 2.5) in methanol for analysis of serum. Mobile phase 3 for analysis of urine was prepared by mixing 150 ml of acetonitrile, 850 ml of water, and 2 ml of 1.8 *M* sulphuric acid. The final composition was acetonitrile—water—1.8 *M* sulphuric acid (15:85:0.2). The mobile phase was deaerated under vacuum before use. The flow-rate of both mobile phase 1 and mobile phase 2 was 2.0 ml/min and that of mobile phase 3 was 1.0 ml/min.

Sample preparation

To a 1.5-ml polypropylene tube (Eppendorf, Hamburg, F.R.G.) containing 300 μl of serum were added 30 μl of 1/15 *M* phosphate buffer (pH 7.0) and 600 μl of ethanol. The mixture was shaken in a vortex mixer, allowed to stand for 5 min, and centrifuged with an Eppendorf centrifuge (Model 5412; Eppendorf) for 1 min. An 80-μl aliquot of the supernatant was injected into the liquid chromatograph.

To a 1.5-ml polypropylene tube containing 1 ml of urine diluted twenty-fold or more, were added 100 μl of 1/15 *M* phosphate buffer (pH 7.0). The mixture was shaken in the vortex mixer and centrifuged with the Eppendorf centrifuge for 1 min; 40 μl of the supernatant were injected into the liquid chromatograph.

Timing of column switching

The timing of automated column switching for determination of I in the serum and urine is shown in Fig. 3. At 0 min, valves 1 and 2 were

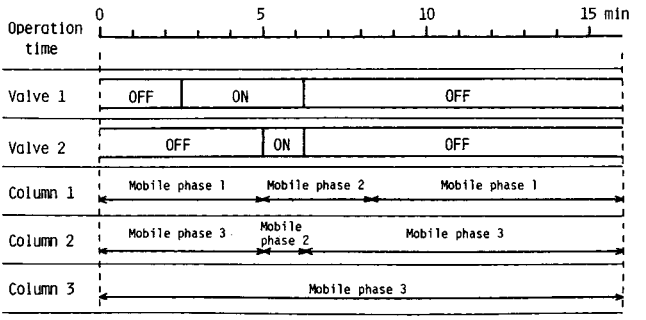

Fig. 3. Timing of automated column switching for analysing I in human serum and urine. Time lag between switching of valve 1 and switching of mobile phase in column 1 arises from a dead space between valve 1 and pump 1 (see Fig. 2.).

positioned (off) so that mobile phase 1 flowed through the anion-exchange column (column 1) to waste and mobile phase 3 flowed through the reversed-phase short column (column 2) and reversed-phase analytical column (column 3) to the UV detector. The sample was injected onto column 1 on which partial separation of I and other substances in the biological fluids was achieved. Valve 1 was switched on at 2.5 min after injection, but column 1 was still eluted with the mobile phase 1 remaining in the dead space between valve 1 and pump 1. At 5 min after injection when I began to desorb from column 1, valve 2 was switched on so that the effluent from column 1 flowed through column 2 to waste. Mobile phase 3 then flowed through column 3 to the UV detector. I was concentrated at the head of column 2 by aqueous mobile phase 2. At 6.25 min after injection, valves 1 and 2 were turned back to the original position (off), and mobile phase 3 then flowed through columns 2 and 3 where further separation of I and other substances in the biological fluid was achieved. Column 1 was then eluted with mobile phase 2 to wash out the substances remaining in column 1. At 8.25 min after injection, mobile phase 1 flowed through column 1 to give equilibration for the next analysis. The injection interval for this procedure was 16 min.

Quantitation

The procedure was standardized by analysing blank serum or urine samples to which had been added 30 or 100 μl of I standard solution instead of 30 or 100 μl of 1/15 *M* phosphate buffer (pH 7.0) as in the sample preparation. The peak height of I was used to establish the calibration graph for the serum and urine samples. The calibration graph was fitted to a $Y = aX + b$ equation by the least-squares method, and the concentrations in the unknown samples were calculated using the calibration graph.

Clinical study

A clinical study was performed on three healthy volunteers given an oral dose of a 100-mg capsule of I. Serum samples were obtained from blood collected by venipuncture at designated intervals and stored at −20°C until analysed. The total urine output was collected at intervals of 0—2, 2—4, 4—6,

6—8, 10—12, 12—23, and 23—24 h. The urine volumes were measured, and aliquots were kept at −20° C until analysed.

RESULTS

Separation

Typical chromatograms obtained from the human serum and urine samples are shown in Figs. 4 and 5. As shown in Figs. 4A and 5A, the background peaks of blank human serum and urine were few and almost completely separated from those of I. Figs. 4C and 5C show typical chromatograms of the serum and urine samples from a healthy volunteer after an oral dose of 100 mg of I. In these chromatograms there were no interferences at the retention times of I.

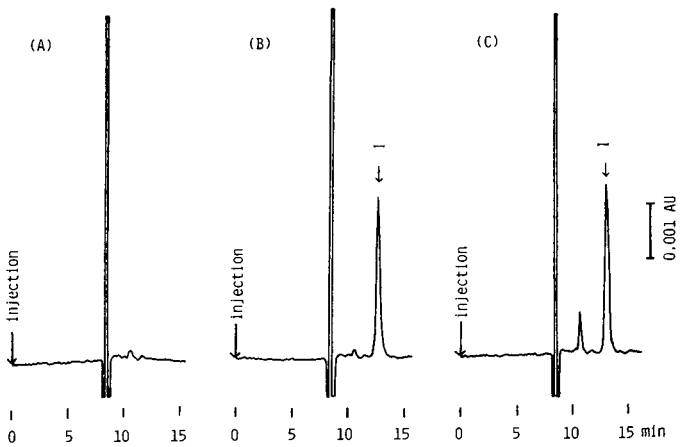

Fig. 4. Chromatograms of (A) blank human serum, (B) human serum containing 1 μg/ml of I, and (C) serum collected from a healthy volunteer after an oral dose of a 100-mg capsule of I (calculated concentration of I was 1.147 μg/ml).

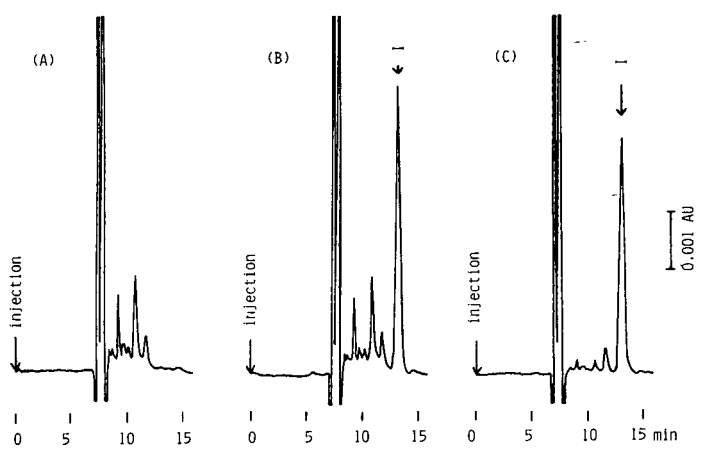

Fig. 5. Human urine (twenty-fold dilution) chromatograms: (A) blank urine, (B) urine containing 25 μg/ml of I, and (C) urine collected from healthy volunteer after an oral dose of a 100-mg capsule of I (calculated concentration of I was 23.3 μg/ml).

Recovery

Sample recovery of I from spiked solutions prepared with biological fluids was compared with that of 1/15 *M* phosphate buffer (pH 7.0). The values obtained from five replicate analyses of spiked samples were 91.8 ± 0.6% (mean ± S.D.) for serum (1 μg/ml of I) and 101.4 ± 0.4% for urine (25 μg/ml of I).

Calibration graph

Typical calibration graphs for human serum and urine are shown in Table I. All calibration graphs show good linearity in each range. The lower limits of sensitivity were 0.05 μg/ml for serum and 0.5 μg/ml for urine, with a signal-to-noise ratio of 3.

TABLE I

TYPICAL REGRESSION DATA FOR CALIBRATION CURVES IN HUMAN SERUM AND URINE

Sample	Concentration range (μg/ml)	Slope	Intercept	Correlation coefficient
Serum	0.05—10.0	7.0568	0.0364	0.9999
Urine	0.50—250	0.5029	0.0312	0.9999

Reproducibility

Reproducibility was evaluated by performing five replicate analyses of spiked serum and urine samples. The results are given in Table II. The coefficients of variation were 0.6% and 0.4% when 1.0 μg/ml and 25.0 μg/ml were spiked in the serum and urine, respectively. The respective values were 6.8% and 2.3% even when 0.05 μg/ml and 1.0 μg/ml were spiked. The actual concentration of I measured by HPLC ranged from 99% to 101% in the ten samples when spiked at higher concentrations in the serum and urine, and from 92% to 108% in the ten samples even when spiked at lower concentrations. This HPLC method for

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF I IN HUMAN SERUM AND URINE

Parameter	Serum	Serum	Urine	Urine
Actual concentration (μg/ml)	0.050	1.000	1.00	25.0
Number of analyses	5	5	5	5
Mean analysed concentration (μg/ml)	0.050	1.002	1.04	25.0
Percentage of actual concentration	100	100.2	104	100
Range (μg/ml)	0.046—0.054	0.994—1.009	1.01—1.06	24.9—25.1
S.D. (μg/ml)	0.003	0.006	0.02	0.1
Coefficient of variation (%)	6.8	0.6	2.3	0.4

the determination of I in human serum and urine thus provides good accuracy and precision even around the lower limit of sensitivity.

Serum levels and urinary excretion of I in man

Serum levels of I after an oral dose of a 100-mg capsule to healthy volunteers are shown in Fig. 6. The drug peaked in the serum (1.023—1.364 μg/ml) within 4—5 h after dosing, and thereafter decreased slowly to a mean of 0.264 μg/ml (0.191—0.305 μg/ml) at 12 h; 19.7—30.1% of the dose was excreted as the unchanged drug in the 0—24 h urine.

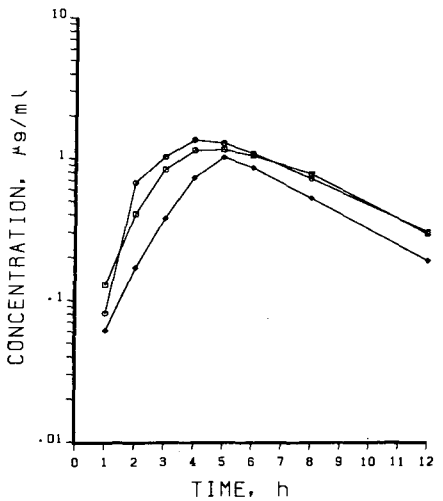

Fig. 6. Serum levels of I in healthy volunteers after an oral dose of a 100-mg capsule of I. (○) Subject A, (□) subject B, (◇) subject C.

DISCUSSION

Early studies on methods for determining I in the serum were carried out by direct injection of deproteinized biological fluids into a reversed-phase HPLC system with UV detection. However, these methods did not separate the background peaks of the biological fluids from that of I. In recent years, HPLC column switching has been found to be a useful technique for the determination of water-soluble materials in biological fluids [15—25]. In our present study, complete separation of the background peaks of biological fluids from that of I (Figs. 4 and 5) was achieved by HPLC column switching using an anion-exchange column and a reversed-phase column. In this system, a reversed-phase short column was placed between the anion-exchange column and the reversed-phase analytical column (Fig. 2) to concentrate the eluent fraction containing I from the anion-exchange column. The use of this short column made it possible to concentrate a large volume (2.5 ml) of the eluent fraction without disturbing the chromatogram on the analytical column (Figs. 4 and 5). The concentration of the large volume of eluent on the short column enhanced the sensitivity and reproducibility of the determination. Only the anion-exchange column has to be changed about every 150 samples. This column can be

easily packed in our laboratory with a small amount of packing material.

The assay was shown to be sufficiently sensitive to quantify I in human serum and urine after oral administration of the clinical dose (Fig. 6). The analysis time of 16 min seems to be acceptable since the method can be used overnight.

ACKNOWLEDGEMENTS

The authors thank Toyo Soda Manufacturing for providing anion-exchange packing material (TSK-IEX540 DEAE). We wish to thank Miss T. Yamashita for her technical assistance during the development of the method.

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Journal of Chromatography, 311 (1984) 347—353
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2269

Note

Whole-cell methanolysis as a rapid method for differentiation between *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*

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Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are Gram-negative, capnophilic, coccobacillary rods indigenous to dental plaque [1]. They have been associated with a number of extraoral infections, e.g. bacterial endocarditis, miscellaneous abscesses, and osteomyelitis (for review, see ref. 2), for which dental plaque often is the source of infection. There are many indications that *A. actinomycetemcomitans* also is implicated as a major pathogen in periodontitis among juveniles (for review, see refs. 2 and 3). The role of *H. aphrophilus* in periodontal disease has not yet been clarified. Unfortunately, the elucidation of this problem is hampered by the fact that there are relatively few tests available in the routine laboratory for distinction between these organisms [4]. To establish more criteria of differentiation, we have previously examined bound cellular fatty acids [5] and fatty acids in whole lipopolysaccharide (LPS) and free lipid A [6] in *A. actinomycetemcomitans* and *H. aphrophilus*, but no marked differences in the fatty acid profiles could be established. Free fatty acids from whole cells differed for some strains [7]. The sugar content of methanolysed and derivatized LPS [8] and whole defatted cells [9] from these species provided more consistent differentiation. Unfortunately, preparation of LPS and whole defatted cells involves rather time-consuming laboratory procedures. The present study, which is based on whole-cell methanolysates, describes a simple and rapid

0378-4347/84/$03.00

TABLE I

PERCENTAGE SUGAR* AND FATTY ACID COMPOSITION OF DERIVATIZED WHOLE-CELL METHANOLYSATES

	Rha	Fuc	Gal	Glc	DD-Hep	LD-Hep	GalN + GlcN	KDO	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:1}$	$C_{16:0}$
Actinobacillus actinomycetemcomitans ATCC 33384 (NCTC 9710)**	0.9	5.0	5.0	11.2	4.7	6.0	2.1	0.4	7.9	5.6	4.9	7.2
Haemophilus aphrophilus ATCC 33389 (NCTC 5906)	1.2	5.1	10.4	23.5	—	5.9	1.8	0.2	10.2	7.0	6.7	7.9

*Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine; GlcN, glucosamine; KDO, 3-deoxy-D-manno-2-octulosonic acid and/or its methanolysis products.
**ATCC, American Type Culture Collection, Rockville, MD, U.S.A.; NCTC, National Collection of Type Cultures, London U.K.

TABLE II

COMPARISON OF RATIOS BETWEEN SELECTED SUGARS* IN EXAMINED BACTERIAL PREPARATIONS

	Whole-cell methanolysates		Whole defatted cells [9]		Lipopolysaccharide [8]	
	Glc/LD-Hep	DD-/LD-Hep	Glc/LD-Hep	DD-/LD-Hep	Glc/LD-Hep	DD-/LD-Hep
Actinobacillus actinomycetemcomitans ATCC 33384	1.9	0.8	2.1	0.8	2.1	0.8
Haemophilus aphrophilus ATCC 33389	4.0		4.0		1.8	

*For abbreviations see Table I footnote.

procedure for accurate distinction between *A. actinomycetemcomitans* and *H. aphrophilus* that is well fitted for the routine laboratory.

MATERIALS AND METHODS

Bacteria

The type specific strains of *A. actinomycetemcomitans* and *H. aphrophilus* were analysed. Sources of isolation and procedures for maintenance and cultivation have been described elsewhere [7].

Methanolysis and derivatization

Whole lyophilized cells were methanolysed by 2 *M* hydrochloric acid in anhydrous methanol for 24 h at 85°C [6] and derivatized with trifluoroacetic anhydride (Fluka) 1:1 in acetonitrile (Rathburn Chemicals, U.K.) [8].

Reference compounds

Sigma (St. Louis, MO, U.S.A.) provided α-D(+)-fucose, D(+)-galactose, α-D(+)-glucose, D(+)-galactosamine, D(+)-glucosamine, D(+)-mannose, and α-L-rhamnose. Natural galactose, glucosamine, L-glycero-D-mannoheptose, mannose, and rhamnose were identified from LPS of *Escherichia coli* [10] and *Salmonella typhimurium* [11] (Sigma). D-Glycero-D-mannoheptose was determined from *Chromobacterium violaceum* [12], provided together with N-glucosamine myristate by Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G. The ammonium salt of 3-deoxy-D-manno-2-octulosonic acid (KDO) (Sigma) was methylated in 2 *M* hydrochloric acid in anhydrous methanol at 85°C for 24 h [6]. Methyl esters of lauric, myristic, palmitic, and palmitoleic acid were obtained from Supelco (Bellefonte, PA, U.S.A.), and 13-methyltetradecanoic acid from Larodan Fine Chemicals (Malmö, Sweden). The methyl ester of racemic 3-hydroxymyristic acid was synthesized [5].

Gas chromatography

This was performed as described previously [8]. The molar response of the trifluoroacetyl derivatives of the detected monosaccharides has previously been given [8].

RESULTS

The distribution of sugars and fatty acids in trifluoroacetyl-derivatized whole-cell methanolysates of *A. actinomycetemcomitans* strain ATCC 33384 and *H. aphrophilus* strain ATCC 33389 is shown in Table I. D-Glycero-D-mannoheptose was detected exclusively in *A. actinomycetemcomitans.* The concentration of galactose and glucose was approximately twice as high in *H. aphrophilus* as in *A. actinomycetemcomitans*, and the amount of KDO and/or its methanolysis products twice as high in *A. actinomycetemcomitans* as in *H. aphrophilus*. In *A. actinomycetemcomitans* the ratio between glucose and L-glycero-D-mannoheptose was 1.9, and between D-glycero- and L-glycero-D-mannoheptose 0.8 (Table II), which agreed fairly well with corresponding

data from whole defatted cells and LPS from this species. The ratio between glucose and L-glycero-D-mannoheptose was 4.0 in both whole-cell methanolysates and in whole defatted cells from *H. aphrophilus*, and 1.8 in LPS from this species.

A. actinomycetemcomitans and *H. aphrophilus* contained the same major fatty acids (Table I). Only small differences were recorded in their quantitative distribution.

The separation of sugars and fatty acids in derivatized whole-cell methanolysates of *A. actinomycetemcomitans* and *H. aphrophilus* is demonstrated on the same chromatogram in Figs. 1 and 2.

Fragmentation of fatty acids and sugars were in agreement with previously published results obtained using gas chromatography—mass spectrometry [7, 8].

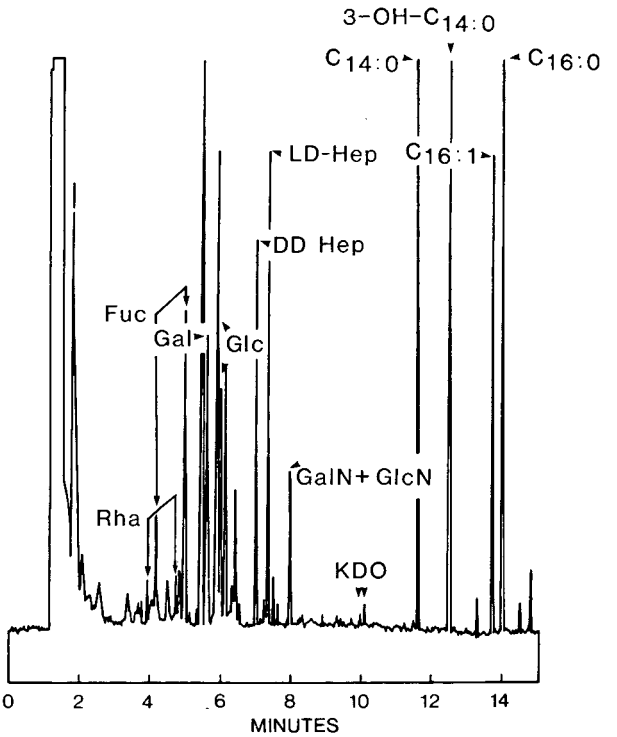

Fig. 1. Gas chromatogram of sugars and fatty acids recovered from trifluoroacetyl-derivatized whole-cell methanolysates of *A. actinomycetemcomitans* strain ATCC 33384 (NCTC 9710). Abbreviations as in Table I footnote.

DISCUSSION

Whole-cell methanolysis, which represents a relatively new approach [13—16], has previously been found useful in taxonomic studies on *Moraxella* and *Neisseria* where fatty acids served as the best differentiating criteria [17—21]. In the whole-cell methanolysates from *A. actinomycetemcomitans* strain ATCC 33384 and *H. aphrophilus* strain ATCC 33389, the composition

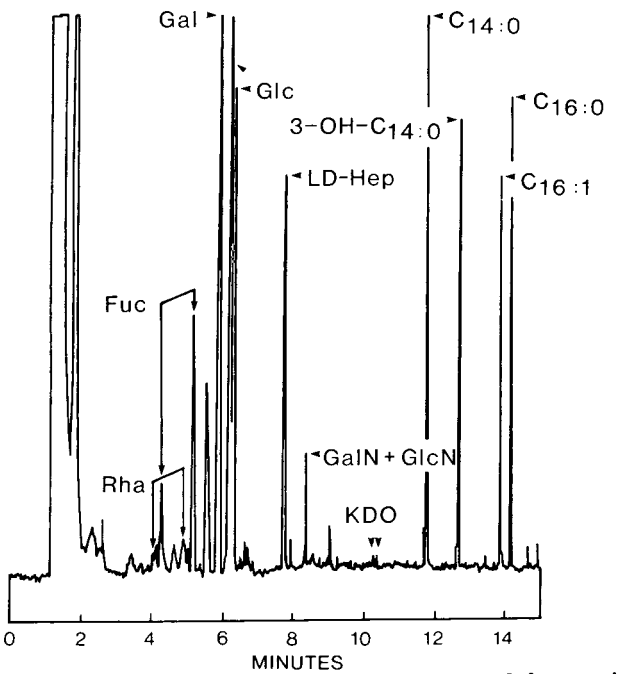

Fig. 2. Gas chromatogram showing sugars and fatty acids recovered from trifluoroacetyl-derivatized whole-cell methanolysates of *H. aphrophilus* strain ATCC 33389 (NCTC 5906). Abbreviations as in Table I footnote.

of cellular fatty acids did not differ markedly. This agreed with our previous observations on bound cellular acids [5], and fatty acids in whole LPS and free lipid A from the same strains [6]. Trifluoroacetyl derivatives of methyl glycosides, on the other hand, provided excellent criteria of differentiation. Whereas *A. actinomycetemcomitans* contained both D-glycero- and L-glycero-D-manno-heptose, *H. aphrophilus* contained exclusively L-glycero-D-mannoheptose. This was in agreement with our previous findings on the sugar composition of LPS [8] and of whole defatted cells [9] from a series of reference as well as laboratory strains of *A. actinomycetemcomitans* and *H. aphrophilus*. D-Glycero-D-mannoheptose may therefore serve as a marker for taxonomic differentiation between these bacteria. Our findings on the distribution of sugars in LPS [8], in whole defatted cells [9], and in whole-cell methanolysates, as well as free cellular fatty acids [7] support the establishment of *A. actinomycetemcomitans* as a species distinct from *H. aphrophilus* in the 1984 edition of Bergey's Manual of Systematic Bacteriology [22].

Methanolic hydrochloric acid is assumed to be a mild and effective agent for cleaving oligosaccharides (for review, see refs. 23 and 24). However, trifluoroacetic acid may attack the methylene group between double bonds causing loss of polyunsaturated components [25]. This may affect cyclopropane fatty acid, which was detected in whole cells [5] and whole LPS and free lipid A [6] of *A. actinomycetemcomitans* and *H. aphrophilus* but not in their whole-cell methanolysates.

The ratio between glucose and L-glycero-D-mannoheptose was approximately the same in whole-cell methanolysates, whole defatted cells, and whole LPS

prepared from *A. actinomycetemcomitans* and in LPS made from *H. aphrophilus* [8, 9]. This suggested that LPS is the primary source of this aldoheptose in *A. actinomycetemcomitans* and *H. aphrophilus*. The higher concentration of galactose and glucose in *H. aphrophilus* than in *A. actinomycetemcomitans* agreed with previous results obtained with whole LPS and whole defatted cells [8, 9].

LPS and whole defatted cells, enabling differentiation by means of D-glycero-D-mannoheptose, seem to be excellent preparations for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*, but they involve rather time-consuming laboratory procedures. Whole-cell methanolysis would therefore be preferable in the routine laboratory. With our experimental set up, little material, i.e. less than 1 mg of lyophilized cells, was needed and both methanolysis and derivatization could be performed rapidly. The peak ratio reproducibility and the stability of the derivatives were good [8, 9].

CONCLUSIONS

(1) D-Glycero-D-mannoheptose may serve as a marker for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

(2) Gas chromatography of trifluoroacetylated whole-cell methanolysates may serve as a rapid method for differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

(3) Establishment of *A. actinomycetemcomitans* in current taxonomy as a species distinct from *H. aphrophilus* is supported.

ACKNOWLEDGEMENTS

This work was supported in part by the Anders Jahres Fond til Vitenskapens Fremme and by the Proprietær Christopher Øverland and Hustru Ingeborg Øverlands Legat. The authors are also indebted to Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G., for generous supply of *Ch. violaceum* and N-glucosamine myristate.

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Journal of Chromatography, 311 (1984) 354—360
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2264

Note

Isocratic analysis of 3-methoxy-4-hydroxyphenyl glycol, 5-hydroxyindole-3-acetic acid and 4-hydroxy-3-methoxyphenylacetic acid in cerebrospinal fluid by high-performance liquid chromatography with amperometric detection

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The study of cerebrospinal fluid (CSF) amine metabolites is important for the clinical investigation of diseases of the central nervous system (CNS). A direct measure of CNS metabolism is essential to test the hypothesis concerning putative neurotransmitter abnormalities in various neurological and psychiatric diseases. The main metabolites found in detectable amounts in the CSF are 3-methoxy-4-hydroxyphenyl glycol (MHPG), 5-hydroxyindole-3-acetic acid (5-HIAA) and 4-hydroxy-3-methoxyphenylacetic acid or homovanillic acid (HVA). These metabolites are derived from noradrenaline (NA), 5-hydroxytryptamine (5-HT), and dopamine (DA), respectively.

Abnormalities in the amounts of metabolites found in CSF have been observed in various psychiatric and neurological conditions. For instance, decreased 5-HIAA accumulation has been observed mainly within a subgroup of depressions [1]. Low CSF 5-HIAA and HVA concentrations are also found in Parkinson's disease [2] and are consistent with the low DA and 5-HT concentration in the basal ganglia from such patients. Altered 5-HIAA and HVA accumulation has also been reported in presenile dementia of the Alzheimer type [3] and epileptic children [4] although the literature is conflicting.

0378-4347/84/$03.00

Clearly there is a need for a sensitive and accurate method of analysis for catecholamine metabolites in order to obtain useful data in such studies.

High-performance liquid chromatography (HPLC) has been widely used to separate catechol- and indoleamines using reversed-phase ion-pair systems. These are described in recent reviews [5, 6], which also deal with the methods of detection. Electrochemical detection provides a high sensitivity which is essential for the detection of low-level endogenous amine metabolites in CSF. There have been numerous reports on the separation of monoamine metabolites in urine [7], rat brain [8], and CSF [9], but many of these required the use of gradient elution with complex buffers. In these studies extraction of the metabolites was also usually necessary before chromatographic analysis.

This paper describes a simple isocratic method for the separation of the main metabolites using a simple eluent and with minimal sample pre-treatment.

EXPERIMENTAL

Materials and reagents

Reference catecholamine metabolites were obtained from Sigma (Poole, U.K.) and were of the highest purity available. Ammonium acetate and glacial acetic acid were AnalaR grade from BDH (Poole, U.K.). Solutions of reference compounds and the mobile phase were prepared in distilled deionized water. Reference compounds were kept on ice during use and were freshly prepared each day.

Apparatus

A Pye Unicam (Cambridge, U.K.) LC3-XP pump and an LCA15 electrochemical detector (EDT Research, London, U.K.) were used. The detector, which is of the wall jet type, employed a glassy carbon working electrode and an Ag/AgCl reference electrode. Samples were injected via a Rheodyne 7125 injector fitted with a 50-μl loop. The chromatographic column (250 mm × 5 mm) was slurry-packed with ODS Hypersil (5-μm Spherical silica, chemically bonded with a monolayer of octadecylsilyl groups) from Shandon Southern Products (Runcorn, U.K.).

Chromatographic conditions

The column was washed with 40% methanol for 30 min and then equilibrated with the mobile phase consisting of 0.1 *M* ammonium acetate, adjusted to pH 5.15 with 3 *M* acetic acid, and 0.27 m*M* EDTA. Mobile phases of pH values ranging from 4.80 to 5.70 have been investigated for the separation. The flow-rate was 1.5 ml/min and the temperature ambient. The mobile phase was continuously degassed with a stream of helium. The peaks were detected amperometrically at a detector sensitivity of 3 nA. The effect of varying the oxidation potential on sensitivity was investigated at pH 5.15.

Samples

Human lumbar CSF were obtained from patients with primary depression diagnosed by the criteria used in the Psychiatry Department. Lumbar punctures were performed between 8.00 and 10.00 a.m. after at least 10 h of fasting and

bed rest in a standardized manner. Spinal fluid (13 ml) was drawn and immediately placed on ice. Spinal fluid was also obtained from patients receiving routine lumbar punctures having various neurological disorders. This study was approved by the Hospital Ethical Committee.

All samples were divided into aliquots for storage at −70°C. Prior to the analysis an aliquot was ultrafiltered [10] through an Amicon PM 10 membrane (NMW 10,000), and then injected directly into the HPLC system.

RESULTS AND DISCUSSION

High-performance liquid chromatography

There are few HPLC techniques capable of simultaneously separating MHPG, 5-HIAA, and HVA from other endogenous compounds in human CSF [11, 12]. One of the major problems is that column efficiency is often poor when traditional buffers, such as phosphate buffers, are used. To overcome this problem acidic amine buffers have been suggested [13] as alternatives for the separation of catecholamine metabolites.

It has been shown [14] that ammonium acetate is a superior masking agent for the residual silanol groups of reversed-phase packings leading to greatly improved column efficiency. It is also compatible with electrochemical detection, being an electrolyte with relatively high electrochemical inertia. Thus, by using ammonium acetate buffer at pH 5.15, efficient separation with sensitive detection of MHPG, 5-HIAA, and HVA can be achieved (Fig. 1A). The applicability of this simple isocratic system is demonstrated by the analysis of CSF samples. The three major amine metabolites are clearly separated from other endogenous substances (Fig. 1B). The parent compounds noradrenaline, adrenaline, and dopamine, as well as 3,4-dihydroxyphenylacetic acid, were eluted before the compounds of interest and were well resolved from these metabolites. We could not obtain similar resolution with phosphate buffers. Solvent degassing is important in electrochemical detection. A reversed-phase system not requiring organic modifier is advantageous as on-line degassing leads to gradual evaporation of the organic modifier resulting in a change of mobile phase composition. The retention of the compounds can be simply controlled by pH and buffer concentration adjustments of the mobile phase without the need for inclusion of organic modifer. EDTA, included in the mobile phase to protect the electrode from metallic contamination, did not affect the retention or resolution of the amines. It can be omitted when an ultraviolet or a fluorescence detector is used.

Effect of pH on retention

The retention of the two acidic metabolites, 5-HIAA and HVA, was significantly affected by the pH of the mobile phase. At lower pH ionization of the acid groups was suppressed, and the molecules were therefore more strongly retained. MHPG is without an acid group and was thus virtually unaffected by pH changes.

Effect of buffer concentration on retention

Increasing the molar concentration of ammonium acetate decreased the

capacity ratios (k') of all three metabolites (Fig. 2). This is probably due to the more effective masking of residual silanol groups by NH_4^+ when higher concentrations of ammonium acetate were used. Although hydrophobic interaction is the main retention mechanism, absorption of solutes onto residual silanol sites may also operate to a lesser extent, judging from these retention behaviours. It is possible to use 0.5 *M* ammonium acetate as the eluent without causing problems to the detector, provided EDTA is present.

Optimum detection potential

The voltammograms of the three metabolites are shown in Fig. 3; whereas useful detection begins at +0.70 V for MHPG and HVA, 5-HIAA can be oxidised at lower potentials. An operating potential of +0.85 V was chosen for the simultaneous sensitive detection of all three compounds.

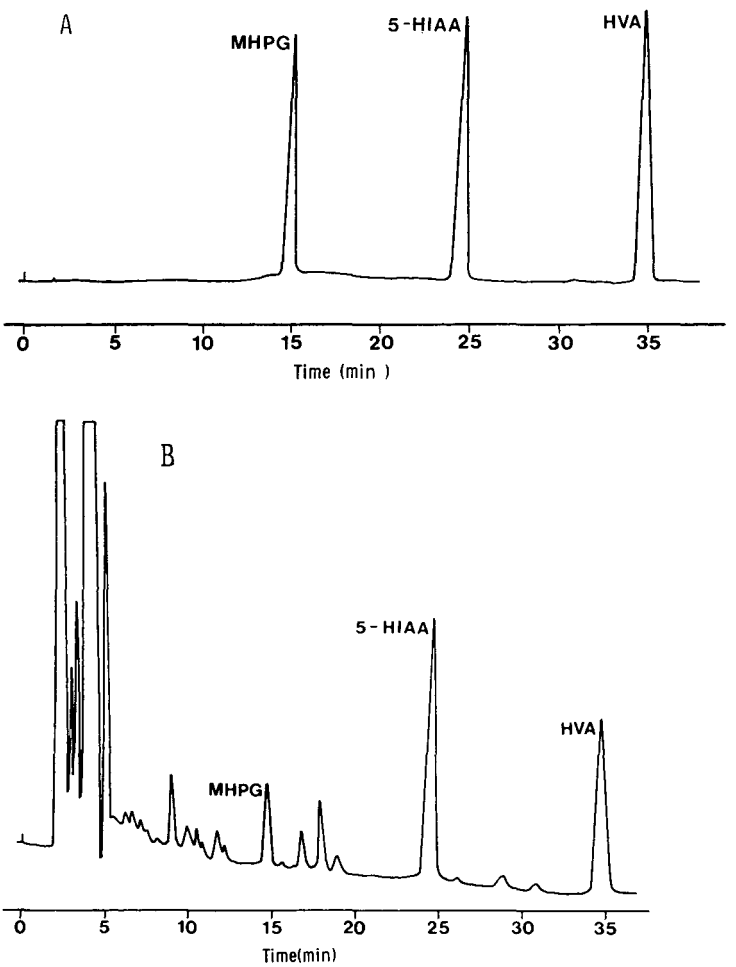

Fig. 1. Separation of MHPG, 5-HIAA and HVA in (A) standard solution of the compounds and (B) cerebrospinal fluid. Column: ODS Hypersil: mobile phase, 0.1 *M* ammonium acetate, pH 5.15.

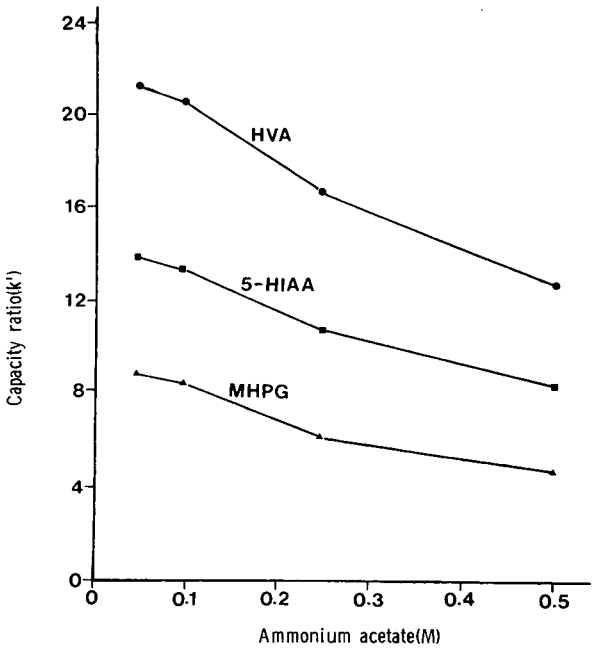

Fig. 2. Effect of molar concentration of ammonium acetate on the capacity ratios (k') of MHPG, 5-HIAA and HVA.

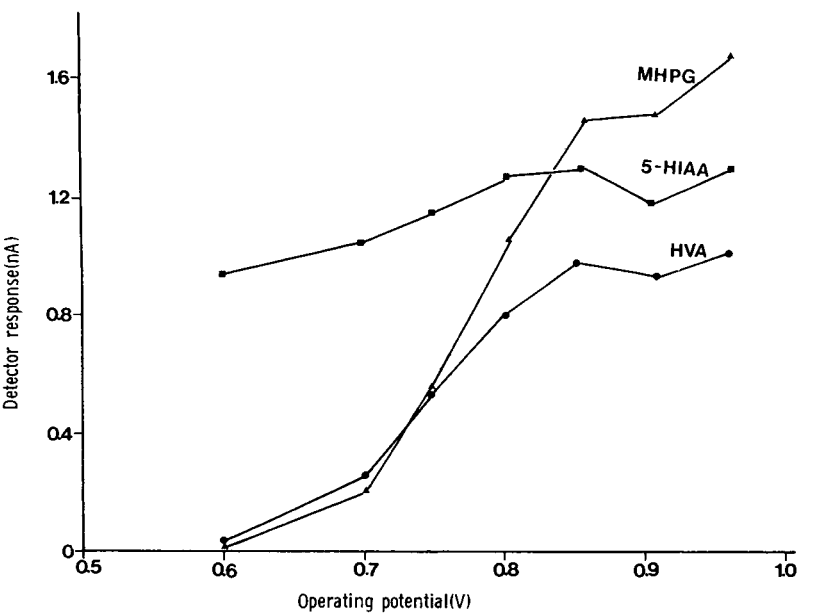

Fig. 3. Voltammograms for MHPG, 5-HIAA and HVA.

Reproducibility, linearity and recovery

The reproducibility of the method was tested by repeated injection (ten times) of a standard solution containing 50 nmol/l of each of the three metabolites. Standard deviations of ± 1% were achieved for peak response and k' values. The use of totally aqueous eluent which is unlikely to suffer from mobile phase composition changes during chromatography is largely responsible for the good reproducibility.

The standard curves were prepared by plotting the peak height against concentration in the range 5—35 nmol/l. Linear regression analysis data indicated no significant deviation from linearity (r = 0.9997). The intercept values also did not differ significantly from zero.

The recovery of a standard solution (50 nmol/l) from ultrafiltration through HPLC separation was also ± 1% rendering the use of an internal standard unnecessary.

The detection limit of MHPG, 5-HIAA and HVA were 30, 34 and 50 pg on column, respectively, based on a signal-to-noise ratio of 3.

MHPG, 5-HIAA and HVA levels in CSF

The catecholamine metabolite levels in CSF of depressed and neurological patients are shown in Table I. The results are well correlated with those previously reported [15]. No attempt was made to relate these levels with the clinical diagnosis of the patients although, as may be expected, there is a greater variation in the levels of 5-HIAA and HVA in the neurological cases than in the depressed patients.

TABLE I

AMINE METABOLITE LEVELS IN HUMAN CEREBROSPINAL FLUID

	MHPG (nmol/l)	5-HIAA (nmol/l)	HVA (nmol/l)
Depressed patients (n = 18)	50.9 ± 21.3	85.2 ± 34.5	162 ± 75
Neurological patients (n = 9)	51.0 ± 16.8	87.3 ± 64.9	293 ± 176

CONCLUSION

MHPG, 5-HIAA, and HVA in CSF can be separated isocratically from the parent compounds by reversed-phase chromatography with ammonium acetate solutions as eluents. The retention behaviour has been studied and it was concluded that effective resolution from all endogenous impurities required a mobile phase of 0.1 M ammonium acetate at pH 5.15.

ACKNOWLEDGEMENTS

We thank Dr. R. Martin and Dr. H. Nissenbaum for the clinical specimens, and Dr. E.H. Reynolds for advice. T. Bottiglieri is supported by a grant from Bioresearch, Liscate, Milan, Italy.

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Journal of Chromatography, 311 (1984) 361—368
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2277

Note

Method for total 3-methoxy-4-hydroxyphenylglycol extraction from urine, plasma and brain tissue using bonded-phase materials: comparison with the ethyl acetate extraction method

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(First received April 25th, 1984; revised manuscript received July 19th, 1984)

Over the past two decades convincing evidence has accumulated indicating that 3-methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of brain norepinephrine (NE) activity [1, 2]. Any biochemical investigation of central NE activity involves MHPG analysis. This analysis has been used in NE agonist and antagonist pharmacological studies [3], in classifying affective disorders [4, 5] and in predicting an individual's treatment response to tricyclic antidepressant drugs [6]. Such studies require a rapid and accurate method for isolating and quantifying MHPG.

The methods currently available involve ethyl acetate extraction [7]. These methods include lengthy handling time, and concentration of the sample, which also leads to the concentration of non-desirable materials and to a decrease of accuracy.

The following method uses Bond-Elut® extraction columns which are specifically adsorbent surface-modified materials. This has the advantage of minimal handling time and very clean sample extraction with good recovery. Compared to the liquid—liquid extraction method, it is better suited for high-performance liquid chromatographic (HPLC) analysis and essentially addresses the problem of sample extraction for HPLC analysis.

Later on in the analysis, the classical fluorimetric method of detection is tedious because it requires derivatization. HPLC analysis combined with amperometric detection complements this improved preparative method and facilitates routine applications.

0378-4347/84/$03.00

EXPERIMENTAL

Instrumentation

The preparative step required: Bond-Elut columns, with 1 ml PH-bonded phase (Analytichem International, Harbor City, CA, U.S.A.), a micropartition system MPS-1® with YMB® membrane (Amicon, Danvers, MA, U.S.A.), a brain homogenizer Polytron® (Kinematica, Luzern, Switzerland), vacuum flasks, ultracentrifuge tubes, etc. The analytical step consisted of the HPLC system: a Model 6000A solvent-delivery system and Model U6K injector equipped with a μBondapak RP-18 column, all from Waters Assoc. (Milford, MA, U.S.A.) and a LC-4A amperometric detector (BAS, West Lafayette, IN, U.S.A.).

Quantification was achieved with a Data Module® integrator (Waters Assoc.). The method used was that of external standard where peak areas were computed using the width at half height.

Chromatographic conditions

The mobile phase consisted of 0.05 *M* citrate—phosphate buffer (pH 3.5)—methanol (10 : 1) filtered through a Millipore membrane (0.22 μm), degassed, then pumped through the column at a flow-rate of 1 ml/min. The electrode was a glassy carbon electrode TL-5 held at 0.85 V versus the Ag/AgCl reference electrode. The sensitivity was 20 nA/V.

Standards and reagents

HPLC-grade methanol and acetonitrile were obtained from Merck (Rahway, NJ, U.S.A.). Double-distilled deionized water was used to prepare aqueous solutions. MHPG hemi-piperazine salt, crude sulphatase (type H-2) and pure sulphatase (type H-1) were purchased from Sigma. MHPG sulphate was obtained from Fluka (Buchs, Switzerland).

Sample preparation

Brain tissue. The rat brain was removed and dissected into various structures. About 100—200 mg of brain tissue were homogenized with a Polytron in 0.2 *M* perchloric acid (1 ml per 100 mg of fresh tissue) containing 0.1% EDTA. After ultracentrifugation (15,000 *g*, 15 min), the supernatant was adjusted to pH 6 for enzymatic hydrolysis which was achieved with 50—100 μl of crude sulphatase at 41°C in 2 h.

Plasma. A 1-ml volume of plasma was isolated by centrifugation and adjusted to pH 6 with 1 ml of 0.1 *M* acetate buffer containing 0.1% EDTA. Enzymatic hydrolysis was achieved with 50 μl of crude sulphatase as for brain tissue. After hydrolysis, pools of plasma were spiked with known quantities of MHPG and then ultrafiltered on a YMB membrane using the MPS-1 micropartition system. This yielded 400—500 μl of ultrafiltered plasma (per ml of plasma) after 30 min of centrifugation at 3000 *g*.

Urine. Urine samples (5 ml) were collected in 10-ml tubes containing an antioxidant (1 mg of sodium metabisulphite per ml of urine). Then 2 ml of a saturated barium chloride solution were added to remove phosphate and sulphate salts, and the sample was centrifuged at 3000 *g* for 10 min. The supernatant was adjusted at pH 6 with 3 ml of 0.1 *M* sodium acetate, and enzymatic

hydrolysis was achieved with 100 μl (per ml of urine) of crude sulphatase at 41°C in 3 h.

Hydrolysis efficiency

Due to the fact that MHPG exists in free and conjugated form it was not suitable to use an internal standard before hydrolysis. We estimated enzymatic hydrolysis by an external standard method, and the internal standard was added at the time of extraction. The efficiency of enzymatic hydrolysis was determined by using solutions of MHPG sulphate. The concentration of the standards and the number of enzyme units were similar to those of the biological samples. Pure and crude sulphatase were used and a comparative investigation of the two forms of enzyme was made.

Extraction procedure

A 1-ml PH Bond-Elut column for each sample was inserted into a 500-ml vacuum flask which was connected to a vacuum source. With the vacuum on, each column was washed with 1 ml of methanol, then with 1 ml of distilled water. The column was not kept dehydrated during the procedure. With the vacuum off, we loaded 400—500 μl of spiked or blank plasma ultrafiltrate (corresponding to 1 ml of total plasma) or 200 μl of homogenized urine or 1—2 ml of brain tissue extract (corresponding to 100—200 mg of brain tissue) onto the column. For each type of sample, parallel reference samples containing known quantities of added MHPG were made for extraction recovery calculation.

After applying the sample to the column, we washed the Bond-Elut with 200 μl of distilled water and eluted the sample with 100 μl of 2 m*M* potassium dihydrogen phosphate—acetonitrile (3 : 1). The pH of all samples was kept at about 5. At this point, the samples were ready for HPLC analysis.

We also assayed samples using the already known ethyl acetate extraction method for comparison. Ethyl acetate was previously washed and saturated with sodium chloride as described by Oishi et al. [8]: 3 vols. of ethyl acetate were used three times to extract the sample. The ethyl acetate was then evaporated at 30°C under reduced pressure. The sample was reconstituted with mobile phase to a volume smaller than the original volume.

RESULTS AND DISCUSSION

Recovery from enzymatic hydrolysis: comparison between pure and crude sulphatase activity

Crude sulphatase (50 μl of type H-2) and pure sulphatase (15 mg of type H-1) equivalent to 250 and 270 units, respectively, were assayed to 50 ng of MHPG sulphate. One unit of sulphatase is defined as the amount of enzyme which can hydrolyse 1 μmol of *p*-nitrocatechol sulphate per h at pH 5.0 and 37°C. MHPG sulphate (50 ng) was dissolved in sodium acetate buffer (0.1 *M*, 2 ml), pH 6.0, containing 0.1% EDTA. The 50 ng of MHPG sulphate were supposed to yield 50 ng of free MHPG. We also checked the presence of MHPG in each lot of enzyme freshly received in order to avoid any MHPG contamination from the enzyme.

TABLE I

HYDROLYSIS OF MHPG SULPHATE BY TWO TYPES OF SULPHATASE

MHPG sulphate (50 ng) was dissolved in 2 ml of 0.1 *M* sodium acetate buffer containing 0.1% EDTA, at pH 6, and was expected to yield 50 ng of free MHPG. Results are the means of four independent experiments and are expressed in ng.

Enzyme	Hydrolysed MHPG sulphate (ng)			
	Incubation period (h)			
	1	2	3	16
Crude sulphatase (H-2, 250 units)	50.6	49.7	50.1	49.1
Pure sulphatase (H-1, 270 units)	3.1	3.8	3.4	22.7

As seen in Table I, crude sulphatase was more efficient than pure sulphatase. With the latter, more than 50% of MHPG sulphate was still not hydrolysed after 16 h of incubation, whereas with crude sulphatase 50 ng of free MHPG were produced after 1 h. This result is not surprising. The high efficiency of crude sulphatase has already been pointed out by Karoum et al. [9]. These authors recommended the use of crude sulphatase with biological materials and incubation for 1 h at 40°C. Such a result explains why many researchers are still incubating for 16 h or more for hydrolysis. However, we think that this depends on the type of enzyme used. Yet Karoum et al. [9] attributed this to the differences between enzyme subunits. From our experience, conditions for each lot of enzyme must be checked after its receipt in order to optimize hydrolysis. From such a result, no other recovery from enzymatic hydrolysis was necessary, since with 250 units of crude sulphatase all MHPG conjugates in 1 ml of plasma or 200 mg of brain were hydrolysed in 1 h. For absolute certainty we always incubated for 2 h. For urine, we slightly increased the time of hydrolysis since MHPG conjugates are more accumulated. We found 3 h to be sufficient.

Recovery and precision from Bond-Elut extraction

Ethyl acetate extraction was used parallel to Bond Elut extraction which allowed the two methods to be compared. Percentage recovery was determined

TABLE II

PERCENTAGE RECOVERIES OF MHPG FROM BIOLOGICAL MATERIALS USING BOND-ELUT AND ETHYL ACETATE EXTRACTION

Values are the means of four independent experiments.

Samples	Percentage recovery (± C.V.)	
	Bond-Elut	Ethyl acetate
MHPG standard	75 ± 0.9	98 ± 12
Urine	80 ± 2.4	93 ± 16
Plasma	60 ± 2.9	94 ± 9.7
Rat brain extract	80 ± 1.9	97 ± 9.9

for all samples and standard solutions using the standards addition method.

Table II shows the various percentage recoveries. It can be seen that the ethyl acetate extraction method yields the highest percentage recovery but that the Bond-Elut method shows better precision. The within-run precision of the Bond-Elut method was good as shown by a coefficient of variation never exceeding 3%, whereas with the ethyl acetate method the coefficient of variation reached 16%.

Linearity and sample clean-up

The linearity of this method is represented by an example which shows plasma MHPG extraction, with known levels of added MHPG ranging from 5 to 20 ng/ml (Fig. 1). This use of parallel references with added MHPG helps to validate the recovery over a large range of plasma MHPG levels. The discrepancy between levels of MHPG found in the same pool of plasma is due to the different recoveries obtained with the two methods. Although ethyl acetate shows the highest recovery, the best linearity was given by the Bond-Elut method.

Although not too high, the percentage recovery was sufficient to recommend this method.

The x-intercept represents the endogenous MHPG (Fig. 1).

The other argument in favour of the Bond-Elut method is given by the very clean chromatogram yielded by this method (Fig. 2C and D). The ethyl acetate extracted MHPG peak is not baseline-resolved and non-desirable peaks fused with the MHPG peak. When working with automatic integration, using computing areas, well resolved peaks are better than fused peaks as the reproducibility

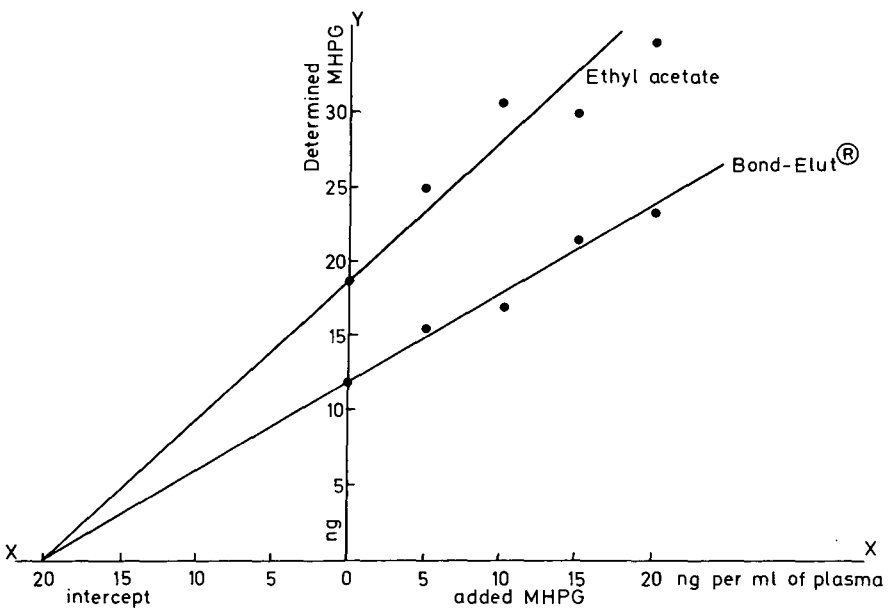

Fig. 1. Comparison between pooled plasma (1 ml) spiked with 5—20 ng of MHPG and extracted by the ethyl acetate or the Bond-Elut method to show the linearity of the two techniques. The x-intercept represents the total endogenous plasma MHPG.

is improved. Fig. 2A and B shows chromatograms from plasma and rat brain extracts, separated by the Bond-Elut method. The sample clean-up is clearly shown by these chromatograms. Finally, these preparative columns can be reused. Where plasma or brain tissue is concerned, we have seen that Bond-Elut columns can be reused one more time, after a good clean-up with 4—5 ml of distilled water, without affecting recovery. This recovery is sharply reduced when the column is used a second time for total urinary MHPG.

Recovery from the YMB membrane

Initially, we thought that the YMB membrane was responsible for the low percentage recovery from plasma. We determined the recovery of MHPG using the MPS-1 system with the YMB membrane. After hydrolysis, 2 ml of plasma were separated into two parts: 1 ml was ultrafiltered on MPS-1 with the YMB membrane and the ultrafiltrate was extracted in the same volume of ethyl acetate as the non-ultrafiltered plasma. The recovery of plasma MHPG in the YMB ultrafiltrate was 96.2% (±1.2). This result indicated that MHPG does not bind to the plasma proteins as Murray et al. [10] suggested occurs in dog plasma. Furthermore, MHPG passes through the YMB membrane very well. The relatively low recovery with Bond-Elut extraction could be due to the ionic environment of the plasma, and not to the low ultrafiltration of MHPG through the YMB membrane as previously thought.

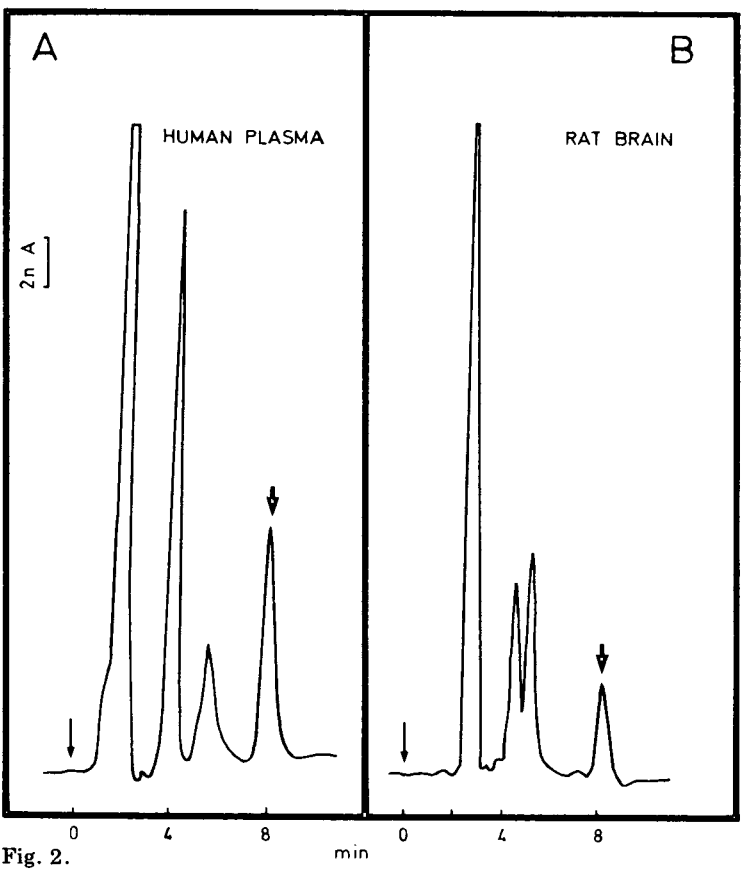

Fig. 2.

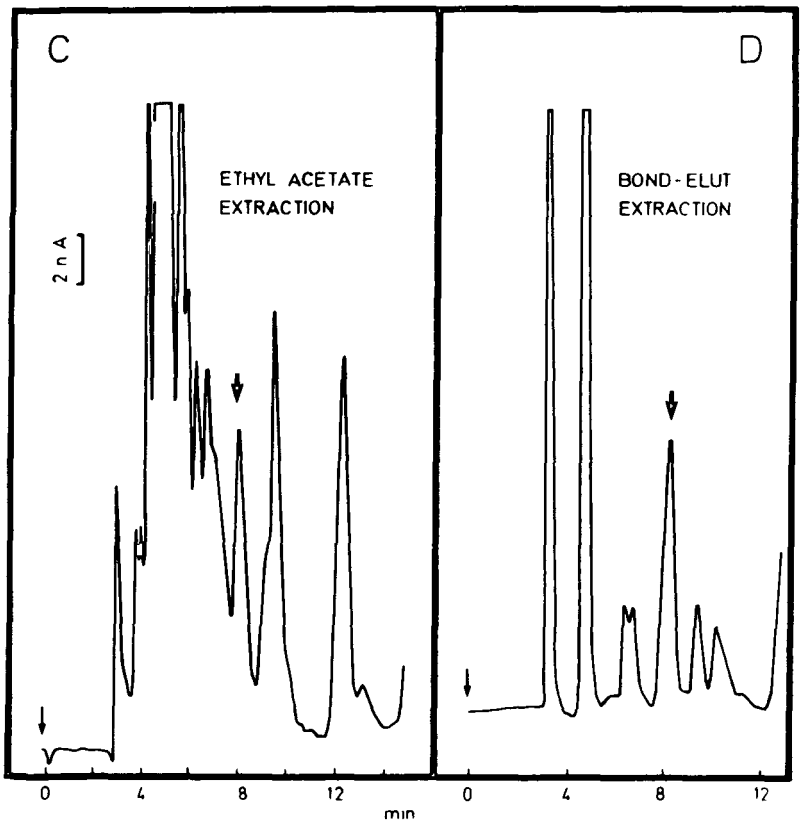

Fig. 2. (A and B): Typical chromatograms using the reversed-phase HPLC system of actual plasma (A) and rat brain (B) extract carried through the Bond-Elut extraction procedure. For chromatographic conditions see Experimental. (C and D): Typical chromatograms using the reversed-phase HPLC system of actual urine carried through the ethyl acetate (C) and Bond-Elut (D) extraction procedures. For chromatographic conditions see Experimental.

Comparison with other techniques

Table III presents results from our technique compared to data reported in the literature. Although this kind of comparison is not suitable for several reasons (differences in condition techniques, type of rat strain or monoamine

TABLE III

RESULTS OF MHPG EXTRACTED WITH BOND-ELUT COLUMNS COMPARED TO THOSE OF THE DIFFERENT TECHNIQUES FOUND IN THE LITERATURE

	MHPG extracted			
	Plasma (ng/ml)		Urine (μg per 24 h)	Rat brain (ng/g)
	Men	Women		
Bond-Elut	18.8 ± 4.5	15.6 ± 3.1	1600—2200	92 ± 10
Literature	22 ± 0.6 [11]	15.7 ± 2.0 [12]	1873 [11]	108 [13]

diet of subjects), nevertheless it does indicate that our results are similar to those reported in the literature. Furthermore, the difference between MHPG levels in men and women shown in Table III argues for the sensitivity of the method.

CONCLUSIONS

The aim of this study was to find a rapid, low-cost and precise method of determining MHPG, as this might be helpful in the diagnosis of certain forms of psychopathology. Although the ethyl acetate extraction method gives a relatively high recovery, the method is not precise enough, is time-consuming, and requires repeated assays in order to show mean data.

Bonded-phase materials for chromatography have existed for a number of years and seem to improve the isolation. It was Analytichem who first suggested isolating urine MHPG with PH-Bond-Elut columns [14]. In this study I was able to improve the conditions for isolation of MHPG from plasma and rat brain and to adapt the Analytichem method for total MHPG extraction. We believe that this method is well suited for HPLC analysis because of its precision and the clean sample chromatograms, and because it is simple enough to be reliable in routine use.

ACKNOWLEDGEMENTS

The author is grateful to Mrs. W. Rudolph and Mrs. C. Lambercy for their skilled technical assistance.

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Journal of Chromatography, 311 (1984) 369—374
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2273

Note

Direct determination of urinary vanillylmandelic acid and homovanillic acid by high-performance liquid chromatography on an anion-exchange column

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(First received April 17th, 1984; revised manuscript received July 16th, 1984)

Neuroblastoma is the commonest tumour of childhood [1]. The prognosis of this tumour closely depends on the patient's age at diagnosis [1]. Therefore, early diagnosis is an important factor in the prognosis. It is favourable when asymptomatic infants who are less than one year of age are detected.

Measurements of vanillylmandelic acid (3-methoxy-4-hydroxymandelic acid, VMA) have been advocated in the detection of patients with neuroblastoma [2—4]. But high levels of urinary VMA are excreted in only 75% of patients with this disease [5]. If measurements of urinary homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA) along with VMA are performed, approximately 95% of patients with this tumour will be detected [5].

Recent methods for the determination of urinary VMA and HVA are based on the use of high-performance liquid chromatography (HPLC). There are methods for the simultaneous determination of both acids [6—12] in addition to the methods for VMA [13—17] and HVA [18—20] separately. The method that uses ultraviolet (UV) detection [6, 7] involves sample concentration and clean-up procedures because sensitivity is low and many UV-absorbing constituents exist in urine. The method with fluorometric detection [8] is more selective than with UV detection. However, sample concentration is

0378-4347/84/$03.00

necessary because of low sensitivity. The method with derivatized VMA and fluorometric detection was successful in the analysis of VMA, but not of HVA [17]. The methods with electrochemical detection [9—12] allow the direct use of urine samples because of high selectivity and sensitivity. Because it is difficult to separate VMA from other urine constituents in these methods which employ an octadecylsilica (ODS) column, paired-ion chromatography [9], gradient elution [10] or organic extraction from acidified urine [11, 12] are used.

This note describes a rapid and simple HPLC method which employs an anion-exchange chromatographic column, electrochemical detection, direct on-column injection of urine and isocratic elution. This method was applied to the determination of the VMA and HVA contents of urine from six-month-old infants.

EXPERIMENTAL

Reagents

Acetonitrile, concentrated hydrochloric acid, formic acid, and ethylenediaminetetraacetic acid disodium salt (EDTA · 2Na) were purchased from Wako (Osaka, Japan). VMA and HVA were purchased from Sigma (St. Louis, MO, U.S.A.). Analytical-reagent grade chemicals were used without further purification. All solutions were prepared from distilled, deionized water. For standard samples, VMA and HVA were dissolved in 0.1 *M* hydrochloric acid.

Instrumentation

A Yanaco (Kyoto, Japan) Model L-4000W high-performance liquid chromatograph with an LA-100 column oven was used. The stainless-steel column (50 × 4.0 mm) packed with Yanaco NB-5801 (anion-exchange, particle size 12 μm) was used. The mobile phase was 3% formic acid, containing 50 μ*M* EDTA for masking iron ion, delivered at a constant flow-rate of 0.9 ml/min. A VMD 101 glassy carbon electrode (Yanaco) with a silver/silver chloride reference electrode was used to oxidize the compounds of interest at 0.80 V potential versus the reference electrode. The resulting signal was recorded at 10 mV using an R3-201 twin-pen recorder (Yanaco). The column was used at 65°C and the column inlet pressure was about 25 kg/cm^2.

Collection of urine samples

The urines were obtained from twenty normal infants (about six months of age) of both sexes and two patients with neuroblastoma. After collection, the pH of the urine was adjusted to less than 2 with 6 *M* hydrochloric acid and refrigerated until assayed.

Sample preparation

Test urine and acetonitrile were pipetted into glass tubes in a ratio of 1:1. Standards were prepared similarly using the mixture of VMA and HVA in 0.1 *M* hydrochloric acid. Routinely, standards in the range 25—110 μmol/l were used. Each tube was stoppered, shaken for 2 min by hand, and centrifuged at

550 *g* for 5 min. The supernate was injected directly into the column. The sample volume (in μl) of injection is approximately given by 44/(urinary creatinine, mmol/l). Prepared samples can be stored for as long as one week at 4° C.

RESULTS AND DISCUSSION

Chromatography

Chromatograms resulting from the analysis of urine from a normal infant (six months of age) and from a patient with neuroblastoma are shown in Fig. 1. VMA and HVA were eluted from the column at about 10 and 15 min, respectively, as asymmetric peaks.

The retention time was inversely dependent on the concentration of formic acid in the mobile phase; increasing the concentration from 1.0 to 10.0% resulted in shorter retention times. The flow-rate of the mobile phase affected the separation of the peaks.

All samples in which VMA or HVA was above the normal range were re-run with the same mobile phase at a flow-rate of 0.5—0.7 ml/min.

The following compounds were checked and found not to interfere in the method: 3-methoxy-4-hydroxyphenylglycol, dihydroxyphenylacetic acid, dihydroxymandelic acid and 3-methoxy-4-hydroxybenzoic acid (vanillic acid).

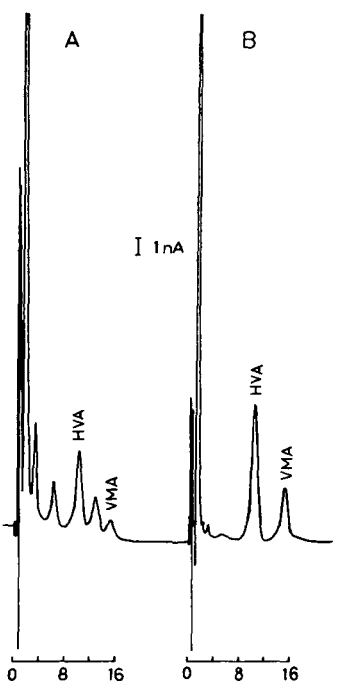

Fig. 1. Chromatograms of urine from a normal infant (A) and a patient (B). Concentrations of HVA and VMA in samples (μmol/l): (A) HVA 18.1, VMA 7.6; and (B) HVA 65.9, VMA 64.1.

Linearity

Calibration graphs for VMA and HVA were rectilinear up to 1 nmol; the detection limits were 50 and 14 pmol, respectively.

Choice of mobile phase

At an early stage of this study, acetate buffer (3 *M*, pH 3.7) was used as the mobile phase. Although the results were satisfactory, this mobile phase has not been used routinely because the concentration of the buffer was too high.

Formic acid was chosen as the mobile phase because it results in a favourable retention time for VMA and HVA, and its concentration is suitable as the mobile phase.

Recovery

Recovery studies were performed by adding specified quantities of VMA and HVA to aqueous solutions of urines obtained from eight normal infants (Table I). The recoveries of VMA and HVA were 96.6% and 94.8%, respectively.

Reproducibility

Reproducibility of the method was determined using two urine samples (Table II). The maximum coefficient of variation (C.V.) was 7.0%.

TABLE I

RECOVERY OF VMA AND HVA FROM URINE

Results obtained by analysis of eight urines from normal six-month-old infants.

	n	Concentration range (μmol/l)	Added (μmol/l)	Recovery (mean ± S.D., %)
VMA	7	5.0—18.9	40	96.6 ± 3.8
HVA	8	6.1—33.3	44	94.8 ± 2.4

TABLE II

REPRODUCIBILITY OF THE METHOD

Results obtained by analysis of ten urine replicates at two concentrations on one day.

	n	Concentration (mean ± S.D., μmol/l)	C.V. (%)
VMA	10	63.5 ± 1.90	3.0
	10	11.2 ± 0.79	7.0
HVA	10	65.8 ± 0.92	1.4
	10	19.8 ± 0.82	4.1

Correlation with a comparison method

Twenty urine samples from normal six-month-old infants and from two patients with neuroblastoma were assayed by the present method and by the method of Miyagawa [8], which employs an ODS column, fluorescence detection and sample extracted with ethyl acetate. The two methods were run in parallel; the results are plotted in Figs. 2 and 3. The data for VMA fit the

regression equation $Y = 0.86X + 0.17$, where X is the method of Miyagawa, with a correlation coefficient of 0.992. The data for HVA fit the regression equation $Y = 0.98X + 0.49$, with a correlation coefficient of 0.998.

The extractability of VMA from aqueous solution into ethyl acetate is low, being lower from dilute hydrochloric acid than from acidified urine [12]. Low extractability causes a large variation in the results. Therefore, VMA in aqueous solution must be extracted three times with ethyl acetate [6, 7] or the method of standard addition (spiking) must be used for each urine [12]. Direct on-column injection of urine samples was studied to solve this problem in our laboratory.

An ODS column has generally been used, but the affinity of VMA for ODS is weak and thus it is difficult to separate VMA from other urine constituents. The difference in affinity between VMA and HVA is large for ODS. Therefore,

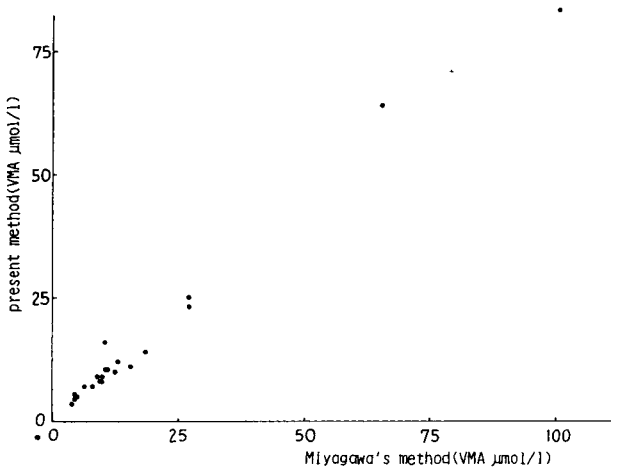

Fig. 2. Correlation between the present method and the method of Miyagawa for VMA assay.

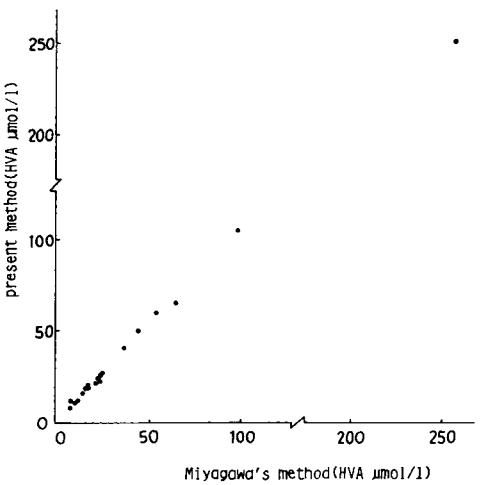

Fig. 3. Correlation between the present method and the method of Miyagawa for HVA assay.

the method reported previously required pre-treatment which extracts VMA (and HVA) with ethyl acetate [11, 12], paired-ion chromatography [9] or gradient elution [10]. An ion-exchange column was studied to solve this problem in our laboratory.

To enable use of a dilute urine sample, electrochemical detection, which has high sensitivity, was studied.

Thus isocratic elution and direct urine sample can be used, and the results obtained by this method were satisfactory.

CONCLUSION

The method described was applied to the determination of the VMA and HVA content of urine from normal infants and patients with neuroblastoma. The method shows the following advantages: short analysis time, selectivity and the possibility to be automated.

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Journal of Chromatography, 311 (1984) 375—379
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2280

Note

Rapid and sensitive amino acid analysis of human collagens using high-performance liquid chromatography

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(First received April 25th, 1984; revised manuscript received June 6th, 1984)

A fluorescence detection system has been used for the highly sensitive analysis of amino acids after reaction with *o*-phthalaldehyde (OPA) [1, 2]. By using the post-column fluorescence-labelling method with OPA after converting imino groups into amino groups in alkaline sodium hypochlorite [3, 4], the determination of proline and hydroxyproline is possible by high-performance liquid chromatography (HPLC). This report describes a rapid and highly sensitive amino acid analysis by HPLC, using post-column fluorescence labelling with OPA and sodium hypochlorite, of human collagens types I, II, III and V, which contain distinct amino acid compositions as well as large amounts of imino acids compared to other proteins.

0378-4347/84/$03.00

EXPERIMENTAL

Chemicals

L-Hydroxyproline and L-hydroxylysine of special reagent grade for amino acid analysis were obtained from Azinomoto (Tokyo, Japan). Commercial 10% sodium hypochlorite was purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan). The solution of standard amino acids and sodium citrate for the amino acid autoanalyser, *o*-phthalaldehyde (OPA) and all the other reagents of analytical grade were purchased from Wako (Osaka, Japan).

Equipment

The apparatus, a Model LC-3A HPLC system manufactured by Shimadzu (Kyoto, Japan) for amino acid analysis, was used with a step gradient programmer Model SCR-1A. A sulphonated polystyrene cation-exchange resin column, 150 × 4 mm I.D. (Shimadzu gel ISC-07/S1504, particle size 7 μm), together with a Dowex 50W-16 resin column 250 × 4 mm I.D. (Shimadzu gel ISC-50) as the precolumn [5], were used for the separation of amino acids.

Procedure

The elution buffer system and the time programme are shown in Table I. After elution with the fifth buffer, the column was recycled by use of the sixth buffer for 15 min and then the first buffer for 60 min or more. The flow-rate was 0.4 ml/min and the pressure was 10.8—11.8 MPa (60—70 kg/cm^2) at 55°C. OPA solution was prepared by mixing 400 mg of OPA in 7 ml of ethanol with 500 ml of 0.5 *M* borate—sodium carbonate buffer (pH 10.2), 1 ml of 2-mercaptoethanol and 2 ml of 10% Brij 35. Sodium hypochlorite solution was prepared by adding 0.5 ml of the 10% sodium hypochlorite to 500 ml of 0.5 *M* borate—sodium carbonate buffer (pH 10.2). The flow-rates of the hypochlorite solution and the OPA solution were set at 0.2 ml/min with a minipump Model PRP-2A. The length of the oxidative reaction coil and that of the fluorogenic reaction coil was 1 m and 2 m, respectively. The reaction temperature was set at 55°C. The results were displayed on a Model C-R1A recording integrator.

TABLE I

BUFFER SYSTEMS AND TIME PROGRAMMES FOR AMINO ACID ANALYSIS

Step	pH	Buffer*	Time (min)
1	2.70	0.067 *M* Sodium citrate—perchloric acid in 7% ethanol	20
2	3.25	0.067 *M* Sodium citrate—perchloric acid	10
3	4.25	0.067 *M* Sodium citrate—perchloric acid	32
4	6.50	0.27 *M* Sodium citrate—perchloric acid	30
5	9.00	0.27 *M* Sodium citrate—sodium hydroxide and 0.016 *M* boric acid	20
6		0.2 *M* Sodium hydroxide	15
Recycle		Buffer 1	60

*Buffers 1—5 contain 0.01% *n*-caprylic acid.

Sample preparation

Human collagens, type I, type III and type V were obtained from placenta by pepsinization and salt fractionation. Human type II collagen was extracted from cartilage by pepsin treatment following 4 *M* guanidine—hydrochloric acid treatment [6]. About 100 μg of each type collagen were hydrolysed in 1 ml of 6 *M* hydrochloric acid under vacuum at 110°C for 24 h. Dried samples were dissolved in 1 ml of buffer 1. The sample solution was passed through a Millipore filter, then 10 μl of the sample were injected.

RESULTS AND DISCUSSION

Typical chromatograms of the mixture of standard amino acids and human type I collagen hydrolysate are shown in Fig. 1. It took about 120 min for the analysis of one sample. Table II shows the amino acid composition of the human collagens (type I, II, III and V) obtained using the post-column labelling method with sodium hypochlorite and OPA, together with the reported data obtained by a conventional automated amino acid analyser with ninhydrin reaction. The values for human type I collagen were calculated from the data

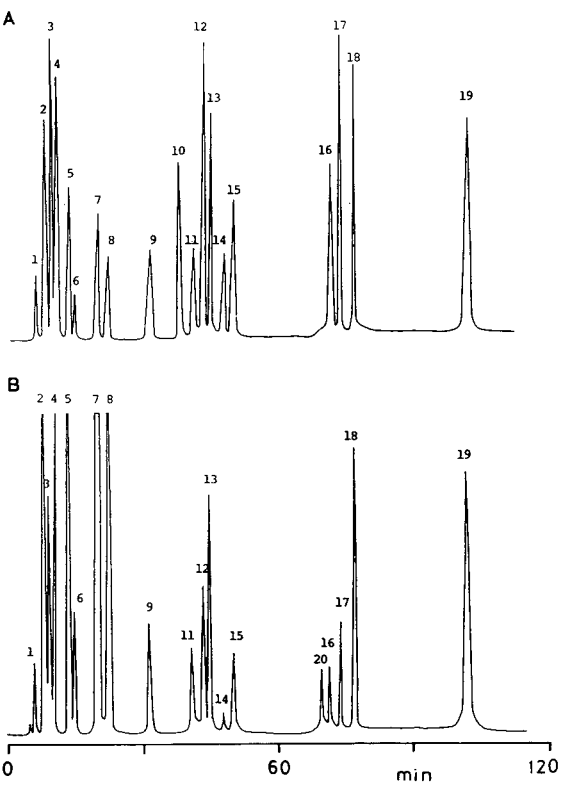

Fig. 1. Chromatograms of (A) a mixture of standard amino acids and (B) human type I collagen hydrolysate. Amino acid analysis was performed as described in the text. The mixture of standard amino acids (each at a concentration of 0.25 nmol) contained: 4-Hyp (1), Asp (2), Thr (3), Ser (4), Glu (5), Pro (6), Gly (7), Ala (8), Val (9), Cys (10), Met (11), Ile (12), Leu (13), Tyr (14), Phe (15), His (16), Hyl (17), Lys (18), Arg (19), and NH_3 (20). For abbreviations see Table II.

TABLE II

AMINO ACID COMPOSITION OF HUMAN COLLAGEN

Results are expressed as the number of residues per 1000 total residues.

Amino acid*	Type I		Type II		Type III		Type V	
	OPA method§	Ninhydrin method [7]	OPA method§	Ninhydrin method [8]	OPA method§	Ninhydrin method [9]	OPA method§	Ninhydrin method
3-Hyp	Trace	1	ND**	2	Trace	NR***	Trace	1
4-Hyp	85 (106)	109	81 (101)	99	111 (125)	125	86 (107)	101
Asp	42	45	41	42	45	42	46	50
Thr	12	18	15	20	9	13	17	23
Ser	35	33	31	27	39	39	26	26
Glu	70	74	91	89	70	71	90	96
Pro	114	115	109	121	109	107	113	118
Gly	367 (337)	325	368 (332)	333	377 (331)	350	383 (345)	325
Ala	113	113	101	100	89	96	37	46
Val	22	24	14	18	12	14	21	24
Cys	ND	ND	ND	NR	Trace	2	1	1
Met	8	5	19	9	7	8	11	8
Ile	9	11	9	9	13	13	17	19
Leu	25	24	27	26	23	22	40	42
Tyr	2	2	1	1	1	3	2	2
Phe	13	12	12	13	9	8	12	11
Hyl	5	11	14	14	5	5	31	38
Lys	26	24	18	22	30	30	8	7
His	5	6	2	2	7	6	15	14
Arg	48	49	46	51	46	46	44	52

*3-Hyp = 3-hydroxyproline, 4-Hyp = 4-hydroxyproline, Asp = asparate, Thr = threonine, Ser = serine, Glu = glutamate, Pro = proline, Gly = glycine, Ala = alanine, Val = valine, Cys = cysteine, Met = methionine, Ile = isoleucine, Leu = leucine, Tyr = tyrosine, Phe = phenylalanine, Hyl = hydroxylysine, Lys = lysine, His = histidine, Arg = arginine.

**ND = not detected.

***NR = not reported.

§Numbers in parentheses are the values after correction for glycine and hydroxyproline using correction factors obtained experimentally for type I collagen by ninhydrin and OPA methods (see text).

of Burgeson et al. [6] on α1 (I) and α2 (I) chains (two-thirds α1 + one-third α2).

The contents of glycine and hydroxyproline residues were about 10% higher and 25% lower, respectively, than those previously reported (Table II). The values for the other amino acids were comparable within the error by the present analysis. Even though relatively high errors in glycine and hydroxyproline occurred, the overall amino acid composition determined by the hypochlorite—OPA method clearly indicates that the purified protein is collagenous. It also demonstrates a distinct difference in amino acid compositions between interstitial collagens (type I—III) and type V collagen. That is, the latter collagen contains fewer than 50 alanine residues, about 100 hydrophobic amino acid residues (Val, Met, Ile, Leu, Tyr and Phe) and 30—40 hydroxylysine residues, while collagens I—III contain about 100 alanine residues, about 80 or less hydrophobic amino acid residues and fewer than 15 hydroxylysine residues. In this respect, the highly sensitive amino acid analysis by OPA reaction after sodium hypochlorite treatment was useful. Improvement of the method for the determination of glycine and hydroxyproline by increasing the oxidation reaction by sodium hypochlorite is now under investigation.

For practical usage in determining the amino acid composition of a collagenous protein which contains about 10% hydroxyproline and 33% glycine, correction factors which are experimentally determined for the same sample of type I collagen by both the conventional ninhydrin method and the present hypochlorite—OPA method should be used. The correction factors for glycine and hydroxyproline under the present condition are 0.9 and 1.25, respectively. The corrected values for the contents of type I, II, III and V collagens are listed in parentheses in Table II. The data are reasonably consistent with the previously reported values.

The reason that the quantification by the OPA method after sodium hypochlorite reaction gives rise to larger errors in the glycine and hydroxyproline content may be as follows. The overestimation of glycine may be due to a relatively smaller number of amines being degraded during the oxidation by sodium hypochlorite under the present conditions. The underestimation of hydroxyproline, on the other hand, may be due to relatively less conversion into amines during hypochlorite oxidation. A high hydroxyproline content which becomes reactive with OPA only after hypochlorite oxidation may have not been completely converted into primary amines.

Other than the high sensitivity for amino acid analysis of collagenous proteins which contain abundant imino acids, the HPLC method has the further advantage of shortening the analysis time (120 min for one sample).

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Journal of Chromatography, 311 (1984) 380—384
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2274

Note

High-performance liquid chromatography of 5-hydroxyindole-3-acetic acid in urine with direct sample injection

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(First received March 6th, 1984; revised manuscript received July 26th, 1984)

Early detection of carcinoid tumors occurring in the small intestine is essential for successful surgical treatment. Since the tumors release large amounts of 5-hydroxytryptamine, an elevated level of 5-hydroxyindole-3-acetic acid (HIAA), the major metabolite of 5-hydroxytryptamine, in the urine is a reliable indicator of carcinoid tumors [1—3]. A number of methods have been described for quantitative determination of HIAA in urine. Udenfriend et al. [2] originally described the widely used nitrosonaphthol colorimetric method which was later modified by Goldenberg [4]. This method is subject to error owing to interfering substances in the urine as well as error introduced by incomplete extraction [5]. Several high-performance liquid chromatographic (HPLC) procedures have been described with increased sensitivity and specificity for HIAA with electrochemical or fluorescence detection [6—11]. Most of these procedures require sample extraction prior to analysis to eliminate interfering peaks. Wahlund and co-workers [8, 9] have reported a direct injection method for HIAA in urine; however, a complex chromatographic system which incorporates tributyl phosphate in the stationary phase is required. We are describing a rapid and simple fluorescence HPLC method with direct injection on an unmodified reversed-phase chromatographic system. The sensitivity and reproducibility of the method are sufficient for routine quantitative screening of urine for HIAA.

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0378-4347/84/$03.00

EXPERIMENTAL

Apparatus

Chromatography was performed with a Waters isocratic liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.). The system included a manual injector, an M6000 pump, a 50 × 3.9 mm guard column packed with Vydac RP (Varian, Synnyvale, CA, U.S.A.), and a 300 × 3.9 mm μBondapak C_{18} analytical column (Waters Assoc.). The effluent was continuously monitored with an Aminco Bowman Model J4-8960A variable-excitation- and -emission-wavelength spectrophotofluorometer (Aminco Bowman, Silver Spring, MD, U.S.A.) with an HPLC flow cell.

Reagents

Anhydrous sodium acetate and glacial acetic acid were reagent grade. Methanol and water were HPLC grade. Acetate buffer was prepared by adjusting a 0.1 *M* sodium acetate solution to pH 4.5 with acetic acid. The acetate buffer and methanol were filtered through 0.45- and 0.5-μm filters, respectively, and degassed prior to use. Standard solutions of HIAA (Sigma, St. Louis, MO, U.S.A.) and the internal standard, 5-hydroxyindole-2-carboxylic acid (HICA) (Aldrich, Milwaukee, WI, U.S.A.) were prepared at 500 μg/ml in water and adjusted to pH < 3 with acetic acid.

Procedure

Urine samples were initially centrifuged to remove solid material. Sample aliquots of 100 μl were mixed with an equal volume of the internal standard and 20-μl aliquots were injected for HPLC. The chromatography was performed at room temperature with a mobile phase of methanol—acetate buffer (14:86), and a flow-rate of 1.4 ml/min. The effluent was continuously monitored at an excitation wavelength of 295 nm and emission wavelength of 345 nm. Positive samples above the calibration range were diluted with water and rerun.

For calibration aqueous dilutions of HIAA at concentrations ranging from 1 to 30 μg/ml were substituted for the urine samples. Peak height ratios relative to the internal standard were used for quantitation.

RESULTS

Fig. 1 is a typical chromatogram of a 10 μg/ml HIAA standard. The retention times for the internal standard and HIAA were 5.5 and 7.5 min, respectively. The retention times for other compounds tested for interference were: 5-hydroxytryptophan, 3.5 min; 5-hydroxytryptamine, 4.8 min; tryptophan, 6.1 min; and indol-3-acetic acid, not detected.

Calibration curves with aqueous standards were linear from 1 to 30 μg/ml with an intercept at 0. The minimum detection limit based on a signal-to-noise ratio of 3:1 was 0.2 μg/ml using the dilutions and injection volume described in the procedure. Calibration curves prepared by spiking negative urine samples with HIAA were identical with those obtained with aqueous standards when the HIAA originally present in the urine was subtracted from each point.

The within-run coefficient of variation at a level of 1.4 μg/ml was 9.2% (n = 10) and 2.3% (n = 10) at a level of 433 μg/ml. The day-to-day coefficient of variation was 6.7% (n = 20) at 1.4 μg/ml and 3.2% (n = 20) at 433 μg/ml.

All samples were finally calculated as μg HIAA per mg creatinine. A level of 3.2 ± 1.7 μg HIAA per mg creatinine (± S.D., n = 47) was obtained for patients without carcinoid tumors. Fig. 2 is an example of the chromatograms obtained for negative samples. The unidentified peaks in Fig. 2 were present in all negative patient chromatograms and varied in intensity. The retention times of these peaks differed from the standard compounds tested. The results for five patients with carcinoid tumors were: 323, 267, 25, 63, and 226 μg HIAA per mg creatinine. Fig. 3 shows the a chromatogram of a positive sample with 433 μg/ml HIAA or 323 μg HIAA per mg creatinine.

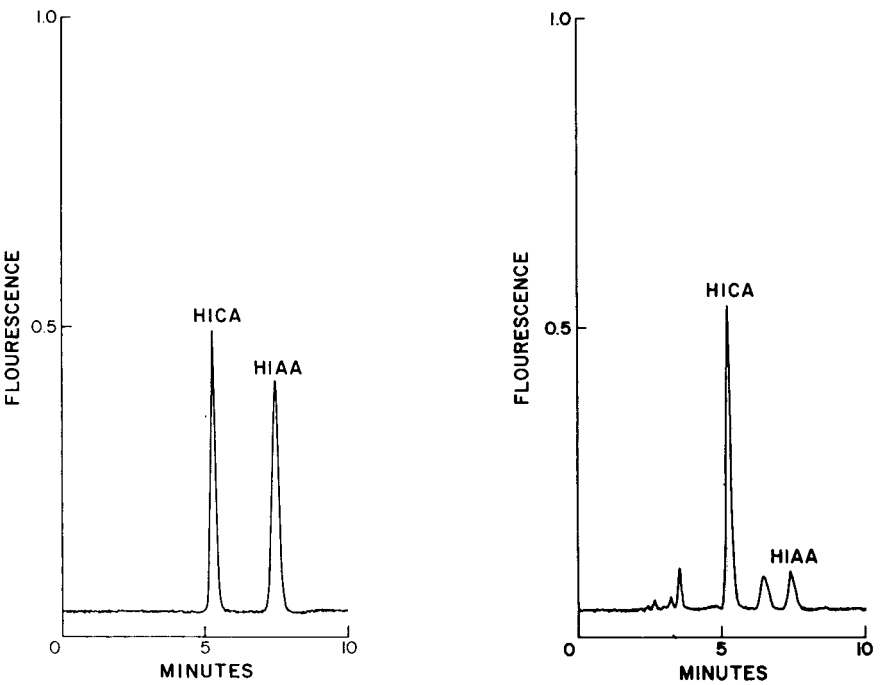

Fig. 1. Chromatogram of an aqueous HIAA standard and the internal standard, HICA. Initial concentrations were: HIAA, 10 μg/ml; HCA, 500 μg/ml. A mixture containing 10 μl of each was injected.

Fig. 2. Chromatogram of a negative patient urine containing a 1.4 μg HIAA/ml.

DISCUSSION

Direct injection of biological fluids offers significant advantages over extraction methods by considerably shortening the sample preparation time and eliminating errors that may arise from variable extraction recoveries. In many cases, however, direct injection is not practical owing to numerous interfering peaks and a decrease in the life of the analytical column. We have minimized

these effects by using a variable-wavelength spectrofluorometer for detection and maintaining a small sample injection volume. The pH of the mobile phase and the excitation and emission wavelengths were optimized to enhance the HIAA and HICA fluorescence relative to other fluorescent substances present in the urine. The retention time of HIAA varied with pH as a single peak with no evidence of interference. The selectivity is significantly improved with a variable-wavelength detector. In comparison, chromatograms under similar conditions with a filter fluorescence HPLC detector contained a number of interfering peaks.

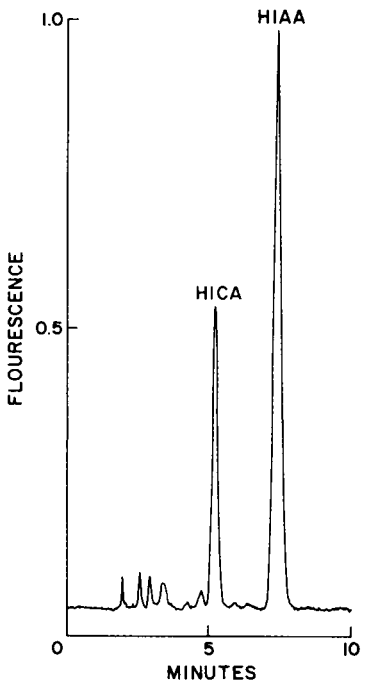

Fig. 3. Chromatogram following a 1:20 dilution of urine from a patient with carcinoid tumor. The undiluted concentration of HIAA was 433 μg/ml.

The guard column was routinely repacked each month when the assay was performed daily. There was no observed change in retention times, increase in column pressure, or any apparent decrease in analytical column life. The procedure described minimizes the problems associated with direct injection an provides a rapid and simple quantitative measurement of HIAA in urine. The procedure has good reproducibility and sufficient sensitivity for routine quantitative screening of urine for HIAA.

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Journal of Chromatography, 311 (1984) 385—389
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2275

Note

Simultaneous determination of tryptamine and its metabolites in mouse brain by high-performance liquid chromatography with fluorometric detection

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(Received May 8th, 1984)

The indoleamines tryptamine (TRM) and 5-hydroxytryptamine (5-HT) are formed by decarboxylation from tryptophan (TRP) and 5-hydroxytryptophan, respectively. The presence of TRM in the mammalian brain has been demonstrated [1—3], although the level is very low compared with that of 5-HT.

It is well known that 5-HT is a neurotransmitter in the central nervous system (CNS) and that it plays an important role in physiological functions such as sleep or thermoregulation. Recently, there has been increasing interest in the physiological and pharmacological effects of TRM. It has been suggested that TRM may play a specific role in neurotransmission and neuromodulation in the CNS [4—7]. In behavioural studies, Dewhurst [8] has reported that TRM acts as an excitatory amine in young chickens and recently it has been proposed that TRM induces myoclonus in guinea pigs treated with the monoamine oxidase (MAO) inhibitor [9].

The involvement of TRM in neurological disorders has also been implied. An elevation of TRM excretion in the urine has been shown to occur in Parkinson's disease and schizophrenia [10, 11].

The turnover rates of trace amines are known to be rather rapid and a large increase in these amines has been observed in the rat brain after MAO inhibition [12, 13]. It has been reported that the half-life for TRM is less than 1 min which is extremely rapid compared to 77 min for 5-HT [14]. TRM is immediately metabolized to indoleacetaldehyde by MAO and is consequently converted to indoleacetic acid (IAA) or tryptophol (TOL).

The occurrence of IAA in the rat brain has been reported [15, 16]. IAA is a major metabolite of TRM and Young et al. [17] suggested that the concentra-

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tion of IAA in the cerebrospinal fluid can be used as an index of TRM turnover. Recently, we demonstrated that TOL is present in the mouse brain as a TRM metabolite [18]. In addition, TOL has been shown to have pharmacological properties [19, 20], and an increase in the IAA level has been observed in several neurological disorders [21, 22]. It is suggested that the measurement of indoles in the TRM pathway may be significant in neurochemical studies.

Until now, the determination of TRM has been performed using fluorometry or gas chromatography—mass spectrometry [1, 23]. These techniques require complicated extraction procedures. Recently, high-performance liquid chromatography (HPLC) has been developed and applied for the determination of biological samples. We have previously reported the simultaneous determination of TRP and its metabolites by HPLC with fluorometric detection [24].

In this paper, we show a simple, sensitive and simultaneous method for the determination of indoles in the TRM pathway, i.e. TRM, IAA and TOL, in mouse brain by HPLC with fluorometric detection.

MATERIALS AND METHODS

TRP and IAA were purchased from Nakarai (Kyoto, Japan). TOL was obtained from Sigma (St. Louis, MO, U.S.A.) and TRM from Wako (Osaka, Japan). The other chemicals were reagent grade. The standard solution was prepared in 0.1 *M* perchloric acid.

Male ddY mice weighing 20—25 g were obtained from Shizuoka Laboratory Animals (Shizuoka, Japan). Mice were killed by decapitation and brains were rapidly removed and weighed. TRM was dissolved in saline and injected intraperitoneally at 50 mg/kg. The mice treated with TRM were killed 2 min after the injection. The brains were stored at −40°C until analysis.

Each brain was extracted according to the method detailed in our previous report [24]. The brain was homogenized in 4 ml of 0.1 *M* perchloric acid containing 0.02% ascorbic acid. The homogenate was centrifuged at 15,000 *g* for 10 min at 0°C. The supernatant was transferred to a sample tube and the residue was resuspended and centrifuged again. The supernatants were pooled. After filtering through a 0.45-μm filter, 20 μl of supernatant were injected directly into the HPLC system.

The chromatography was performed with a Shimadzu (Kyoto, Japan) LC-3A liquid chromatograph. A Zorbax C_8 reversed-phase column (10 μm particle size, 250 mm × 4.6 mm I.D.) was used for the separation of the indoles. The mobile phase consisted of 50 m*M* acetate buffer (pH 5.0) containing 35% methanol. The fluorometric detection was performed with an RF-530 spectrophotofluorometer (Shimadzu, Japan). The excitation and emission wavelengths were set at 280 and 350 nm, respectively. The flow-rate was 0.7 ml/min. The column temperature was maintained at 24 ± 1°C during the analysis.

The concentrations of indoles were determined from peak heights compared with external standards. The retention times and peak heights were obtained using a Chromatopac C-R1B data processor (Shimadzu).

RESULTS AND DISCUSSION

A large number of methods for determining indole compounds, including gas chromatography—mass spectrometry, fluorometry and HPLC, have been described [25]. HPLC with fluorometric detection offers high specificity for the measurement of indoles using their native fluorescence. Recently, we also reported a sensitive method for the determination of TRP and its metabolites using HPLC with fluorometric detection [24]. However, there are few research reports about the sensitive determination of TRM and its metabolites [1, 15]. We developed the assay and applied it for the sensitive and simultaneous determination of TRM and its metabolites by HPLC with fluorometric detection.

Fig. 1 shows the chromatogram of the standard solution. TRP, TRM, IAA and TOL were clearly separated by the C_8 reversed-phase column. In addition to the three indoles in the TRM pathway, TRP, the precursor amino acid of the indoleamines, was eluted simultaneously.

The retention times and detection limits of the indoles are summarized in Table I. The limit of sensitivity is low, in the range 10—20 pg. The standard curves of peak height for the indoles were linear over the range 0.1—50 ng.

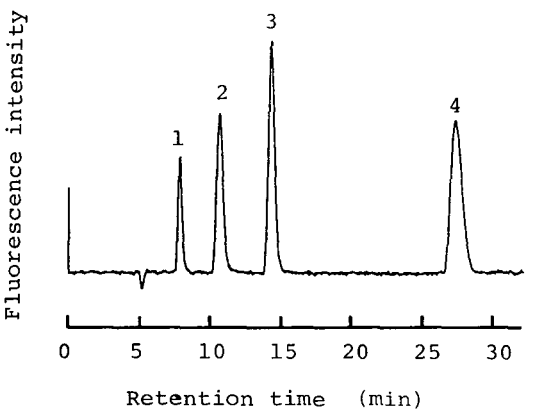

Fig. 1. Chromatogram of a standard mixture of 1 ng of each indole. Peaks: 1 = TRP; 2 = TRM; 3 = IAA; 4 = TOL. For chromatographic conditions, see text.

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF INDOLES

For chromatographic conditions, see text.

Compound	Retention time (min)	Detection limit (pg)*
Tryptophan	8.10	20
Tryptamine	10.92	15
Indoleacetic acid	14.57	10
Tryptophol	28.02	15

*Injected quantity giving a signal-to-noise ratio of 2.0.

Reproducibility was examined by repeated injections of 1 ng standard solution. Standards were determined with the coefficients of variation in peak heights and retention times being 0.9—3.6%.

In Fig. 2A a typical chromatogram of a normal mouse brain sample is shown. TRP was monitored at 8.1 min. The mean concentration of TRP was 3497 ± 42 ng/g (mean ± S.E., n = 7), which agrees with previous reports [26, 27].

Fig. 2B shows the chromatogram of brain of a TRM-treated mouse. TRM was injected at 50 mg/kg and the mouse was killed 2 min after the treatment. TRP, TRM, IAA and TOL were detected and the concentrations for the sample shown in Fig. 2B were 4568 ng/g TRP, 325.3 ng/g TRM, 779.4 ng/g IAA and 66.6 ng/g TOL.

The recoveries for the brain (mean ± S.E.) were: TRP 90 ± 3.4%; TRM 87.1 ± 0.9%; IAA 82 ± 2.2%; TOL 86 ± 1.3%.

Identification of peaks was made by changing the percentage of methanol and pH in the mobile phase. The sample peaks were coeluted with the standards.

The half-life of TRM is known to be very short and it has been thought that TRM is mainly metabolized to IAA. Recently, we reported the occurrence and formation of TOL as a metabolite of TRM [18]. In this study, we have further confirmed that both IAA and TOL are formed from TRM.

It has been suggested that TRM is a neuroactive amine and that it possibly plays a role as a neurotransmitter or neuromodulator in the CNS [4—7]. In

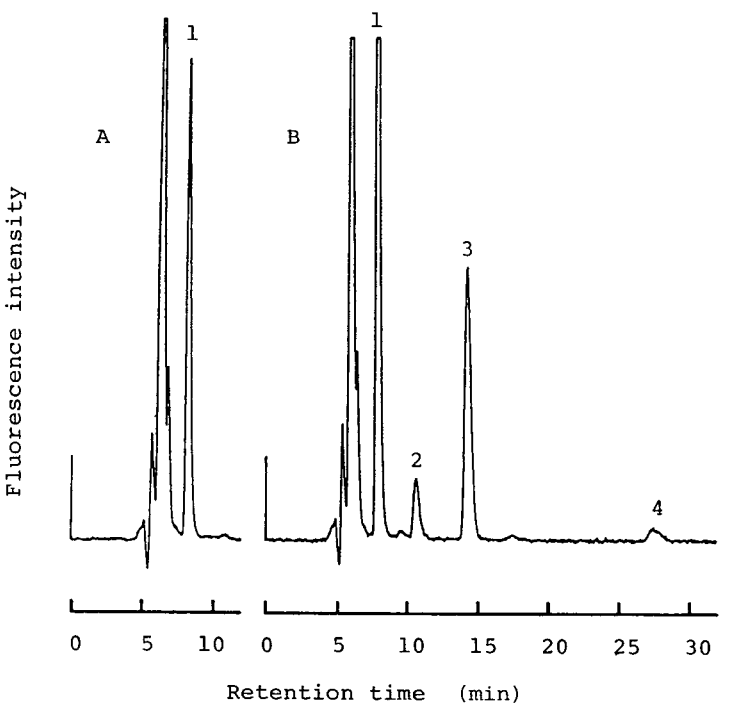

Fig. 2. Chromatograms of mouse brain samples: (A) normal mouse brain; (B) brain from mouse treated with 50 mg/kg TRM. Peaks: 1 = TRP; 2 = TRM; 3 = IAA; 4 = TOL. For chromatographic conditions, see text.

clinical studies, too, there are some indications that TRM may act in this way in neurological disorders [10, 11]. It has also been suggested that, besides TRM itself, the metabolites of TRM, IAA and TOL are involved in neurochemical functions [19—22]. Therefore, it would seem useful for neurochemical studies to elucidate the TRM metabolism.

In conclusion, the described method is quite simple, accurate and sensitive. This is the first report allowing the simultaneous determination of indoles in the TRM pathway. Our assay will be useful for follow-up investigations of TRM metabolism and offers great advantages to pharmacological and neurochemical studies of TRM.

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Journal of Chromatography, 311 (1984) 390—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2278

Note

Assay for histidine decarboxylase in rat stomach and brain by high-performance liquid chromatography with fluorescence detection

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Histidine decarboxylase (HDC, EC 4.1.1.22, L-histidine carboxylase) plays an important role in the formation of histamine in tissues, and it catalyses the decarboxylation of histidine to histamine in the presence of pyridoxal-5′-phosphate (PALP). The enzyme is found in many tissues such as fetal rat liver [1], mouse mastocytoma [2], mammalian brain [3], rat stomach [4] and hamster placenta [5]. Although the activity in these tissues is usually very low, its assay has been required for biological studies on the biosynthesis, storage and release of histamine.

The activity has been assayed by radiochemical and conventional fluorimetric methods. Although the radiochemical methods [6—12] are highly sensitive, they are rather complicated and require expensive substrates. The fluorimetric methods [13, 14], based on the reaction of enzymatically formed and endogenous histamine with *o*-phthalaldehyde (OPA) in alkaline medium to produce a fluorescent product, permit the assay of the activity in partially purified enzyme preparations but they are not very well suited for the assay in crude en-

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0378-4347/84/$03.00

zyme preparations which are rich in various biogenic amines. The methods are insensitive and so require relatively large amounts of biological samples.

We have previously presented a selective and sensitive method for the assay of histamine in urine and plasma by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection, based on the precolumn derivatization of the amine with OPA [15, 16].

This present paper describes an HPLC method for the assay of HDC in biological materials. Histamine formed from the substrate histidine under the optimum conditions for the enzyme reaction is separated from unreacted histidine by ion-exchange chromatography and then determined by the HPLC method. The enzyme preparations from rat stomach and brain were employed as those with relatively high and extremely low activities of HDC, respectively, to establish the assay procedure.

EXPERIMENTAL

Materials and methods

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. Amberlite CG-50 (Na^+; 200—400 mesh; Rohm and Haas, Philadelphia, PA, U.S.A.) (50 g) was washed successively with 500 ml of 2 *M* sodium hydroxide (three times), 500 ml of water (three times), 500 ml of 2 *M* hydrochloric acid (three times) and 500 ml of water (three times), and finally equilibrated with 0.2 *M* phosphate buffer (pH 6.5). The amberlite CG-50 column was prepared by packing 0.25 ml of the resin in a glass tube (100 × 4 mm I.D.). An HPLC column (150 × 4 mm I.D.) of TSK-GEL LS-410 ODS SIL (particle size 5 μm; Toyo Soda, Tokyo, Japan) [16] was used. The column can be used for more than 1000 injections when washed with aqueous methanol (1 : 1, v/v) at a flow-rate of 0.5 ml/min for ca. 30 min after every analyses.

HDC preparations from rat stomach and brain

Rat stomach and brain were obtained from male Wistar rats (5—6 weeks of age; weight 160—200 g) and male Sprague—Dawley rats (6—7 weeks of age; weight 250—300 g), respectively. The rats were killed by a blow on the neck and subsequently decapitated. The stomach was cut open along the greater curvature and the mucosal surface was cleaned thoroughly with ice-cold saline. The corpus ventriculi area of the stomach was scraped off and homogenized in 8 vols. of 0.1 *M* acetate buffer (pH 5.5) containing 0.2 m*M* dithiothreitol and 1.0% (v/v) polyethyleneglycol (average molecular weight, 300). The homogenate was centrifuged at 10,000 *g* for 30 min in a refrigerated centrifuge and the supernatant was used as the HDC preparation. The whole rat brain was rapidly removed, blotted and homogenized with 20 ml of the acetate buffer in the same way. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant was used for the assay. The whole procedure was carried out at 0—5°C. The protein concentration in both enzyme preparations was adjusted to ca. 100 μg per 0.1 ml with the acetate buffer. The protein concentration was measured by the method of Lowry et al. [17] using bovine serum albumin as a standard protein.

Apparatus

A Shimadzu LC-3A high-performance liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Shimadzu RF-530 fluorescence spectromonitor fitted with a 12-μl flow-cell operating at an emission wavelength of 450 nm and an excitation wavelength of 360 nm. The sensitivity of the spectromonitor was set in general at the ranges 2 and 256 of the high level in the assay for HDC in stomach and brain, respectively. Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 × 10 mm); spectral band-widths of 5 nm were used in both the excitation and emission monochromators.

Procedure

Cofactor solution was 0.2 *M* phosphate buffer (pH 6.8) containing 12.5 μM PALP for the assay of HDC in stomach or 37.5 μM for the assay in brain, 250 μM dithiothreitol, 1.25% (v/v) polyethyleneglycol and 12.5 μM aminoguanidine. To 0.8 ml of the solution was added 0.1 ml of the HDC preparation. The mixture was preincubated at 37°C for 10 min and then incubated again at 37°C for 60 min for HDC assay in stomach and for 180 min for the assay in brain, after addition of 0.1 ml of 25 m*M* histidine (substrate solution). The reaction was terminated by adding 0.2 ml of 2.4 *M* perchloric acid. The mixture was centrifuged at 7000 *g* for 5 min. To the supernatant (1.0 ml) were added 0.2 ml of 1.8 *M* potassium hydroxide and 1.0 ml of 0.5 *M* phosphate buffer (pH 6.5). The mixture was placed in an ice-bath for ca. 10 min, and then centrifuged at 1500 *g* for 10 min. The supernatant (2.0 ml) was poured on to an Amberlite CG-50 column. Histidine in the column was washed twice with 1.0 ml of 0.2 *M* phosphate buffer (pH 7.0) and then twice with 1.0 ml of water. The adsorbed histamine was eluted with 1.0 ml of 0.4 *M* sodium hydroxide. To the elute, 50 μl of 74.6 m*M* OPA in methanol were added and the resulting mixture was allowed to stand at 25°C for exactly 3 min to develop the fluorescence. Sulphuric acid (1.5 *M*, 100 μl) was added to stop the reaction. The reaction mixture (100 μl) was subjected to HPLC. The mobile phase was a mixture of 0.2 *M* sodium chloride, methanol and 0.1 *M* hydrochloric acid (55 : 60 : 1.25, v/v/v). The flow-rate was 0.5 ml/min. The column temperature was ambient (20—25°C).

For the blank, the same procedure was carried out except that the order of addition of the substrate solution and the perchloric acid solution was reversed. The net peak height in the chromatogram was used for the quantification of histamine. The amount of histamine was calibrated by means of the standard addition method: the substrate solution (0.1 ml) in the procedure for the blank was replaced with histamine standard solution (500 and 17.5 pmol per 0.1 ml for rat stomach and brain, respectively) dissolved in the substrate solution.

RESULTS AND DISCUSSION

The conditions of the fluorescence reaction and HPLC were almost the same as described previously [15, 16].

Fig. 1 shows typical chromatograms obtained with the brain HDC preparation prepared according to the procedure. The peak due to histamine was ob-

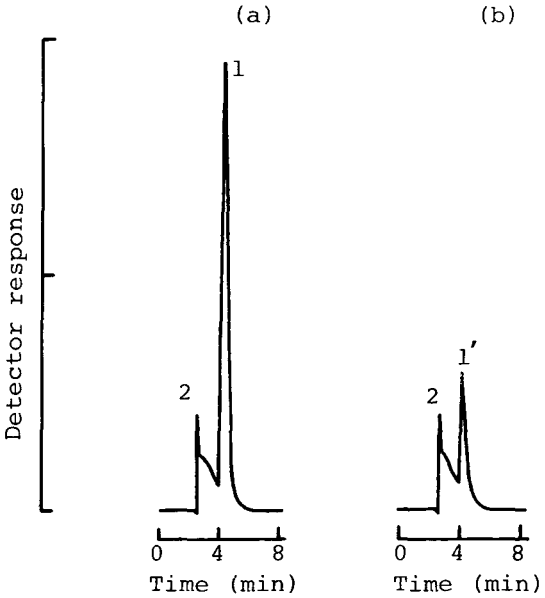

Fig. 1. Chromatograms obtained with (a) the HDC preparation from rat brain and (b) the blank carried through the procedure (see text). Peaks: 1 = histamine formed enzymatically plus endogenous histamine; 1′ = endogenous histamine; 2 = biogenic amines other than histamine. The activity of HDC was 0.79 pmol per min per mg of protein. The HDC preparation used contains 109 μg of protein and 5.5 pmol of endogenous histamine in 0.1 ml.

served at the retention time of 4.5 min in each of the chromatograms (peaks 1 and 1′). The eluates from peaks 1 and 1′ have the same fluorescence excitation (maximum 360 nm) and emission (maximum 450 nm) spectra. Peak 1′ in the chromatogram of the blank increases in height in proportion to increasing amount of protein in the HDC preparation, and is due to the endogenous histamine in the preparation. Peak 2 increases in height with increasing amount of protein in the preparation, and is ascribable to biogenic amines other than histamine.

The patterns of chromatograms obtained with the HDC preparation from stomach were virtually identical with those of Fig. 1. However, the levels of both the enzyme activity and endogenous histamine in stomach are very much greater than in brain, and, therefore, compared to the peak for histamine, the peak for biogenic amine (corresponding to peak 2 in Fig. 1) was relatively very small.

Since the substrate histidine forms a fluorophore which shows similar chromatographic and fluorescence spectral behaviours to that of histamine when it reacts with OPA (structures of the fluorophores of histamine and histidine remain unknown), adequate separation of histamine from unreacted histidine is required before derivatization of histamine with OPA. This was successfully carried out by chromatography on a small column of Amberlite CG-50. The recovery of 1.0 nmol of histamine in the presence of 1.0 μmol of histidine was 98.2 ± 2.4% [mean ± standard deviation (S.D.), $n = 15$].

A maximum and constant HDC activity was obtained in the presence of 2.0—5.0 m*M* histidine in the incubation mixture with an observed Michaelis

constant of 0.40 m*M*; 2.5 m*M* was used as a saturating concentration for the enzyme reaction. HDC was most active at pH 6.8 in the presence of 2.5 m*M* histidine in the incubation mixture. Maximum activity was attained in phosphate buffer at concentrations of 0.15—0.3 *M* in the incubation mixture; 0.2 *M* phosphate buffer (pH 6.8) in the cofactor solution was employed. PALP in the incubation mixture provided a maximum activity in the concentration ranges of 5—20 μM and 20—40 μM for the assay of HDC in stomach and brain, respectively; 10 and 30 μM respectively, were selected for the standard procedure. The prescribed concentrations of dithiothreitol and polyethyleneglycol, stabilizers of HDC, in the incubation mixtures were optimal [14].

Histaminase present in the enzyme preparation catalyses the conversion of histamine to imidazoleacetaldehyde and thus a histaminase inhibitor aminoguanidine is used in the HDC assay procedure [14].

Aminoguanidine in the incubation mixture gave a maximum activity of HDC at concentrations of 8—20 μM, 10 μM was used in the procedure. Histamine is also converted to N^{τ}-methylhistamine by histamine N-methyltransferase-mediated reaction. The effect of N-methyltransferase on the amount of histamine formed was examined using 10 μM histamine solution in place of the substrate solution in the procedure. The amount of histamine did not vary with or without incubation for 180 min. Therefore, it seemed that N-methyltransferase does not affect the assay of HDC.

The enzyme activity was linear with time up to at least 3 and 6 h for the enzyme preparations from stomach and brain, respectively, when incubated at 37° C. The amounts of histamine formed for the prescribed incubation times were proportional to the amount of protein in the HDC preparations (0.1 ml) up to 1.5 mg. The HDC preparations were adjusted to contain ca. 100 μg of protein per 0.1 ml, respectively, in the procedure.

A linear relationship was obtained between the peak height of histamine and the amount of the amine added in the range of 2.0 pmol to 5.0 nmol to the enzyme reaction mixture. The recoveries of histamine added to the reaction mixtures of the blanks in HDC assay of stomach and brain in the amounts of 500 and 10 pmol were 93.2 ± 3.0% and 95.3 ± 2.4% (mean ± S.D., n = 12 in each case), respectively.

The lower limits of detection for histamine formed enzymatically were 110 pmol per assay tube in stomach HDC assay and 1 pmol per assay tube in brain HDC assay. The limit was defined as the amount giving 1.2 times the height of the peak in the blank for the reason that the amounts of enzymatically formed and endogenous histamine can be measured fairly precisely (coefficient of variation below 3%).

HDC activities in the preparations from rat (male, Wistar, 5—6 weeks of age) stomach and rat (male, Sprague—Dawley, 6—7 weeks of age) brain were 80.5 ± 9.1 and 0.79 ± 0.15 pmol per min per mg of protein, respectively (mean ± S.D., n = 10 in each case). The values were similar to those obtained by other workers [6—14].

This study provides the first HPLC method with fluorescence detection for the assay of HDC. The method is highly sensitive and so requires only 100 μg of protein from rat stomach or brain. The method may permit the assay of HDC in preparations from other tissues. Therefore, it should be useful for biological investigations of HDC in place of radiochemical methods.

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Journal of Chromatography, 311 (1984) 396—399
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2276

Note

Rapid analysis of adenosine, AMP, ADP, and ATP by anion-exchange column chromatography

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(Received March 10th, 1984)

We have developed a simple conventional anion-exchange column chromatographic method for the determination of nucleotides [1, 2]. The present study demonstrates that the analysis of adenosine, AMP, ADP, and ATP can be accomplished with speed, sensitivity, and accuracy by simply modifying the elution mode and using a smaller column. This method is therefore suitable to many areas of application, such as rapid determination of adenylate energy charge [3], enzymatic activity involving adenine nucleotides, and the content of adenine nucleotides in human erythrocytes. High-performance liquid chromatography (HPLC) has been used to monitor adenine nucleotides in human blood [4], and to determine the profile in the erythrocyte [5]. The chromatographic method described in this report is simpler and more convenient than that of HPLC.

EXPERIMENTAL

Materials

Materials used were described previously [1].

Column chromatography

A column packed with AG MP-1 resin, 10 × 3 mm, was eluted with a programmed hydrochloric acid gradient established by using a GP-250 gradient programmer equipped with two P-500 pumps (Pharmacia, Piscataway, NJ, U.S.A.); one pumps distilled water, the other 0.192 *M* hydrochloric acid. The gradient programme comprised four steps: distilled water for 1 min, 6% of

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0.192 *M* hydrochloric acid for 1 min, a linear gradient from 6% to 100% of 0.192 *M* hydrochloric acid for 2 min, and then 0.192 *M* hydrochloric acid for 2 min. A column flow-rate of 2 ml/min or 1 ml/min was used. The column was washed with distilled water for 1 min at a flow-rate of 10 ml/min to regenerate the column. The effluent was monitored at 257 nm, and the peaks were integrated by a minigrator of Spectra Physics (Piscataway, NJ, U.S.A.), and the amount of solute in each peak was calculated according to the following equation:

$$\text{Amount (nmol)} = \frac{Int \cdot Av \cdot \text{a.u.f.s.} \cdot F \cdot 1066}{E_{257}}$$

The equation is derived from the equation previously described [1]. *Int* is the integration value in mV-min; *Av* is the signal output of the monitor, and is equal to 0.2 absorbance units/mV; a.u.f.s. is the sensitivity setting of the monitor in absorbance units at full scale; *F* is the flow-rate in ml/min; 1066 is the equation constant; and E_{257} is the molar extinction coefficient at 257 nm. The extinction coefficients are $15.4 \cdot 10^3$ for adenosine, $15.0 \cdot 10^3$ for AMP and ADP, and $14.7 \cdot 10^3$ l mol^{-1} cm^{-1} for ATP.

Preparation of samples

Blood or packed erythrocytes (25 µl) which were packed by centrifugation at 400 *g* for 10 min was pipetted into 225 µl of cold distilled water. After vigorous agitation on a vortex mixer, 250 µl of cold 6% trichloroacetic acid (TCA) were added and thoroughly mixed. The precipitate was cleared by centrifugation and 250 µl of the supernatant solution were neutralized with 50 µl of 2 *M* tris(hydroxymethyl)aminoethane (Tris). The solution (100 µl) was then injected into the column for chromatography.

RESULTS AND DISCUSSIONS

As shown in Fig. 1, resolution of adenosine, AMP, ADP, and ATP is accomplished rapidly using the programmed elution mode. Two chromatograms are shown in the figure, one is eluted at a constant flow-rate of 2 ml/min (A); the other at 1 ml/min (B). In A, the analysis took only 4 min; therefore, the last step of the gradient programme could be eliminated so that a cycle of analysis required only 5 min. Decreasing the flow-rate to 1 ml/min as shown in B, a total of 7 min was required to run a sample; however, the sensitivity increased two-fold.

In this study, a small column was used. This has three advantages; it reduces the retention time and speeds up the chromatography; a relatively small volume of distilled water is required to regenerate the column; and a relatively lower flow-rate can be used to increase the sensitivity.

The sensitivity of the method is about 30 pmol. However, the reliable ranges which yield a linear relationship between the amounts and the integration values were obtained at higher concentrations. These ranges are listed in the second column of Table I, and were obtained by monitoring the effluent at 0.2 a.u.f.s. and at a constant flow-rate of 2 ml/min. Table I also shows the reproducibility and accuracy of the chromatography. The results were obtained

from eight repeated runs of a mixture containing the four authentic compounds.

A typical chromatogram of blood nucleotides is shown in Fig. 2B. A blank which contains no blood but TCA and Tris is shown in A. It shows two ultraviolet-absorbing peaks; one is due to Tris and the other to TCA. The latter peak co-eluted out with AMP; therefore, the amount was subtracted from that of AMP in the subsequent analyses. It should be noted that solutes were retained slightly longer than the standard samples. This is due to the presence of excess amount of Tris used to neutralize the TCA extract. The peak shown by a broken line is the position of adenosine, indicating that the presence of excess amount of Tris base causes a slight increase in the retention time but

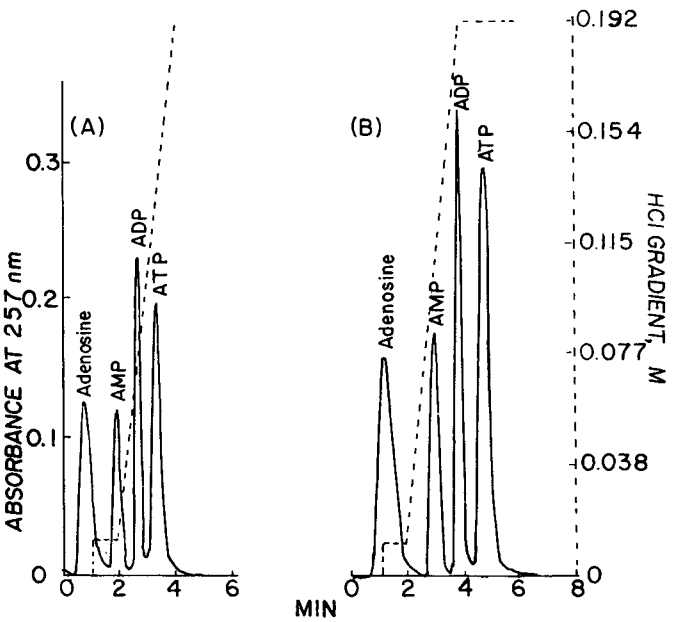

Fig. 1. Resolution of adenosine, AMP, ADP and ATP at flow-rates of 1 ml/min (A), and 2 ml/min (B). The hydrochloric acid gradients are indicated by the broken lines.

TABLE I

LINEAR RANGES, ACCURACY AND REPRODUCIBILITY OF THE CHROMATOGRAPHIC METHOD IN THE DETERMINATION OF ADENOSINE, AMP, ADP, AND ATP AT A COLUMN FLOW-RATE OF 2 ml/min

Means were obtained from eight runs of the same mixture.

Compound	Linear range (nmol)	Mean (nmol)	Standard deviation	Coefficient of variation (%)
Adenosine	0.6—155	11.81	0.17	1.44
AMP	0.5—116	10.49	0.37	3.53
ADP	0.5— 87	13.34	0.04	0.30
ATP	0.5—130	19.89	0.18	0.91

without interfering with the resolution. The levels of adenine nucleotides represented by the figure were 46 nmol of AMP, 84 nmol of ADP, and 538 nmol of ATP per ml of blood.

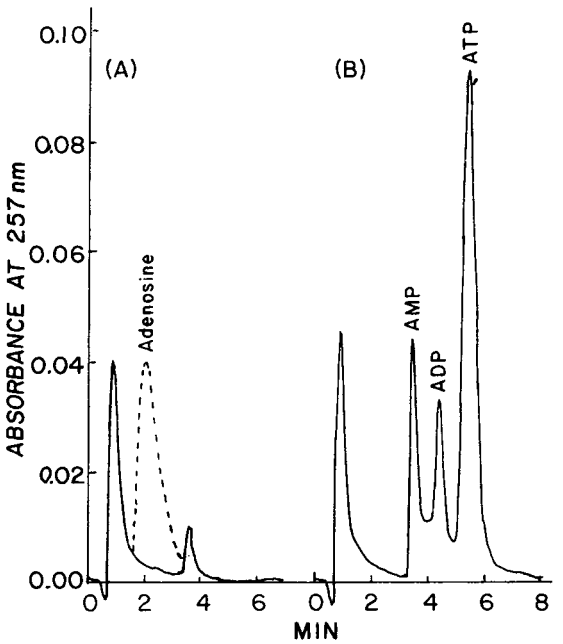

Fig. 2. Chromatogram of adenine nucleotides in human blood. The column flow-rate was 1 ml/min. A is a blank containing TCA and Tris. The position of adenosine peak obtained at another run with external adenosine is indicated by the broken line. A typical blood adenine nucleotide profile is shown in B.

Analyses of eight samples of packed human erythrocytes from hospitalized patients showed that the total adenine nucleotide concentrations varied from 1153 to 1497 nmol/ml packed erythrocytes with the ratios of ATP:ADP:AMP comparable to the values reported [5].

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Journal of Chromatography, 311 (1984) 400—404
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2268

Note

Thin-layer chromatography of hydroxylysine for collagen analysis

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(First received April 10th, 1984; revised manuscript received July 16th, 1984)

Ultramicro methods of amino acid analysis based on thin-layer chromatographic (TLC) separation of dansylated derivatives [1, 2] provide a simple and convenient procedure for analyzing the amount and specific radioactivity of picomolar quantities of amino acids [3]. The application of this simple but sensitive technique has allowed analysis of minute quantities of amino acids associated with subcellular amino acid pools and individual protein bands eluted from analytical polyacrylamide gels, as well as a number of other applications [4]. In a previous publication outlining optimal approaches to methodologies, we pointed out that the technique would also be well suited for routine determination of modified amino acids such as hydroxyproline [4]. We report here on the use of this radioisotope dilution method to rapidly and specifically quantitate hydroxylysine. The method is based on the derivatization of free amino acid with [^{14}C]Dns chloride to form [^{14}C]di-Dns-hydroxylysine which can be readily separated from other amino acids by TLC on polyamide plates. Indirect quantitation of non-radiolabeled hydroxylysine in acid hydrolysates of crude tissue and body fluid samples is carried out by mixing these with a precise amount of [^{3}H]hydroxylysine prior to dansylation. The degree to which the specific activity of the latter isotopic species is lowered allows quantitation of picomolar quantities of hydroxylysine. Because previously available methods of analysis of this amino acid are based on liquid chromatography or multi-step analytical procedures (for review, see ref. 5), the application of TLC provides significant improvement in convenience while increasing the sensitivity to the picomolar (10^{-10} to 10^{-12} *M*) range.

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EXPERIMENTAL

[^{3}H]Hydroxylysine was prepared by custom catalytic exchange in tritiated aqueous medium (Amersham, Arlington Heights, IL, U.S.A.) using δ-hydroxylysine hydrochloride obtained from Sigma (St. Louis, MO, U.S.A.). The resulting tritiated hydroxylysine (generally but not uniformly labelled) had a specific radioactivity of 32 dpm/pmol as determined by dansylation and confirmed gravimetrically. We obtained methyl[^{14}C]Dns chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) from Research Products International (Mount Prospect, IL, U.S.A.), diluted it with [^{12}C]Dns chloride to a specific radioactivity of 7.2 dpm/pmol, and stored it in acetone at 4° C until used. The concentration of the final Dns chloride solution was 4.2 m*M*. Standard mixtures of nineteen amino acids with or without hydroxylysine were obtained from Pierce (Rockford, IL, U.S.A.).

To quantitate hydroxylysine content of urine and crude tissue, samples were hydrolyzed overnight in redistilled hydrochloric acid (Ultrex, J.T. Baker, Phillipsburg, NJ, U.S.A.) at 110° C under vacuum, evaporated to dryness, taken up twice in deionized water and redried. The dried sample was brought up in deionized water. A 10-μl aliquot was mixed with 10 μl (300 pmol unless otherwise stated) of [^{3}H]hydroxylysine ("probe"), dried under vacuum and solubilized in 20 μl of 1 *M* sodium carbonate—sodium bicarbonate buffer (pH 9). The pH of the resulting sample was tested, and 10 μl additional buffer were added if the pH remained less than 9.

An equal volume of [^{14}C]Dns chloride solution in acetone was added to the sample and reacted at 37° C in a humidified atmosphere for 30 min or until the yellow color disappeared. The reacted sample was dried down and extracted three times in 100 μl of water-saturated ethyl acetate. The pooled samples in ethyl acetate were dried down and the sample reconsitituted in 10 μl deionized water. Chromatography was performed as previously described [3]. In brief, the reaction mixture was applied near the corner of a 7.5 × 7.5 cm^2 polyamide plate (Cheng Chin Trading, Taiwan, or Accurate Chemical and Scientific Company, Hicksville, NY, U.S.A.) by repeated spotting. The plates were developed first by ascending chromatography in covered glass tanks in formic acid (88%)—water (2:100), then dried. The plates were turned 90° and rechromatographed in benzene—glacial acetic acid (90:10). For optimal separation of hydroxylysine, chromatography was repeated in the second dimension with the same solvent system. Development in each tank was continued until the solvent front ran all the way to the top of the plate. Dns-amino acids were visualized under short-wave ultraviolet light (see Fig. 1), and identified based on patterns published in the literature [3, 4]. Individual dansylated derivatives were cut from the plate and placed in 7-ml scintillation vials to which were added 0.15 ml fresh NCS solubilizer (Amersham) and 3 ml Econofluor (New England Nuclear, Boston, MA, U.S.A.). Radioassay was performed on a Packard Tri-Carb liquid scintillation spectrometer with average counting efficiencies of 0.253 for ^{3}H and 0.505 for ^{14}C. The tritium-specific radioactivity of hydroxylysine (dpm/pmol) was calculated as:

$$\frac{^{3}\text{H dpm} \times 2}{^{14}\text{C dpm} \div 7.2 \text{ dpm/pmol}} \quad (1)$$

where 7.2 dpm/pmol is the specific radioactivity of [^{14}C]Dns chloride. The two-fold correction is made because of the didansylated nature of the hydroxylysine species isolated.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatographic separation of didansylated hydroxylysine from other amino acids. Under the conditions of chromatography chosen, most other amino acids were allowed to migrate further from the origin in both dimensions in order to provide optimal separation of di-Dns-hydroxylysine from Dns-hydroxide and other neighboring derivatives.

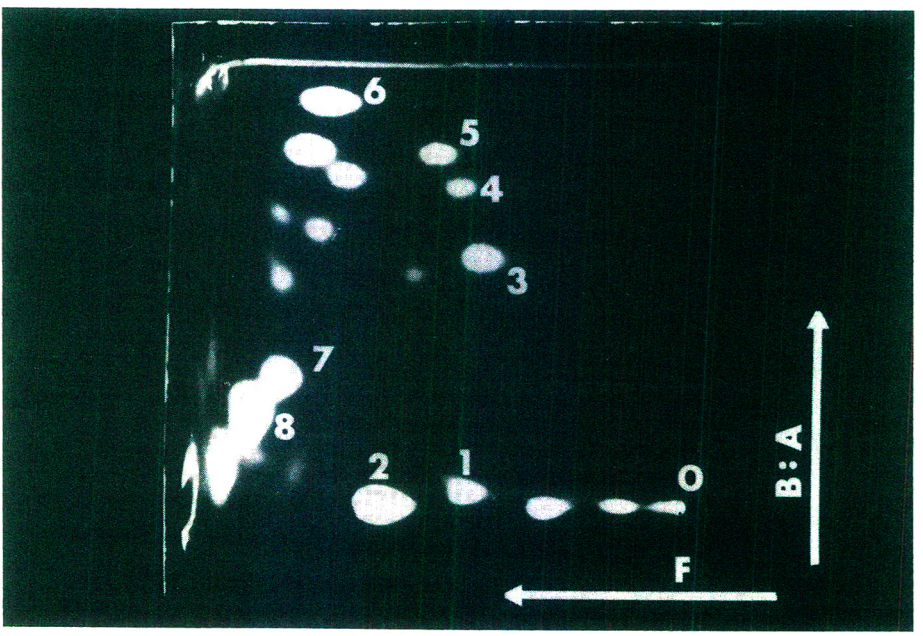

Fig. 1. TLC of Dns-amino acid derivatives. Identification of individual spots is based on patterns provided in previous publications [3, 4]. Individual dansylated amino acids are readily visualized under short-wave ultraviolet light by their yellow fluorescence. Approx. 100 pmol of hydroxylysine were chromatographed in the presence of a mixture of twenty other amino acids. 0 = Origin, 1 = di-Dns-hydroxylysine, 2 = Dns hydroxide, 3 = Dns-phenylalanine, 4 = Dns-leucine, 5 = Dns-isoleucine, 6 = Dns-proline, 7 = Dns-glycine, and 8 = Dns-hydroxyproline. Arrows indicate ascending chromatography in formic acid (F) and benzene—acetic acid (B:A).

Eqn. 1 shows how the specific radioactivity of hydroxylysine can be determined. Fig. 2 shows how hydroxylysine in non-radioactive samples can be quantitated by mixture with [^{3}H]hydroxylysine (probe) or known specific activity. Known amounts of non-radioactive hydroxylysine were added to a fixed amount (300 pmol) of [^{3}H]hydroxylysine probe. The solid line shows

the expected reduction in specific radioactivity as increasing amounts of non-labeled hydroxylysine are added, and can be expressed by the equation:

$$\frac{\text{SA (probe + standard)}}{\text{SA (probe)}} = 1 + \frac{\text{pmol (standard)}^{-1}}{\text{pmol (probe)}} \quad (2)$$

where SA is specific radioactivity of hydroxylysine. We set up the assay in such a way that unknown samples containing 300 pmol of hydroxylysine fell on the middle of the curve. The observed data (closed circles) were obtained by adding known amounts of non-labeled hydroxylysine and closely fit this expected curvilinear relationship.

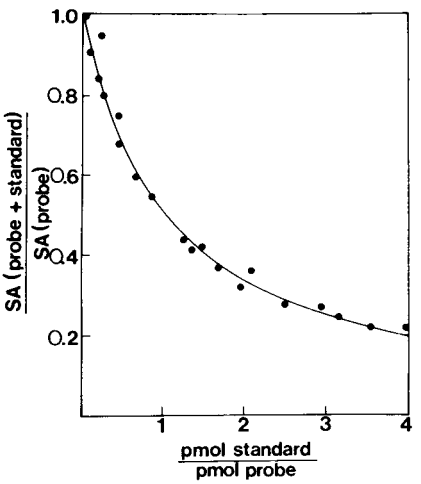

Fig. 2. Standard curve for quantitation of hydroxylysine. The progressive decrement of [^{3}H]hydroxylysine probe specific activity as increasing amounts of non-labeled hydroxylysine are added is shown. The solid line indicates the expected relationship between specific activity and amount of added hydroxylysine. Closed circles represent individual data points. The assay as set up is accurate for samples containing 100—1000 pmol of hydroxylysine.

Based on analysis of fifteen standard samples containing 0.5 to 3.0 times as much unlabelled hydroxylysine as the probe, we found that the measured value was 0.988 ± 0.041 (mean ± S.D.) of the expected hydroxylysine content. Because the relationship between specific radioactivity and amount is non-linear, we routinely dilute all unknown samples empirically such that they contain 0.5 to 3.0 times as much hydroxylysine as found in the probe. Where necessary, the amount of probe can similarly be varied.

To test whether the assay for hydroxylysine was similarly accurate in the presence of other amino acids, we constructed a standard curve by adding increasing amounts of a standard mixture of nineteen amino acids to 300 pmol of [^{3}H]hydroxylysine. The composition of the mixture was such that 38 pmol of amino acids were present for each pmol of hydroxylysine. Eqn. 3 describes the close linear relationship between the amount of hydroxylysine added (X) and the amount measured by radiodilution assay (Y):

$$Y = 0.015 + 0.997X \; (r^2 = 0.999, p < 0.001) \quad (3)$$

Approx. 19% of the starting hydroxylysine which was reacted with Dns chloride could be recovered in the carefully cut di-Dns-hydroxylysine spot. However, because quantitation is based on specific radioactivity determinations and because radiolabeled probe is added to the unknown sample before dansylation and separation, complete recovery is not necessary for accuracy.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Jill Soltys and Patty Waitkus and the services of the Word Processing Center. William S. Stirewalt, Ph.D., provided helpful insight into experimental approaches. Supported by Grants PHS 5429-22-9 from the National Institutes of Health and from the Health Effects Institute.

Research described in this article is conducted under contract to the Health Effects Institute, an organization that supports the conduct of independent research and is jointly funded by the U.S. Environmental Protection Agency and automotive manufacturers. Publication here implies nothing about the view of the contents by HEI or its research sponsors. HEI's Health Review Committee may comment at any time and will evaluate the final report of the project. Additionally, although the work described in this document has been funded in part by the U.S. E.P.A. under assistance agreement with HEI, the contents do not necessarily reflect the views and policies of the agency; nor does mention of trade names or commercial products constitute endorsement or recommendations for use.

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Journal of Chromatography, 311 (1984) 405—410
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2281

Note

Fluorometric evaluation of sarcosine in urine and serum

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(First received May 2nd, 1984; revised manuscript received July 18th, 1984)

Sarcosine (N-methylglycine) is a metabolic derivative of choline. Sarcosine is normally demethylated into glycine by an oxidative process necessitating tetrahydrofolate and catalysed by the flavoenzyme sarcosine dehydrogenase [1—3]. This enzyme is present in the human liver but not in fibroblasts [4—6]. Some rare examples of inactive sarcosine dehydrogenase have been described, characterized by hypersarcosinuria and hypersarcosinaemia [5, 7—13]. If one excludes some cases that were corrected by injections of folic acid [14], a genetic origin appears probable, as an autosomal recessive character [13].

The evaluation of sarcosine has, up to now, been realized by several adaptations of the amino acid ion-exchange chromatographic technique initially described by Spackman et al. [15]. Separation and evaluation are satisfactory as regards urinary sarcosine, but do not permit the evaluation of blood sarcosine except in cases of very large increases. The normal concentration of sarcosine in blood is below the limit of detection and remains undetermined.

In our laboratory, we have been engaged for several years in the evaluation of picomole amounts of proline and hydroxyproline by a technique of derivatization with a fluorogenic compound, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), followed by thin-layer chromatography of the fluorescent derivative and scanning fluorometry [16—18].

Adapting the thin-layer fluorometric recording technique used for hydroxyproline, we set up the following evaluation methods for sarcosine in urine and blood.

MATERIALS AND METHODS

Reagents

When not specifically mentioned, chemicals were purchased from Prolabo,

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Paris, and were of reagent grade. Chloroform, triethylamine, 2-mercaptoethanol, methanol and formic acid were bought from E. Merck (Darmstadt, F.R.G.). *o*-Phthaldialdehyde was obtained from Sigma (St. Louis, MO, U.S.A.) and NBD-Cl from Aldrich (Beerse, Belgium).

Standard sarcosine and 4-hydroxyproline were bought from Sigma. A mixture of eighteen reference amino acids was purchased from Beckman (Gagny, France) for the purpose of amino acid analyser standardization. A standard of 3-hydroxyproline was prepared in our laboratory [19].

Preparation of urine samples

Deproteinization was performed by addition of an equal volume of absolute ethanol at 4°C. After several minutes of stirring, the mixture was centrifuged and 4 ml of supernatant were evaporated to dryness under a stream of nitrogen at ordinary temperature.

The urine residue was dissolved in 0.4 ml of a 0.2 mol/l sodium citrate buffer pH 2.2 and clarified by centrifugation at 1000 *g* for 10 min. An aliquot of 0.2 ml of supernatant was chromatographed as described previously [18].

Derivatization reaction

The eluate, after concentration to dryness, was dissolved in 0.1 ml of water. To this solution was added 0.2 ml of a 0.06 mol/l *o*-phthaldialdehyde solution [18]. After 1 min standing at room temperature, 0.2 ml of a 0.03 mol/l solution of NBD-Cl in ethanol was added. The preparation of the NBD-Cl solution has been previously described [16]. The mixture of amino acids, *o*-phthaldialdehyde and NBD-Cl was then incubated for 30 min at 65°C in a stoppered test tube. The NBD-amino acid derivatives were ready for thin-layer chromatography.

Preparation of blood serum samples

To 2 ml of serum was added 1 ml of a 20 g per 100 ml sulphosalicylic acid solution. After stirring, the mixture was centrifuged at 1000 *g*. A 1-ml volume of supernatant was diluted with 0.5 ml of 0.2 mol/l sodium citrate buffer, pH 2.2; 1 ml of this mixture was layered at the top of a 52 × 0.9 cm column of M 82 resin in a Multichrom B Beckman amino acid analyser connected to a fraction collector. The amino acids were eluted by 0.2 mol/l sodium citrate buffer pH 3.2 at 39°C for 40 min and then at 65°C for a further 40 min period at a flow-rate of 70 ml/h. Fractions of 7 ml were collected. Sarcosine was eluted between glutamine and glutamic acid with a retention time of 66 min and was obtained quantitatively in fraction 11.

This fraction was deionized on a 6.4 × 1.1 cm column of Dowex 50W-X2 and processed as described in the case of urinary sarcosine for the derivatization reaction.

Thin-layer chromatography

The system has been fully described in several previous papers [16–18]. In the case of sarcosine, two solvents were alternatively used: solvent I, chloroform–methyl ethyl ketone–formic acid (75:20:5, v/v), and solvent II, chloroform–acetone–toluene–methanol–triethylamine (40:20:20:15:5, v/v).

The development time was 70 min with solvent I and 45 min with solvent II at 20°C. Both solvents gave satisfactory separations of sarcosine. The plates were dried for 10 min at 65°C. They can be stored in the dark up to three days prior to spectrofluorometric evaluation [16].

RESULTS

The various steps of the evaluation were checked for recovery and reproducibility with known concentrations of control sarcosine. After the preliminary column chromatography, the recovery, measured in four separate experiments, was 99 ± 2% of the amount deposited. The reproducibility of the complete method was checked by eight separate measurements of the same sample of urine supplemented with sarcosine in order to reach a concentration of 100 pmol in every final deposit on thin layer. Recovery was 95.5 ± 2%. For assessing the reproducibility, a sample of urine was evaluated eight times and gave the results (mean ± 1 S.D.) 7.88 ± 0.22 μmol per 24 h.

A photo of a thin-layer chromatogram of urine samples viewed under ultraviolet light is shown in Fig. 1. The clearing effect of *o*-phthaldialdehyde is demonstrated by the differences between lanes 7 and 8. A fluorometric record of a urine sample is shown in Fig. 2.

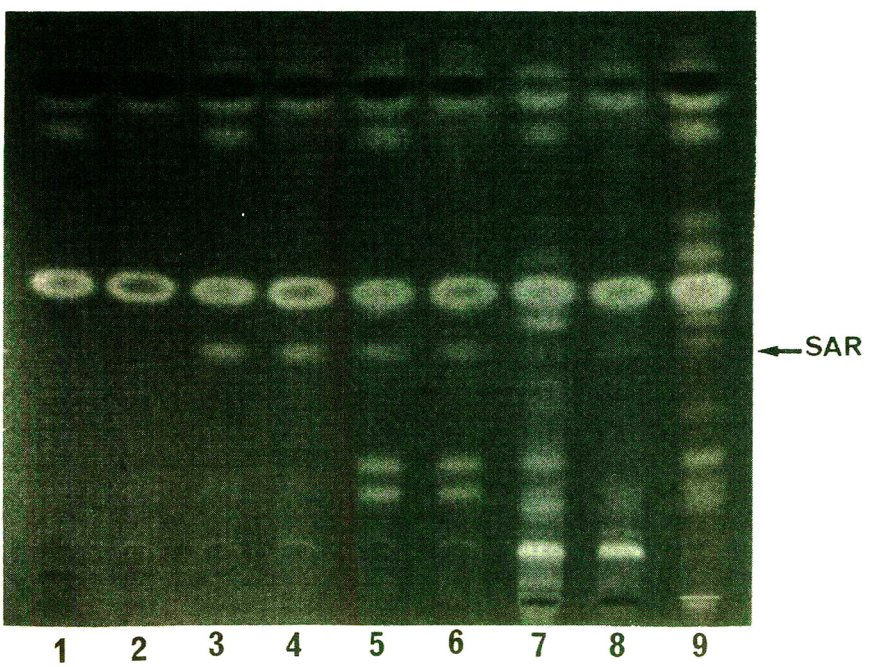

Fig. 1. Photo under ultraviolet light of a thin-layer chromatogram of NBD-Cl derivatives from control and urinary amino acids. Lanes 1, 3, 5, 7, 9: direct reaction with NBD-Cl. Lanes 2, 4, 6, 8: reaction with *o*-phthaldialdehyde prior to derivatization with NBD-Cl. Lanes 1, 2: blank reagents. Lanes 3, 4: control sarcosine. Lanes 5, 6: control sarcosine, 3-hydroxyproline and 4-hydroxyproline. Lanes 7, 8: urine samples. Lane 9: mixture of control amino acids. Solvent: formic acid—ethyl methyl ketone—chloroform (5:20:75, v/v). SAR = sarcosine.

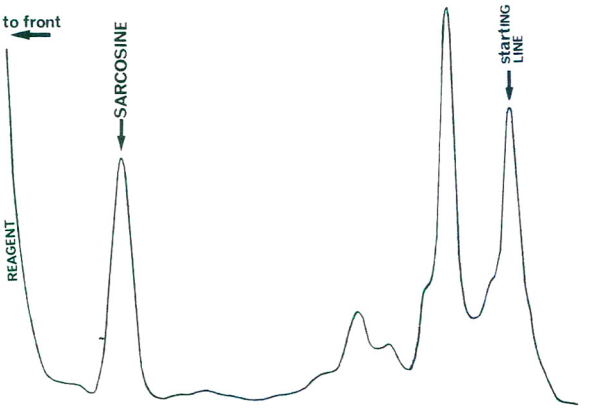

Fig. 2. Scan of a thin-layer chromatogram showing the peak of NBD-sarcosine (114 pmol) from a sample of urine. Solvent: formic acid—ethyl methyl ketone—chloroform (5:20:75, v/v). Spectrofluorometer Farrand Mark I, sensitivity 0.3, scan speed 150 mm/min.

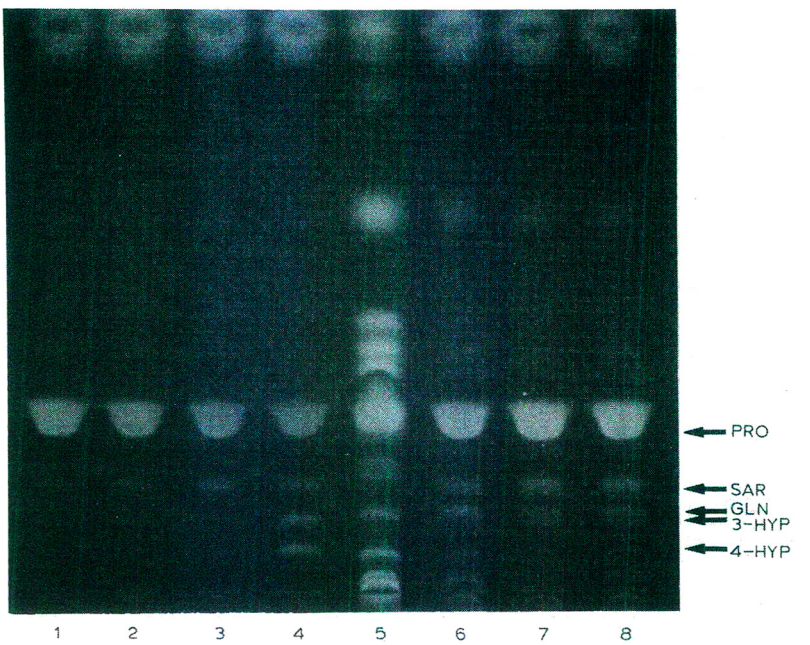

Fig. 3. Photo under ultraviolet light of a thin-layer chromatogram of NBD-Cl derivatives from control and serum amino acids. Lanes 1, 2, 3: control sarcosine in 50, 100 and 200 pmol amounts. Lane 4: control 4-hydroxyproline, 3-hydroxyproline and sarcosine. Lane 5: mixture of amino acids. Lane 6: fraction 11 eluted from a Beckman amino acid analyser and fraction collector, from a sample of serum containing 200 pmol of sarcosine in the deposit. Lanes 7, 8: same fraction 11 obtained from two other samples of serum. Solvent: chloroform—acetone—toluene—methanol—triethylamine (40:20:20:15:5, v/v). PRO = proline, SAR = sarcosine, GLN = glutamine, 3-HYP and 4-HYP = 3- and 4-hydroxyproline.

Serum sarcosine

The elution of sarcosine from the Beckman amino acid analyser was checked in four separate experiments using samples of the same serum each supplemented with 200 pmol of control sarcosine. The recovery was 97.1 ± 5.3% of the amount deposited. All the sarcosine was found in fraction 11, between glutamine and glutamic acid, as shown in Fig. 3.

On the thin-layer chromatograms there is a linear relationship between the surface of the NBD-sarcosine peaks and the amount of NBD-sarcosine deposited on the plates at concentrations ranging from 0 to 400 pmol. The fluorescence spectrum of NBD-sarcosine is the same as that of NBD-hydroxyproline [16].

Results of evaluation of sarcosine in urine and serum

This technique was used for the evaluation of sarcosine in the urines of 54 apparently normal subjects. The normal range, defined within the limits of two standard deviations from the mean, was 1.75—20 μmol per 24 h. No sex-related difference was found.

We measured serum sarcosine in fifteen normal subjects and found levels of 1.59 ± 1.08 μmol/l (mean ± 2 S.D.).

DISCUSSION

The method that we propose for sarcosine has been in use in our laboratory for the evaluation of the hydroxyproline isomers for several years. It has proved reliable and very sensitive. We have now extended it to sarcosine because we found that this methylated amino acid reacted with NBD-Cl as well as hydroxyproline. Two preliminary steps of ion-exchange chromatography are necessary because the NBD-Cl derivatization is impaired by mineral ions and the thin-layer separation suffers from interferences by other amino acids when they are in large amounts.

In the case of serum, the use of an amino acid analyser is justified by the fact that it concentrates sarcosine within a few millilitres of effluent and separates it from most of the other amino acids. Direct detection of sarcosine by the ninhydrin reaction in the amino acid analyser is not possible because the amounts involved are 100 times smaller than the lower limit of detection. Direct fluorometric detection of sarcosine in the effluent would probably not work because the concentration would still not be high enough. The advantage of thin-layer chromatography is to concentrate the fluorescent substance in a very restricted area. In our system, picomole amounts are easily quantitated. The NBD-sarcosine spots are perfectly separated from any other fluorescent substance in the proposed system.

Our method gives as reference values for blood serum 0.50 to 2.70 μmol/l. Reference values for serum sarcosine have not previously been given. In the case of urine, the reference values that we found are 1.75—20 μmol per 24 h. The reference values given in the literature [7, 8] are inacurate: "normal range inferior to 22.5 μmol/l". The method does not permit the evaluation of sarcosine conjugates, if they exist in urine. We found that creatine, hydrolysed for 110 h at 105°C in 6 mol/l hydrochloric acid, liberates sarcosine. When

urine is hydrolysed in the same conditions, significant amounts of sarcosine are liberated. It is not certain whether this originates from sarcosine conjugates or from creatine.

Until now, the medical usefulness of sarcosine evaluations has been restricted to the exceptional cases of hypersarcosinaemia of genetic origin (some twenty cases described), in which the amounts of sarcosine in blood and urine exceeded several hundred times the normal range [7, 8, 10, 12, 13]. The more sensitive method that we propose in this paper may help still unknown limited defects of sarcosine metabolism to be discovered.

ACKNOWLEDGEMENTS

We express our thanks to Mr. Chastang for skilled technical help and to Mrs. Debref and Miss Oudard for careful typing of the manuscript.

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Journal of Chromatography, 311 (1984) 411—417
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2271

Note

Determination of the antimalarial mefloquine in human plasma by gas chromatography with electron-capture detection

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(First received April 17th, 1984; revised manuscript received July 16th, 1984)

Mefloquine, DL-*erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinoline-methanol (Ro 21-5998) (I, Fig. 1), is a new antimalarial drug for the treatment of drug-resistant falciparum malaria [1]. Pharmacokinetic studies in humans showed a long biological half-life of the parent drug [2, 3]. The main plasma and urine metabolite is 2,8-bis(trifluoromethyl)-4-quinoline carboxylic acid (II, Fig. 1) [4].

Several methods have been reported for the determination of the unchanged drug in human plasma including thin-layer chromatography (TLC) [5], high-

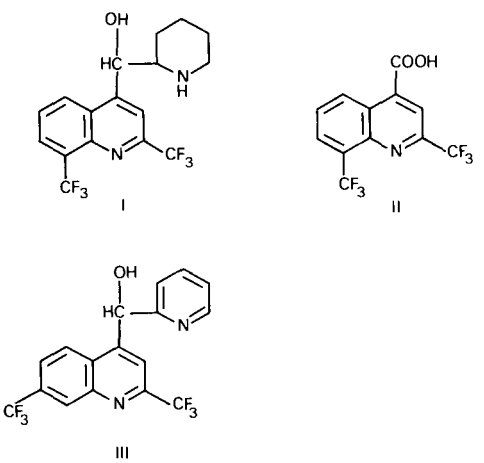

Fig. 1. Structural formulae of mefloquine (I), its metabolite (II) and of the internal standard (III).

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performance liquid chromatography (HPLC) [6] and gas chromatography—mass spectrometry (GC—MS) [7]. All of these methods have some inherent drawbacks. The sensitivity of both the TLC method [5] and the HPLC method [6] is limited and not sufficient for pharmacokinetic studies. Recently a more sensitive HPLC method has been published [8]. However, a three-step extraction procedure was required. GC—MS, on the other hand, is very sensitive but not available in every routine analytical laboratory. Due to the presence of two trifluoromethyl groups in the molecule, gas—liquid chromatography (GLC) with electron-capture detection (ECD) was expected to be more sensitive than TLC or HPLC. A GLC—ECD method for use in animal studies has been reported [9]. The present paper describes a simple and sensitive GLC—ECD method for the determination of mefloquine in human plasma, using packed columns. As the metabolite (II) is pharmacologically inactive, its measurement was not attempted by the present method.

EXPERIMENTAL

Reagents

Dichloromethane p.a. (freshly distilled), methanol p.a., and acetonitrile (LiChrosolv) were all from E. Merck, F.R.G.), as well as tris(hydroxymethyl)-aminomethane (Tris) and 0.1 *M* hydrochloric acid.

Tris—HCl buffer, 0.2 *M*, pH 8, was prepared by mixing equal volumes of a solution of Tris (12.5 g Tris in 500 ml of water) and of 0.1 *M* hydrochloric acid and adjusting the pH to 8.0.

N-Trimethylsilylimidazole (TSIM) was from Pierce Eurochemie.

Internal standard

The structurally closely related compound DL-α-(2-pyridyl)-2,7-bis(trifluoromethyl)-4-quinolinemethanol (Ro 12-9744) (III, Fig. 1) was used as internal standard.

Chromatographic system

The following instruments were used: gas chromatograph Varian 2700 with a 8.5-μCi ^{63}Ni electron-capture detector (Varian, Darmstadt, F.R.G.), modified for pulse mode with a Pye electron-capture amplifier (Philips, Kassel, F.R.G.); autosampler Hewlett-Packard 7660 A (Hewlett-Packard, Böblingen, F.R.G.); integrator SP 4100 (Spectra-Physics, Darmstadt, F.R.G.). A packed column, 150 cm × 4 mm filled with 3% SP-2250 on 80—100 mesh Supelcoport (Art. No. 1-1767, Supelco, Crans, Switzerland) was used. The chromatographic conditions were as follows: flow-rate 50 ml/min (argone—methane), oven 205°C, injector 270°C, detector 320°C. The high detector temperature was chosen to keep the foil clean of contaminations. The chromatographic response itself was independent of the electron-capture detector temperature.

Under these conditions, the following retention times were obtained: internal standard 4.6 min, mefloquine 6.4 min.

Teflon® -faced septa were used in the injector. For the autosampler, 250-μl glass micro-vials (Perkin-Elmer, Ueberlingen, F.R.G.), capped with aluminium foil, prepared in the laboratory, were used.

Plasma standards and internal standardization

Mefloquine base (10 mg) was weighed into a 10-ml flask and dissolved in methanol.

Plasma standards (calibration standards) at the following concentrations were prepared: 2000, 1500, 1000, 500, 250, 125, 62.5, 31.25, and 15.6 ng/ml. The 2000 ng/ml standard was prepared by adding 100 μl of the mefloquine solution to 50 ml of drug-free human plasma. The other standards were then prepared by stepwise dilutions with drug-free plasma. These calibration standards were stored deep-frozen (−18°C) in small portions until needed for analysis.

Internal standard (10 mg) was weighed into a 10-ml flask and dissolved in methanol. After appropriate dilution with water, this aqueous solution was used for internal standardization.

Sample preparation

Plasma samples and calibration standards were thawed at room temperature and then briefly mixed on a Vortex mixer. Aliquots of 0.25 ml of plasma (concentration range 15.6—125 ng/ml) or 0.1 ml of plasma (concentration range 62.5—2000 ng/ml) were transferred into a conical glass tube (15 ml). An equal volume of pH 8 Tris buffer, 100 μl of the aqueous solution of the internal standard and 6 ml of dichloromethane were added and the tube was closed by a glass stopper. The plasma was then extracted on a rotary extractor (10 min, 30 rpm). After centrifugation (5 min, 1000 *g*), the aqueous phase was carefully aspirated and discarded; 5 ml of the organic phase were transferred into a conical glass tube (7 ml volume) and evaporated to dryness under a gentle stream of nitrogen; 100 μl of a 10% solution of TSIM in acetonitrile were then added to the dry residue. The glass tube was stoppered and, after mixing on a Vortex mixer, the solution was maintained at room temperature for 15 min. After this, 400 μl of acetonitrile (concentration range 15.6—125 ng/ml) or 1000 μl of acetonitrile (concentration range 62.5—2000 ng/ml) were added. Samples with concentrations > 2000 ng/ml needed further dilution. The mixture was then transferred to the 250-μl micro-vials by means of a glass pipette and 1 μl was injected for analysis.

Calibration

Four or five calibration standards, covering the anticipated concentration range (15.6—125 or 62.5—2000 ng/ml), were processed as described above and analysed alongside the unknown samples. Peak height ratios of unchanged drug to the internal standard were measured and the calibration was obtained from linear regression of the peak height ratio against concentration. This line was then used to calculate the concentration of the unchanged drug in the unknown samples.

RESULTS

Trimethylsilylation

The GC—MS spectrum of the mefloquine TMS derivative was identical to that previously published [4], and confirmed the formation of the O-silyl

derivative. A 100-μl volume of a 10% solution of TSIM in acetonitrile was sufficient for complete reaction. This was established as follows: the dried residue of 300 ng of mefloquine base (corresponding to a plasma concentration of 3000 ng/ml in this procedure) was derivatized with the 10% TSIM solution; a second 300-ng sample was reacted with 100% TSIM. The same peak height was obtained from both reactions. A silylation time of 15 min was sufficient for complete derivatization. For reaction times of more than 15 min, no measurable change in the peak intensity was observed.

Recovery

Spiked plasma samples of various concentrations were prepared and extracted as described above. Solutions of mefloquine in dichloromethane were added to evaporated extracts of drug-free plasma to give samples in the same concentration range as the spiked plasma. Following evaporation and silylation, injection of these samples provided the "100%" values. The recovery was calculated by comparing the peak heights from these two experiments. The recovery varied between 97% and 107% in the range 20—2000 ng/ml (Table I).

TABLE I

RECOVERY AND INTRA-ASSAY PRECISION

$n = 4$.

Concentration (ng/ml)	Recovery (%) (mean ± S.D.)	Precision (C.V., %)
20	97 ± 2.5	1.2
200	107 ± 3.4	2.7
2000	105 ± 3.6	2.5

Linearity

A linear correlation between peak height ratio and concentration of mefloquine in plasma was found in the range 15.6—125 ng/ml (0.25 ml of plasma, final dilution to 500 μl) and in the range 62.5—2000 ng/ml (0.1 ml of plasma, final dilution to 1100 μl). Plasma samples with higher concentrations needed further dilution in the final step in order to ensure that the chromatographic response fell within the linear range of the detector used ($2 \cdot 10^2$, from 10^{-12} ng to $2 \cdot 10^{-10}$ ng of mefloquine).

TABLE II

INTER-ASSAY PRECISION AND ACCURACY

Added (ng/ml)	Found (ng/ml)	C.V. (%)	Deviation between added and found (%)	Replicates (n)
7.8	7.8	6.0	±0.0	5
62.5	62.2	3.9	—0.5	8
250	249	2.3	—0.4	6
500	501	3.2	+0.2	6
1000	993	2.8	—0.7	6

Precision

Intra-assay precision was calculated from spiked samples which were analysed as replicates during one working day. The mean coefficient of variation was ± 2.1% in the range 20—2000 ng/ml (Table I).

Inter-assay precision was calculated from spiked plasma samples (quality control samples), which were analysed as unknowns on different days using a new calibration each day. The mean coefficient of variation was 3.1% in the range 62.5—1000 ng/ml and 6.0% at the limit of detection (Table II).

Detection limit

Using 0.25 ml of plasma and injecting 1 µl from 500 µl of the final mixture, the limit of detection was 5 ng/ml at a signal-to-noise ratio of 5:1. This corresponds to an injected amount of $2.5 \cdot 10^{-12}$ g of mefloquine. Injecting the pure compound as its TMS derivative, the minimum detectable amount was $0.7 \cdot 10^{-12}$ g. Pharmacokinetic studies undertaken up to now, however, showed that the above detection limit was more than adequate.

Selectivity

The method is selective for mefloquine. No interference from the main metabolite of mefloquine, or as a result of the simultaneous administration of

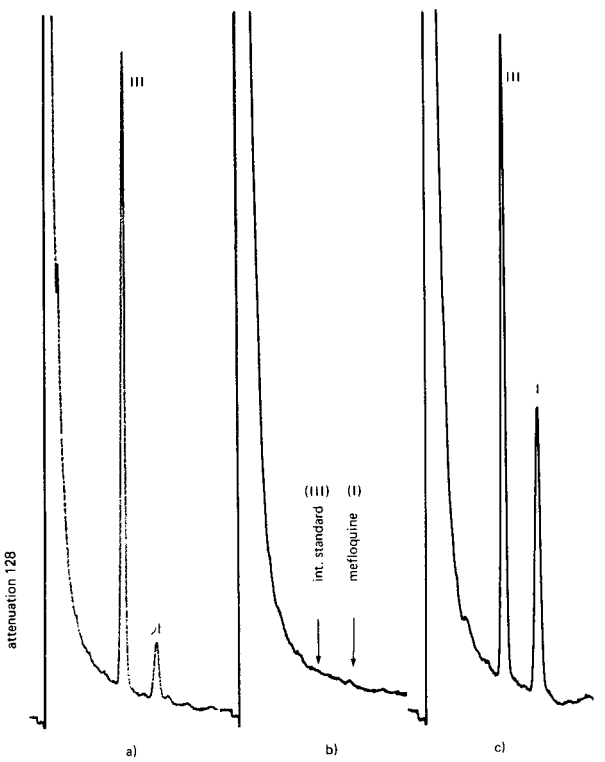

Fig. 2. Chromatograms of plasma extracts (0.25 ml of plasma extracted; injected volume 1 from 500 µl): (a) calibration standard, 15.6 ng/ml; (b) volunteer's pre-dose plasma; (c) volunteer's plasma, eight weeks after oral administration of 750 mg of mefloquine. The peak corresponds to 75 ng/ml mefloquine.

Fansidar® (an antimalarial containing pyrimethamine and sulfadoxine) was observed.

Stability in plasma and in solution

Stability of the drug in biological fluids was described by Schwartz and Ranalder [7]. According to these authors the drug is stable at room temperature for three days and at −20°C for two months. Under the conditions we used, the TMS derivative was found to be stable in the final solution for at least 24 h at room temperature.

Application of the method to biological samples

Plasma from volunteers and patients receiving mefloquine per os has been analysed by this method. Fig. 2 shows chromatograms of plasma extracts from a volunteer after oral administration of mefloquine (750 mg).

Plasma levels of the parent drug are presented in Fig. 3. In this case a terminal elimination half-life of 16 days was calculated.

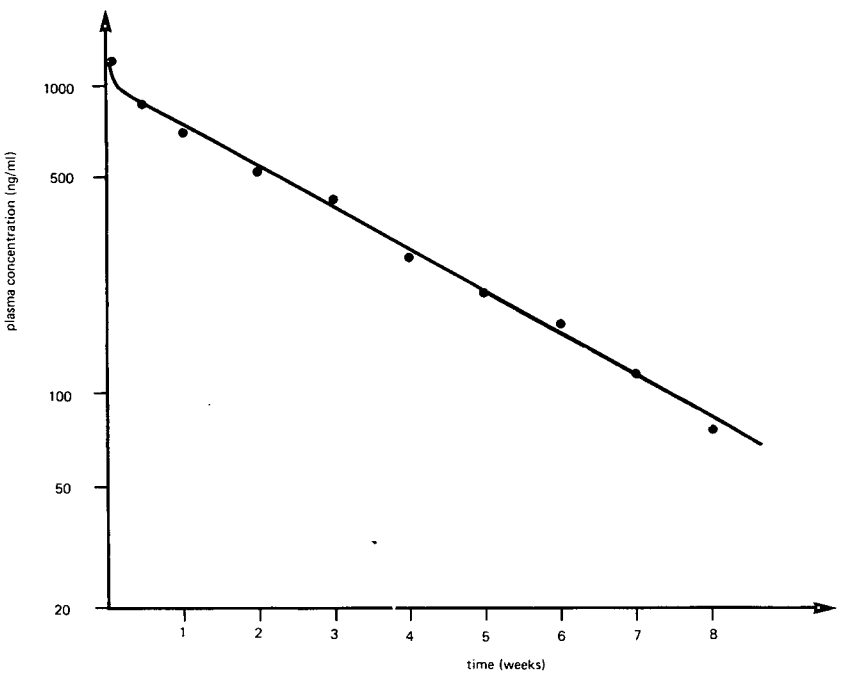

Fig. 3. Plasma levels of mefloquine after oral administration of 750 mg of mefloquine. Terminal half-life, $t_{½\beta}$, = 16 days.

ACKNOWLEDGEMENTS

We thank Dr. Dell for helpful discussions and for revising the manuscript, and Mr. Oesterhelt for having carried out the GC—MS spectra.

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Journal of Chromatography, 311 (1984) 418—423
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2253

Note

Determination of linogliride in biological fluids by high-performance liquid chromatography

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(First received February 1st, 1984; revised manuscript received July 16th, 1984)

Linogliride fumarate, N-(1-methyl-2-pyrrolidinylidene)-N′-phenyl-4-morpholinecarboximidamide (*E*)-2-butenedioate, is a new oral hypoglycemic agent [1, 2], currently undergoing clinical evaluation.

This paper describes the high-performance liquid chromatographic (HPLC) method which has been employed successfully in analyzing biological samples from clinical and non-clinical studies. The assay presented in this paper is sensitive, specific, and simple to perform. Using an automated injector (WISP) and a Lab Automation Computer System (HP-3354), up to 96 plasma samples are easily analyzed during an 8-h working day.

EXPERIMENTAL

Reagents

Piroglíride (Pg), a pyrrolidine analogue of linogliride (Lg) (Fig. 1) was used as the internal standard. Piroglíride and linogliride were obtained as the sulfate and the fumarate salt, respectively (McNeil Pharmaceutical, Spring House, PA, U.S.A.). Plasma used in the preparation of the standard curve was obtained from the Biological Specialty Corporation (Lansdale, PA, U.S.A.). The chromatography solvents (acetonitrile and methanol) were HPLC grade from Fisher

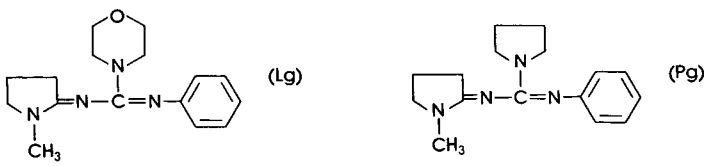

Fig. 1. Chemical structures of piroglíride (Pg) and linogliride (Lg).

0378-4347/84/$03.00

Scientific (Fair Lawn, NJ, U.S.A.). Triply distilled water (Ephrata Mountain Water, Manheim, PA, U.S.A.) was used in the preparation of solutions, buffers and in the mobile phase. The glacial acetic acid, diethyl ether, and potassium hydroxide used were ACS grade (Mallinckrodt, Paris, KY, U.S.A.). The sodium acetate trihydrate was analytical reagent grade from Mallinckrodt.

High-performance liquid chromatography

The HPLC system consisted of a Beckman Model 112 solvent delivery system, and a Beckman Model 160 ultraviolet absorbance detector equipped with a 254-nm wavelength filter. The column was 25 cm × 4.6 mm I.D. packed with 10-μm RP-2 sorbent (Brownlee Labs., Santa Clara, CA, U.S.A.). A 3 cm × 4.6 mm I.D. 10-μm RP-2 guard column from Brownlee Labs. was also used. The mobile phase was composed of acetonitrile—0.1 *M* acetate buffer pH 4.6—methanol (40:40:20, v/v/v). The mobile phase was prepared fresh daily and filtered through a 0.45-μm Millipore® filter (Millipore, Bedford, MA, U.S.A.). The column was conditioned with approx. 100 ml of mobile phase prior to use. After conditioning the column, the flow-rate was maintained at 2.0 ml/min. Retention times were 4.3 min for linogliride and 6.2 min for the internal standard (Fig. 2). Samples were injected using a Waters Intelligent Sample Processor (WISP 710B, Waters Assoc., Milford, MA, U.S.A.).

Plasma standard solutions

Plasma standards (volume 10.0 ml) containing 2—2000 ng/ml linogliride in plasma were prepared as follows: 0.1 ml of linogliride fumarate (conversion factor to linogliride free base is 1.405) solution in methanol containing the appropriate amount (200—200,000 ng equivalent) of the linogliride free base, was added to 9.9 ml of the drug-free plasma.

Equipment

Disposable screw-top bottles (volume 14.5 ml) with polyethylene-lined caps and 12-ml centrifuge tubes (conical bottom) were employed in the extraction. Prior to use, all glassware was soaked in detergent for 2 h, rinsed thoroughly with distilled water and heat-treated for 3 h at 270°C. Polyethylene-lined screw-caps were soaked in *n*-heptane for 1 h and dried at 60°C prior to use.

Extraction procedure

An aliquot of plasma or urine (0.1—1.0 ml) containing linogliride as a standard or as an unknown was placed in a 14.5-ml disposable screw-top bottle. To this were added 0.1 ml of a methanolic internal standard solution containing 1000 ng/ml internal standard, 0.2 ml of 5 *M* potassium hydroxide solution and 3.0 ml of diethyl ether. The capped bottles were shaken for 10 min on a table-top mechanical shaker (Eberbach) at 240 oscillations per minute. The bottles were then placed upright in a dry ice—methanol bath for approx. 2 min or until the aqueous layer was completely frozen. The diethyl ether (2.7—3.0 ml) was then decanted into a 12-ml conical centrifuge tube to which 0.2 ml of 0.1 *M* acetate buffer at pH 4.6 had been added. Each sample

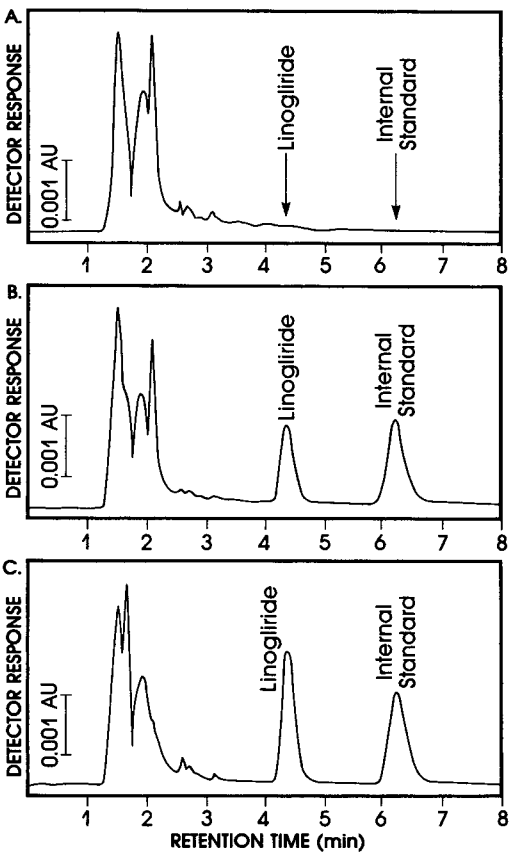

Fig. 2. Typical chromatograms from extracted human plasma samples. (A) Blank. (B) Sample seeded with 50 ng/ml linogliride and 100 ng/ml internal standard. (C) Sample taken 10 h following a 100-mg oral dose; the linogliride concentration was calculated to be 81 ng/ml.

was vortexed for 5 sec using a Vortex-Genie® (Scientific Instruments, Springfield, MA, U.S.A.) at a speed setting of 6. The aqueous and diethyl ether layers were allowed to separate for 1 min, after which the diethyl ether layer was aspirated. All of the acetate buffer layer was transferred to an insert tube which was placed onto the sample carousel of the automatic injector (WISP 710B). A 100-μl aliquot of the acetate buffer layer was injected into the HPLC system.

Quantitation and data handling

Standard curve data were generated by analyzing a series of plasma standards (2—2000 ng/ml). Data were analyzed by linear regression analysis (peak height ratios versus plasma concentrations) using the reciprocal of the variance of the peak height ratios as the weighting factor. Concentrations of linogliride in unknown plasma samples were calculated from the linear regression equation using the measured peak height ratios.

A Hewlett-Packard 3354 Lab Automation System was used for automatic data acquisition, temporary data storage, data analysis and report generation. Calibration curves, calculated linogliride concentrations and final reports were generated using internally developed application software.

RESULTS AND DISCUSSION

Recovery

The recovery of linogliride from plasma/urine was estimated at two concentrations (100 and 1000 ng/ml) with six determinations at each concentration. A mean recovery of 53.9 ± 5.8% was obtained for linogliride in plasma/urine. The recovery of the internal standard from plasma/urine at 100 ng/ml was 54.8 ± 2.0% (twelve determinations).

Stability

Freshly prepared plasma/urine standard solutions were compared to plasma/urine standard solutions frozen at −5°C for one month. The variations in peak height ratios at each drug level between 5 and 1000 ng/ml were insignificant.

Furthermore, linogliride and the internal standard were found to be stable in pH 4.6 acetate buffer at room temperature overnight. Therefore, injection of extracted samples can be performed on the next day without observable changes in peak height ratios.

Sensitivity

Linogliride and the internal standard absorb ultraviolet light strongly at 254 nm in the mobile phase (for linogliride, ϵ_{254} = 21,700). When 1 ng of linogliride was injected into the liquid chromatograph under the stated conditions, a peak with a signal-to-noise ratio of 50 was obtained. It was observed that the ultraviolet absorption of linogliride is twice as strong in a mobile phase composed of acetonitrile—water (1:1, v/v) than in one of methanol—water (1:1, v/v). However, 20% methanol was incorporated into the final mobile phase to reduce the total run time for each injection.

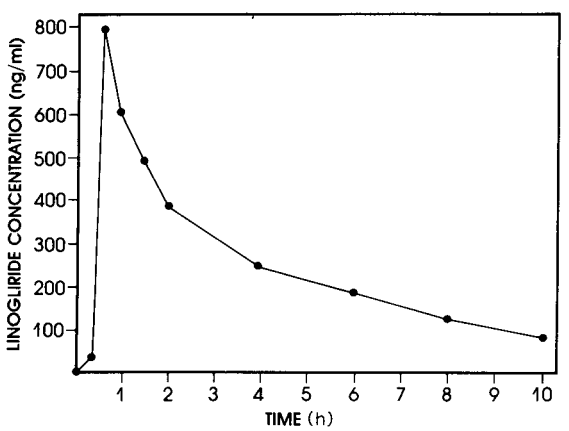

Fig. 3. Plasma concentration—time profile from a subject following oral administration of a 100-mg linogliride dose as a capsule.

The lowest concentration of linogliride that has been determined quantitatively in 1-ml plasma/urine samples is 2 ng/ml. This sensitivity appears more than adequate for clinical studies since the plasma concentration of linogliride in normal volunteers 10 h following oral administration of a capsule dose (100 mg) is approx. 80 ng/ml (see Fig. 3). For smaller sample volumes, assay sensitivity was reduced proportionally. Using 0.1-ml plasma/urine samples, the lower detection limit was 20 ng/ml.

Selectivity

The selectivity of the assay is demonstrated in Fig. 2 which displays chromatograms of processed samples of drug-free plasma, drug-free plasma spiked with known amounts of linogliride and human plasma from a clinical study. Interference from endogenous human plasma/urine components was not observed at the retention times of linogliride and the internal standard.

Other drugs that could potentially be co-administered with linogliride are the antihypertensives, diuretics, and non-steroidal anti-inflammatory agents. Plasma samples spiked with the drugs propranolol, furosemide, hydrochlorothiazide, indomethacin, ibuprofen and tolmetin sodium were analyzed using the present procedure and no interfering peaks were observed except for propranolol. Propranolol was extracted and eluted approx. 0.3 min after linogliride using these assay conditions. In this event, base-line separation of the compounds can be achieved with slight adjustments of the mobile phase composition.

Standard curve

Standard curve data generated by analyzing plasma standard solutions are presented in Table I. Linear regression analysis (peak height ratios versus

TABLE I

SUMMARY OF STANDARD CURVE DATA GENERATED ON FOUR CONSECUTIVE DAYS OF ANALYSIS FOR LINOGLIRIDE IN PLASMA

Linogliride seeded (ng/ml)	*n*	Mean value found* (ng/ml)	Standard deviation (ng/ml)	Precision (%)	Accuracy (%)
2	7	1.9	0.19	9.8	−2.6
5	7	5.1	0.33	6.5	2.0
10	8	10.1	0.53	5.2	1.1
20	8	19.9	1.3	6.4	−0.5
50	8	49.0	2.1	4.2	−2.0
100	7	99.0	6.9	7.0	−1.0
200	8	200.0	9.6	4.8	−0.1
500	7	486.0	33.0	6.9	−2.8
1000	8	1013.0	55.0	5.4	1.3
2000	8	2076.0	110.0	5.3	3.8

*Calculated from the equation: $[\text{Linogliride}] = \frac{\text{Peak Height Ratio} - 0.001}{0.0194}$ where 0.0194 and 0.001 are the slope and intercept of the regression equation. The regression equation was obtained by method of least squares with data weighted by 1/variance.

linogliride plasma concentrations) gave a slope of $19.36 \cdot 10^{-3} \pm 0.14 \cdot 10^{-3}$ S.D., a *Y*-intercept of 0.0013 ± 0.0013 S.D. and a correlation coefficient equal to 0.998 with a Student's *t* of 135. The composite standard curve is linear between 2 and 2000 ng/ml and passes through the origin, within experimental error. Similar standard curve data have also been generated for spiked urine samples. Excellent accuracy and precision were obtained.

Accuracy and precision

Accuracy and precision of the assay were measured by the relative difference between the mean experimental linogliride concentration and the theoretical value, and the relative standard deviation, respectively (Table I). Frozen seeded control samples at three concentrations were blind-coded and analyzed concomitantly with plasma samples from the clinical study over a period of four weeks. The accuracy and precision (inter-run) at the three concentrations were all within 10%.

Application of the procedure to plasma samples

To date the procedure has been used successfully in the analysis of biological samples from clinical and non-clinical studies. Fig. 3 shows the plasma concentration—time profile obtained from one subject following a single oral 100-mg dose of linogliride. Serial blood samples were drawn at selected time points up to 10 h following dosing. The 81 ng/ml plasma concentration observed at the final time point is well above the detection limit of the assay. In summary, this HPLC assay for linogliride is highly sensitive, selective and precise. The extraction procedure is simple and rapid, and the assay has been automated with excellent results.

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Journal of Chromatography, 311 (1984) 424—429
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2254

Note

Improved high-performance liquid chromatographic method for the quantitation of *cis*-thiothixene in plasma samples using *trans*-thiothixene as internal standard

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(First received December 14th, 1983; revised manuscript received July 16th, 1984)

In an earlier paper we reported a high-performance liquid chromatographic (HPLC) method for the separation of *cis* and *trans* forms of thiothixene [1]. We quantitated *cis*-thiothixene in plasma samples using mesoridazine as an internal standard. Since mesoridazine is also a commonly used antipsychotic drug and a metabolite of thioridazine we switched to another internal standard, thioproperazine, with equal success [2]. As reported earlier, setting the ultraviolet (UV) detector at 229 nm gave higher sensitivity [2]. Analysis of over 200 plasma samples using either of the above internal standards did not show any detectable amount of *trans*-thiothixene in any of the patient plasma samples. Thus we established that there is no biotransformation of the active drug, *cis*-thiothixene, into the inactive *trans*-thiothixene in humans. It therefore occurred to us that the non-biological non-drug, *trans*-thiothixene, would be the ideal standard for the quantitation of *cis*-thiothixene in human plasma samples. We now report here a simple HPLC method for the quantitation of *cis*-thiothixene with the *trans* form as an internal stnadard.

MATERIALS AND METHODS

Isoamylalcohol was Fisher-certified grade and all other solvents were HPLC grade from Burdick & Jackson Labs. or J.T. Baker. *cis*-Thiothixene and *trans*-thiothixene maleate standards were obtained from Pfizer Research Labs. through the kind courtesy of Dr. D.C. Hobbs.

Stock standard solution

Standard stock solutions of *cis*- and *trans*-thiothixene were separately

0378-4347/84/$03.00

prepared by accurately weighing 3.0 mg of *cis*-thiothixene and 3.45 mg of *trans*-thiothixene maleate and dissolving in 3 ml of methanol. Working standards were prepared from the stock solution to contain 10 μg/ml by dilution with water. Drug-free plasma was then spiked with *cis*-thiothixene to concentrations of 0, 2, 4, 6, 8, 10 and 20 ng/ml to prepare plasma calibration standards. All standards were stored at −20° C protected from light.

Extraction of plasma samples

Plasma (1 ml) and 10 μl of internal standard, *trans*-thiothixene (100 ng), were mixed with 1.0 ml of 2 *M* sodium carbonate (pH 9.8) and 5 ml of hexane-isoamyl alcohol (98.5:1.5). The mixture was vortexed twice for 15 sec each time, then centrifuged for 15 min at 700 *g*. The upper organic layer was transferred into a 5-ml centrifuge tube and evaporated under nitrogen. Finally, 0.5 ml of the solvent was used to rinse the sides of the tube and the solution evaporated to dryness under nitrogen. The residue was dissolved in 50 μl of mobile phase by vortexing for 15 sec, centrifuged for 5 min and 20 μl were injected into the HPLC system. From the peak height ratio of *cis*-thiothixene and the internal standard *trans*-thiothixene the plasma concentration is determined. A calibration curve from extracted plasma standards containing 0, 2, 4, 6, 8, 10 and 20.0 ng of *cis*-thiothixene and 100 ng internal standard was obtained.

HPLC conditions

A Bio-Rad 1310 HPLC pump and 1306 variable-wavelength UV detector, a Rheodyne 7125 sample injector with a 200-μl sample loop, and a Spherisorb 5-μm cyanopropyl HPLC column, 150 × 4.6 mm (Custom LC, U.S.A.), were used. The detector was set to 229 nm, 0.0025 a.u.f.s. sensitivity, as suggested earlier [1].

The mobile phase was 0.01 *M* potassium dihydrogen phosphate (pH 7.0)—acetonitrile—methanol (400:480:120), at a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Relative retention data for *cis*- and *trans*-thiothixene and some commonly used antipsychotic drugs on our system are given in Table I. Thioproperazine has a relative retention time of 1.11, is distinctly separated as a peak and is distinguishable from *cis*- and *trans*-thiothixene. The percent recovery of *cis*- and *trans*-thiothixene added to plasma samples was the same and the mean percentage recovery of the internal standard (*trans*-thiothixene) was over 90% and ranged between 80% and 100%.

We have not found any interfering drugs or metabolites. Chromatograms of standards in drug-free plasma and patient samples are shown in Fig. 1. The plasma blank with ten different drug-free samples did not show any interference from endogenous compounds. The *trans*-thiothixene used as internal standard is almost free from the *cis* isomer. (Fig. 1, 0 ng). The patient samples (P_1 and P_2) showed 1.0 and 7.3 ng/ml, respectively. Calibration curves were run with every batch of plasma standards and had correlation coefficients (six points) of 0.994 ± 0.002 S.D. (n = 19). For routine analysis, we included in

TABLE I

RELATIVE RETENTION TIMES (RTT) OF PSYCHOTROPIC DRUGS AND METABOLITES TO *cis*-THIOTHIXENE

Compound	RTT	Compound	RTT
cis-Thiothixene	1.00	Trimipramine	1.53
trans-Thiothixene	1.24	Doxepin	2.00
Desmethylthiothixene	2.08	Amitriptylene	2.12
		Imipramine	2.18
Loxapine	0.44	Desmethyldoxepin	2.59
Clozapine	0.47	Notriptylene	2.65
Fluphenazine	0.50	Desipramine	2.88
Haloperidol	0.88	Maprotylene	2.79
Chlorohaloperidol	0.88	Protriptylene	2.94
Thioproperazine	1.11		
Chlorpromazine	1.89	Diazepam	0.26
Mesoridazine	3.00	Desmethyldiazepam	0.26
Thioridazine	3.06	Flurazepam	0.56
		Chlordiazepoxide	0.27
Trazodone	0.32		
Amoxapine	0.80	Benztropine	4.47

TABLE II

DATA ON STANDARD CALIBRATION AND PLASMA CONTROLS RUN ON DIFFERENT DAYS

No.	*r* Value	Low control (ng/ml)	High control (ng/ml)
1	0.9959	1.3	8.75
2	0.9946	0.7	7.4
3	0.9956	1.5	6.5
4	0.9930	0.25	7.5
5	0.9960	2.05	7.2
6	0.9960	1.1	7.3
7	0.9963	0.07	6.95
8	0.9907	1.08	6.8
9	0.9947	0.25	6.5
10	0.9950	0.14	7.4
11	0.9845	1.0	8.5
12	0.9909	1.4	8.2
13	0.9935	2.2	7.4
14	0.9936	2.9	7.6
15	0.9807	1.5	7.4
16	0.9969	1.9	8.9
17	0.9995	1.5	8.6
18	0.9920	2.6	8.6
19	0.9980	1.9	8.1
Mean ± S.D.	0.9937 ± 0.002	1.32 ± 0.8	7.6 ± 0.72

Equation $Y = 0.0092X \pm 0.024$

every batch of patient plasma samples a low and high plasma control as a quality control measure. The results of analysis on different days are shown in Table II. Similarly with two other batches of plasma controls, the assayed batches were 10.0 ± 0.63 and 36.8 ± 3.5 ng/ml (n = 10). To assess the validity of the assay method in a wide range of plasma levels that one may have to deal with in clinical samples we carried out the assay of spiked human plasma samples which were prepared for us at Pfizer Central Research Lab. (Groton,

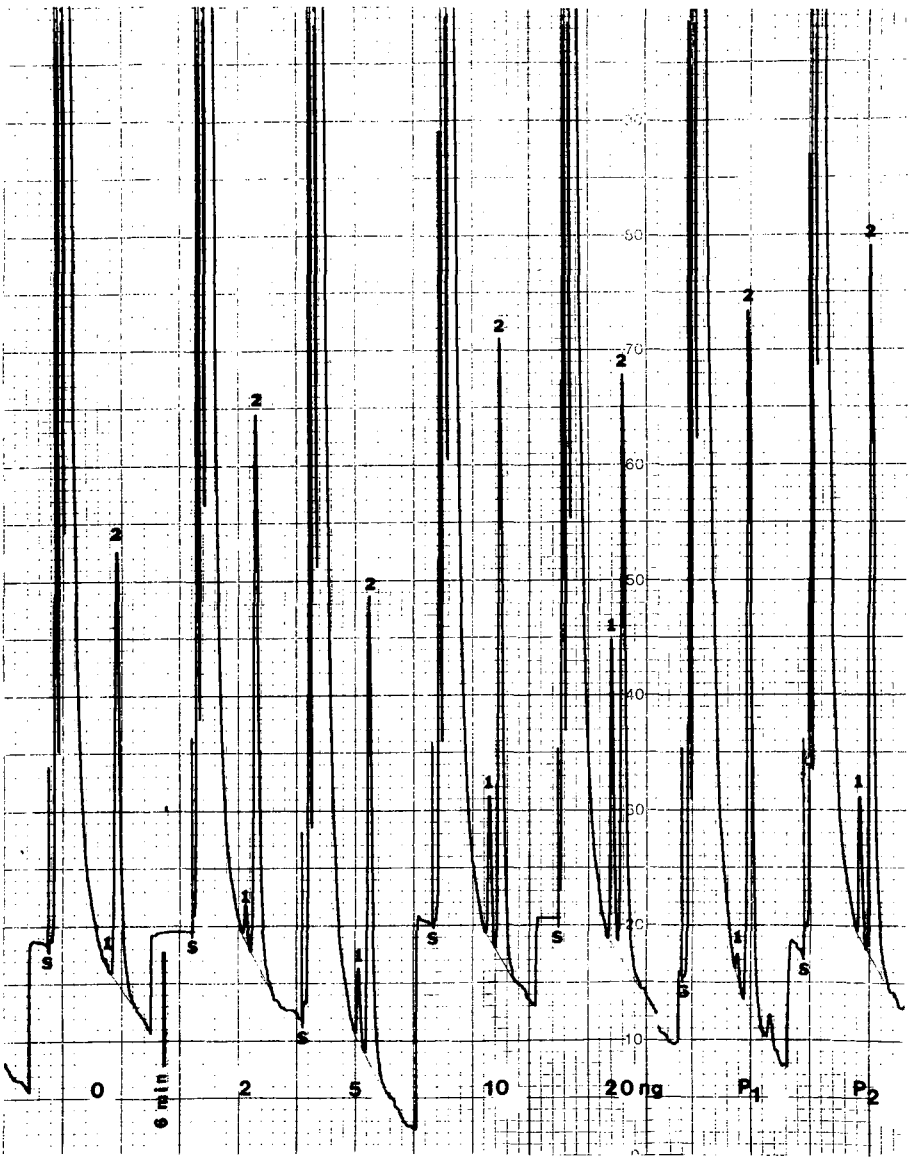

Fig. 1. Chromatograms of standards for calibration and patient plasma samples (P_1 and P_2). S = start: Chart speed 25 cm/h. Peaks: 1 = *cis*-thiothixene, 2 = *trans*-thiothixene (internal standard).

CT, U.S.A.). The samples were coded, frozen and sent to our laboratory for analysis. After the assay results were reported the code was broken and we were informed about the spiked levels and the assayed levels. These results are shown in Table III. These results indicate that reproducibility is good at all concentrations and accuracy is excellent at concentrations $\geqslant$ 12 ng/ml. The accuracy at the low concentrations is also adequate for clinical samples. Further we have not found any false positive in several plasma blanks analyzed. The minimum detectable level was 0.5 ng/ml *cis*-thiothixene. Plasma samples were from different clinical studies of patients receiving 5 to 70 mg thiothixene and from patients at MCV Hospital. Plasma levels ranged from undetectable to 40 ng/ml. This method has been in continuous and successful use in our laboratory for more than a year with adequate quality control procedures.

TABLE III

ASSAY OF THIOTHIXENE IN SPIKED HUMAN PLASMA

Spiked (ng/ml)	Found (ng/ml)	Ratio	Mean (S.D.)
0	0		
0	0		0 (0)
0	0		
0	0		
4	2.8	0.7	
4	2.6	0.65	0.67 (0.06)
4	2.9	0.73	
4	2.4	0.60	
8	7.5	0.94	
8	6.4	0.80	0.85 (0.07)
8	7.0	0.88	
8	6.3	0.79	
12	13.2	1.10	
12	12.9	1.07	1.08 (0.03)
12	12.5	1.04	
12	13.2	1.10	
16	16.4	1.03	
16	14.9	0.93	0.99 (0.06)
16	15.1	0.94	
16	17.0	1.06	
18	17.7	0.98	
18	18.3	1.02	0.95 (0.06)
18	16.3	0.91	
18	16.2	0.90	
35	34.5	0.99	
35	36	1.03	1.0 (0.02)
35	34.9	1.0	
35	34.5	0.99	

The *trans* form of thiothixene is added in excess to compensate for its lower UV absorbance and also to serve as a carrier for better extraction of the drug. We do not add *cis*-thiothixene to each sample, as in our earlier report [1], but upon repeated freeze—thaw cycles of the internal standard solution, a portion isomerises, so a blank plasma sample with added internal standard was included in each run. The isomerisation does not occur rapidly enough to affect results within a run. When we notice more than 0.5 ng of *cis*-thiothixene in our internal standard, we make a fresh internal standard solution.

ACKNOWLEDGEMENT

We thank Dr. D.C. Hobbs and Pfizer Central Research for supplying *trans*-thiothixene, providing coded spiked plasma samples and his interest in this work.

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Journal of Chromatography, 311 (1984) 430—433
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2256

Note

Determination of pindolol in human plasma by high-performance liquid chromatography with amperometric detection

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(First received March 12th, 1984; revised manuscript received July 18th, 1984)

Few methods for the assay of pindolol (Fig. 1), a beta-blocking drug, have been published. The first assay was described by Pacha [1]. This fluorimetric method, after reaction with *o*-phthalaldehyde, lacks specificity and sensitivity because interfering substances can be co-extracted from biological fluids causing serious variations in blank values. More recently, an electron-capture gas—liquid chromatographic (GLC) procedure was proposed [2], but this also shows insufficient specificity depending on reagent impurities. Another electron-capture GLC method, using a wall-coated open tubular column, was described by the same author, but a derivatization step is required which can lead to insufficient specificity [3]. A high-performance liquid chromatographic (HPLC) method with fluorescence detection has been proposed [4] but this involves a time-consuming extraction procedure to eliminate interfering substances and the claimed sensitivity of 2 ng/ml of plasma can rarely be attained in routine use. The lack of sensitivity of the HPLC procedure has been recently confirmed [5]. A simple and highly sensitive HPLC method with amperometric detection is reported in this present paper.

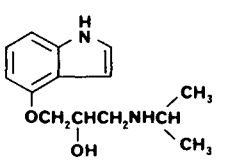

Fig. 1. Structural formula of pindolol.

0378-4347/84/$03.00

EXPERIMENTAL

Reagents

All reagents were of analytical grade. Ethanol was purchased from E. Merck (Darmstadt, F.R.G.); distilled water was HPLC grade; sodium dihydrogen phosphate was obtained from Sigma and perchloric acid from E. Merck; diethyl ether was from E. Merck and pindolol was obtained from Sandoz (Rueil Malmaison, France).

Chromatographic system

A Waters 6000A solvent delivery system fitted with a Rheodyne 7125 sample valve equipped with a 100-μl loop was used in conjunction with a column (15[illegible] × 3.9 mm I.D.) packed with LiChrospher octadecylsilane (particle size 5 μm) The mobile phase consisted of methanol—0.01 *M* perchloric acid (1:4, v/v) maintained at a flow-rate of 2 ml/min (about 70 bars).

The detector was a Metrohm ELCD system composed of a 641 VA detector, a glassy carbon electrode and an Ag/AgCl reference electrode, fixed at a potential of 1.0 V used at a sensitivity of 50 nA full scale.

Standard solutions and blood sample

The standard stock solution (1 mg/ml) of pindolol was prepared by dissolving the compound in perchloric acid (0.01 *M*); drug-free venous blood was obtained from healthy human subjects. Blood samples were obtained from healthy subjects receiving an oral dose of 15 mg of pindolol. Blood was collected into plastic tubes containing lithium heparin and centrifuged at 2000 *g* for 10 min. Plasma was stored at −20° C until assayed.

Extraction of pindolol and estimation

Plasma (1 ml) was placed into a 15-ml stoppered glass tube, to which were added 0.5 ml of 1 *M* sodium hydroxide and 10 ml of diethyl ether. Pindolol was extracted into the diethyl ether by shaking for 20 min. After centrifugation (2 min at 2000 *g*), 9 ml of the ether layer were transferred to a conical tube containing 200 μl of perchloric acid (0.01 *M*) and pindolol was extracted into the aqueous phase by vortexing the solution for 15 sec. After centrifugation, the aqueous phase was frozen at −20° C and the organic phase was discarded; 100 μl of the acidic phase were injected into the HPLC column. A calibration curve was prepared by treating plasma samples containing known amounts of pindolol (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ng/ml) in the same way as the unknown samples.

RESULTS AND DISCUSSION

Pindolol isolated from plasma gave a retention time of 2.4 min and is well separated from any analytical artefacts (Fig. 2). The pindolol peak height is linearly related to its plasma concentration up to a concentration of 200 ng of pindolol per ml of plasma, as indicated by the high correlation coefficient (r = 0.997) of an eleven-point curve. Variations in the calibration curve from day to day were small, the coefficient of variation of the slope being 6.5%.

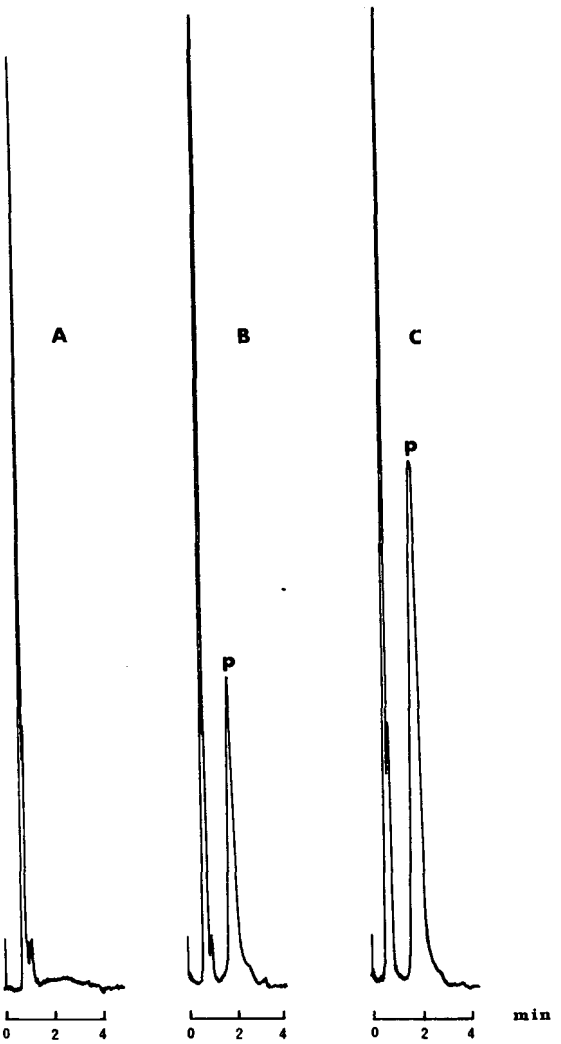

Fig. 2. High-performance liquid chromatograms of extracts from plasma: (A) control drug-free plasma; (B) plasma containing 30 ng/ml pindolol; (C) patient's plasma, 1 h after an oral dose of 15 mg of pindolol (70 ng/ml). p = pindolol.

TABLE I

REPRODUCIBILITY OF THE ASSAY FOR PINDOLOL IN PLASMA

n = 10 at all concentrations.

Pindolol (ng/ml)	Coefficient of variation (%)
1	11.0
10	6.7
80	1.2

Recovery of pindolol from plasma was estimated by comparing the peak height after an injection of pure solution of pindolol with the peak height after the injection of extracted plasma containing the same dose of pindolol. The recovery, at any concentration, was over 95%. The reproducibility of the assay, together with the coefficient of variation, is given in Table I.

The limit of detection of the assay is about 0.5 ng/ml when 1 ml of plasma is used. It did not appear necessary to include an internal standard in the assay.

Plasma samples from patients taking pindolol were analysed. The plasma pindolol concentrations observed in these patients after an oral dose of 15 mg are in agreement with the known pharmacokinetics of this drug and are given in Table II.

TABLE II

PINDOLOL CONCENTRATIONS IN PLASMA OF HEALTHY SUBJECTS AFTER AN ORAL DOSE OF 15 mg OF PINDOLOL

Subject	Pindolol concentration (ng/ml)				
	Time of sampling after dose (h)				
	0	1	3	8	24
1	0	78	40	17	0
2	0	103	67	22	15
3	0	26	15	10	0

In vitro, other drugs were investigated for possible interference: prazosin, chlorothiazide, diltiazem, quinidine, digoxin, propranolol. None of these drugs interfered in the estimation of pindolol.

These results indicate that the HPLC we propose is very sensitive (a three- to four-fold increase in sensitivity over the usual HPLC fluorimetric procedure), and is suitable for use in pharmacokinetic studies and drug monitoring in patients, even for 12-h plasma levels which are frequently less than 2 ng/ml. The method shows good reproducibility, though some care must be taken: it is necessary to clean the working electrode after use to preserve good sensitivity, and it is essential to stabilize the electrode until the response of the detector is constant. In summary, this technique is easy to perform and is inexpensive.

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Journal of Chromatography, 311 (1984) 434—442
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2257

Note

Determination of trilostane and ketotrilostane in human plasma by high-performance liquid chromatography

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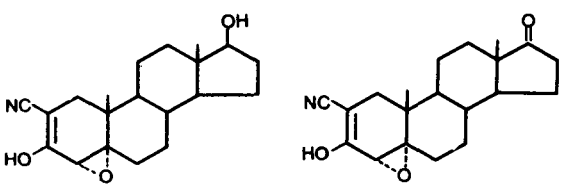
(Received April 24th, 1984)

Trilostane [(4α,5α,17β)-4,5-epoxy-3,17-dihydroxyandrost-2-ene-2-carbonitrile, I, Fig. 1] is a synthetic steroid which has been shown to be a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase—Δ^5-3-oxosteroid isomerase (3β-HSD) enzyme system in laboratory animals [1—3] and humans [4]. The drug has been used to modify adrenal steroidogenesis in conditions such as Cushing's syndrome [4], primary aldosteronism [5], various forms of hypertension [6], and recently it has been found to be of benefit in the treatment of some forms of cancer of the breast [7].

High-performance liquid chromatographic (HPLC) examination of extracts of plasma from dosed healthy male volunteers indicated that chromatograms contained two peaks, due to trilostane and a metabolite, which were absent



(I) (II)

Fig. 1. Structures of trilostane (I) and ketotrilostane (II).

0378-4347/84/$03.00

from extracts of control plasma. This communication reports the isolation and identification of the metabolite as the 17-keto analogue of trilostane (ketotrilostane, II, Fig. 1) using various chromatographic techniques including thin-layer chromatography (TLC), HPLC and gas chromatography—mass spectrometry (GC—MS), and describes an HPLC procedure for the quantitative analysis of trilostane and ketotrilostane in human plasma.

EXPERIMENTAL

Reagents and standards

Modrenal capsules (Modrestane, U.S.A.) containing 60 mg trilostane were supplied by Sterling Research Labs. (Guildford, U.K.). Trilostane was obtained from Sterling Organics (Dudley, U.K.), ketotrilostane from Sterling-Winthrop Research Institute (Rensselaer, New York, NY, U.S.A.) and ethisterone (the internal standard) from Sigma (St. Louis, MO, U.S.A.). The reagent for silylation, N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS), was purchased from Pierce (Rockford, IL, U.S.A.). All other chemicals and solvents were of analytical or chromatographic grade, as appropriate.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 6000A pump, a Waters Model 710B WISP automatic sample injector, a 5-μm Hypersil ODS column (150 × 4.6 mm I.D.) obtained from HPLC Technology (Macclesfield, U.K.), a Waters Model 440 detector operating at 254 nm, and a Spectra Physics SP4100 data system. For quantitative analysis and for monitoring separations the mobile phase consisted of methanol—0.1 *M* formic acid (1:1) at a flow-rate of 2 ml/min. Both column and mobile phase were maintained at 30°C. Under these conditions the retention times for trilostane, ketotrilostane and the internal standard were approx. 6, 7 and 12 min, respectively.

Two additional mobile phases, methanol—water (55:45) and methanol—water—1.3 *M* ammonium acetate (45:40:15), were used for metabolite characterisation.

Thin-layer chromatography

For the examination of plasma extracts and purified metabolite by TLC, samples were spotted on 0.25-mm precoated layers of silica gel G-60 F_{254} (E. Merck, Darmstadt, F.R.G.) together with standards of trilostane and ketotrilostane. Development was carried out at room temperature in the solvent systems chloroform—water—acetic acid—*tert.*-butanol (65:20:10:5, lower phase), chloroform—methanol—formic acid (80:10:10) and dichloromethane—diethyl ether—acetic acid (80:15:5).

The separated components were visualised by fluorescence quenching of 254-nm radiation and by charring at 100°C after spraying with 3.75 *M* sulphuric acid. With the latter procedure trilostane and ketotrilostane yielded characteristic grey—brown-coloured products. The approximate R_F values of trilostane and ketotrilostane in these systems were 0.52 and 0.60, 0.37 and 0.50, and 0.36 and 0.52, respectively.

Gas chromatography—mass spectrometry

The GC—MS system was a Finnigan 4000 gas chromatograph—quadrupole mass spectrometer monitored by a Data General Nova 3 computer. The gas chromatograph contained a 15 m × 0.4 mm I.D. fused-silica capillary column coated with OV 73 (Phase Separations, Deeside, U.K.). For capillary operation the gas chromatographic oven was held at 80°C for 1 min and then programmed to 305°C at 25°C/min. Injection port, separator and transfer line temperatures were set at 260°C, 280°C and 280°C, respectively. Helium was used as carrier gas with a flow-rate of 4.0 ml/min.

Isolation and derivatisation of metabolite

Four male volunteers were each dosed with Modrenal capsules (4 × 60 mg). Blood sampes (50 ml) were taken 2, 3 and 4 h after dosing into tubes containing lithium-heparin as anticoagulant. The plasma samples obtained by centrifugation were pooled and stored below −15°C until required.

A 100-ml aliquot of plasma was adjusted to approx. pH 5 by the addition of 50 ml acetate buffer (0.5 *M*, pH 5.0) and extracted with diethyl ether 3 × 200 ml). Extracts were pooled, dried over sodium sulphate and evaporated to dryness.

The residue, taken up in 0.5 ml chloroform, was applied to a glass column containing a bed of silica gel (Activity III, Woelm Pharma, F.R.G., 100 mm × 13 mm I.D.) made up and eluted with chloroform (25 ml) followed by chloroform—methanol (9:1) at approx. 1 ml/min. Fractions were collected and those found to contain the metabolite were taken down to dryness and re-chromatographed on a 5-μm Hypersil ODS column (250 mm × 10 mm I.D.) at ambient temperature using the mobile phase methanol—water (55:45) at a flow-rate of 4 ml/min.

Those fractions of the eluate containing the metabolite were again pooled, evaporated to dryness under reduced pressure and dried over P_2O_5 for 1 h. The residue was reconstituted in 15 μl toluene and 15 μl BSTFA—TMCS reagent and heated at 60°C for 15 min. A 1-μl aliquot of the reaction mixture was injected directly into the GC—MS system. A small sample of ketotrilostane was derivatised and chromatographed in a similar manner for comparative purposes.

Preparation of analytical standards and samples

Plasma standards were prepared in duplicate by supplementing 2.0 ml of control human plasma (obtained from blood with lithium-heparin as the anticoagulant) with aliquots of solutions of trilostane and ketotrilostane in 1 m*M* potassium hydroxide to produce concentrations of 0 and 0.2—1.0 μg/ml trilostane and 0 and 0.25—2.5 μg/ml ketotrilostane.

Two sets of randomised and coded plasma samples with trilostane and ketotrilostane concentrations within the ranges described above, to be analysed under single-blind conditions, were prepared in a similar manner. One set prepared in quadruplicate was analysed upon preparation, the other prepared in triplicate was stored in the laboratory freezer for seven days before analysis.

Analytical procedure

In a tube containing 2.0 ml of human plasma, were added 1.0 ml acetate buffer (0.5 *M*, pH 5.0) and 10 ml chloroform containing the internal standard (0.2 μg/ml). After mixing and centrifugation the upper aqueous phase was carefully removed by aspiration and discarded, leaving the lower organic phase which was dried over sodium sulphate. An aliquot (5 ml) was evaporated to dryness under a stream of nitrogen in a dry-block heater at 45° C. The residue was dissolved in 50 μl ethanol and 20 μl injected into the liquid chromatograph for analysis.

Extraction efficiency

The percentage recoveries of the extraction procedures for trilostane and ketotrilostane were determined at five concentrations, ranging from 0.2 to 1.0 μg/ml for trilostane and from 0.25 to 2.5 μg/ml for ketotrilostane, by comparing the peak heights obtained from extracted plasma samples with those obtained by injection of unextracted samples.

Calculation of results

Regression analyses of the peak height ratios (trilostane:internal standard, ketotrilostane:internal standard) obtained for the standards were performed to determine the linearity of the response with respect to concentration. The resulting regression lines were used to estimate the concentrations of trilostane and ketotrilostane in the prepared samples. The minimum quantifiable level (MQL) of the assays were estimated as the concentration whose lower 80% confidence limit just encompassed zero [8].

RESULTS AND DISCUSSION

Identification of ketotrilostane in plasma extracts

HPLC examination of extracts of plasma from dosed individuals revealed that chromatograms contained two peaks, assigned to trilostane and a metabolite, which were absent from extracts of control plasma. Typical chromatograms are shown in Fig. 2A and B. Since Mori et al. [9] had previously reported that significant quantities of the 17-keto analogue of trilostane (ketotrilostane) were present in the plasma of rats dosed with the drug it seemed possible that this compound might be responsible for the additional peak noted in extracts of human plasma. This view was strengthened when it was demonstrated that the human metabolite had identical chromatographic properties to ketotrilostane in a range of TLC and HPLC systems.

Confirmation was obtained when it was shown that the trimethylsilyl (TMS) derivatives of the isolated metabolite and ketotrilostane eluted as sharp peaks with similar retention times of around 11.5 min under the GC conditions described in the experimental section and that both gave essentially identical mass spectra (Fig. 3A and B). The molecular ion at *m/e* 399 indicated the formation of the mono-TMS derivative.

Assay of trilostane and ketotrilostane in human plasma

Preliminary investigations suggested that the plasma concentrations of

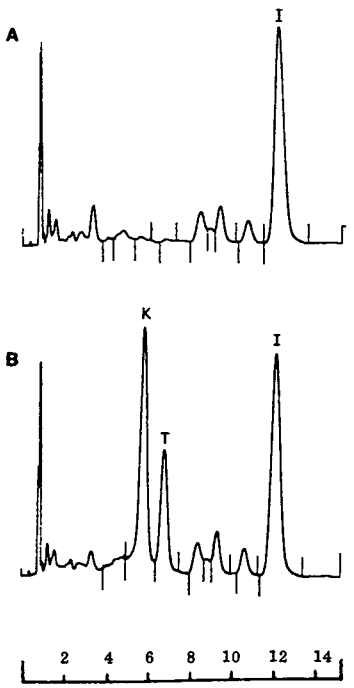


Fig. 2. Typical chromatograms of extracted plasma samples obtained from a volunteer before (A), and after (B) 120-mg oral dose of trilostane (see text for chromatographic conditions) Peaks: K = ketotrilostane (concentration 2.3 μg/ml); T = trilostane (concentration 1.0 μg/ml), I = internal standard (ethisterone, 0.2 μg/ml).

trilostane and ketotrilostane after a standard dose used in volunteer studies (120 mg) were likely to be in the ranges 0—1 μg/ml and 0—2.5 μg/ml, respectively. Regression analysis on the standards indicated a linear relationship between peak height ratio and concentration over these ranges (coefficient of regression > 0.99 in both cases).

The control plasma used to prepare the standards and samples contained an endogenous component which gave rise to a small peak at the same retention time as trilostane. When this contribution was subtracted the slope for trilostane was typically 0.484 and the *Y*-intercept —0.001 μg/ml. For ketotrilostane the slope was 0.463 and the *Y*-intercept —0.006 μg/ml.

The concentrations of trilostane and ketotrilostane in the prepared plasma samples, estimated by inverse prediction from the regression equation, and allowing for the contribution of the endogenous component, are summarised in Tables I and II. The accuracy of the assay, defined by the ranges of the mean percent differences from the nominal concentration values, varied from —6.7% to 7.9% for trilostane and —7.9% to 6.5% for ketotrilostane. The respective MQL values were 0.03 and 0.05 μg/ml. Extraction efficiencies (± S.D.) were 82 ± 6% for trilostane and 76 ± 12% for ketotrilostane and were independent of concentration.

The HPLC assay procedure described in this communication is simple, relatively rapid and has the advantage over radioimmunoassay [10] in that it

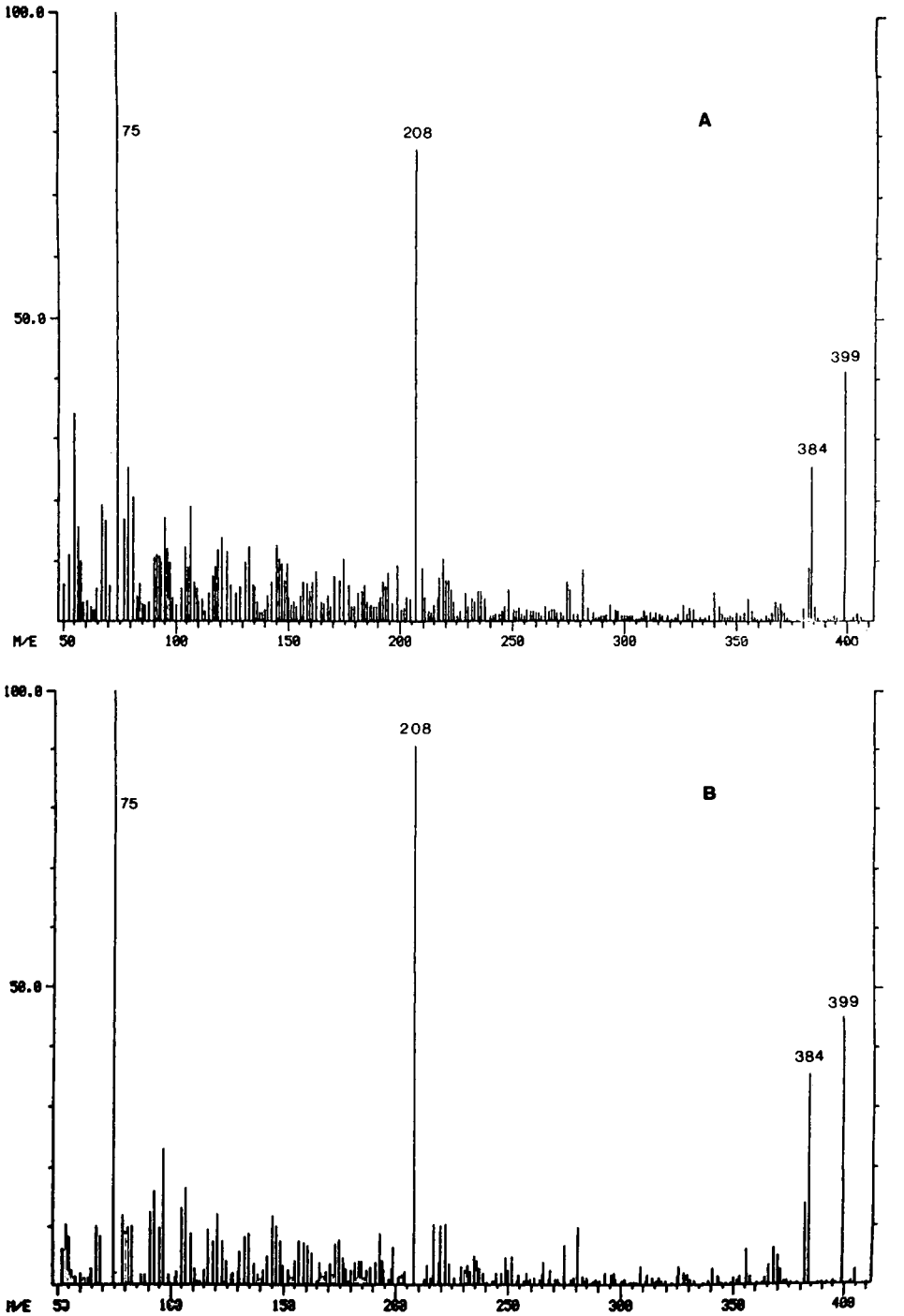


Fig. 3. Mass spectra of the (mono-)TMS derivatives of the metabolite isolated from human plasma (A), and of ketotrilostane (B).

TABLE I

RESULTS OF THE ANALYSIS OF PREPARED HUMAN PLASMA SAMPLES FOR TRILOSTANE

MQL = 0.03 μg/ml.

Concentration added (μg/ml)	Concentration found, fresh* (μg/ml)	Concentration found, frozen** (μg/ml)
0	(0.06)***	
	(0.05)	ND§
	(0.05)	(0.04)
	(0.07)	(0.04)
0.30	0.28	
	0.30	0.30
	0.29	0.30
	0.26	0.32
Mean	0.28	0.31
C.V. (%)	6.1	3.8
Mean percent difference	−6.7	3.3
0.51	0.53	
	0.45	0.50
	0.53	0.45
	0.48	0.50
Mean	0.50	0.48
C.V. (%)	7.9	6.0
Mean percent difference	−2.0	−5.9
0.71	0.69	
	0.71	0.67
	0.67	0.69
	0.69	0.68
Mean	0.69	0.68
C.V. (%)	2.4	1.5
Mean percent difference	−2.8	−4.2
0.91	0.89	
	0.87	0.92
	0.94	0.89
	0.90	0.91
Mean	0.90	0.91
C.V. (%)	3.3	1.7
Mean percent difference	−1.1	0

*Analysed upon preparation.
**Analysed after seven-days storage in a laboratory freezer.
***Small endogenous components in plasma; mean subtracted from subsequent concentration levels.
§ND = not detected.

TABLE II

RESULTS OF THE ANALYSIS OF PREPARED HUMAN PLASMA SAMPLES FOR KETOTRILOSTANE

Concentration added (μg/ml)	Concentration found, fresh* (μg/ml)	Concentration found, frozen** (μg/ml)
0	<MQL***	
	<MQL	<MQL
	<MQL	(0.09)
	<MQL	(0.07)
0.31	0.31	
	0.37	0.29
	0.31	0.29
	0.32	0.28
Mean	0.33	0.29
C.V. (%)	8.8	2.0
Mean percent difference	6.5	−6.5
0.76	0.78	
	0.70	0.72
	0.85	0.67
	0.75	0.72
Mean	0.77	0.70
C.V. (%)	8.1	4.1
Mean percent difference	1.3	−7.9
1.53	1.51	
	1.51	1.40
	1.47	1.45
	1.48	1.43
Mean	1.49	1.43
C.V. (%)	1.4	1.8
Mean percent difference	−2.6	−6.5
2.29	2.17	
	2.14	2.27
	2.43	2.23
	2.24	2.27
Mean	2.24	2.26
C.V. (%)	5.8	1.0
Mean percent difference	−1.8	−1.3

*Analysed upon preparation.
**Analysed after seven-days storage in a laboratory freezer.
***MQL = 0.05 μg/ml.

allows for the simultaneous quantitation of both trilostane and ketotrilostane. The recently developed cytochemical bioassay [11] measures total trilostane-like bioactivity (i.e. inhibition of 3β-HSD activity by trilostane and its bioactive metabolites) but gives no information on their relative concentrations. This

ability to measure trilostane and ketotrilostane separately is important for determining the pharmacokinetics of the drug and for studying the effects of dose, formulation and disease states on metabolism.

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Journal of Chromatography, 311 (1984) 443—448
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2261

Note

Determination of ibuprofen in human plasma by high-performance liquid chromatography

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(First received March 2nd, 1984; revised manuscript received July 16th, 1984)

Ibuprofen, D,L-2-(4-isobutylphenyl)propanoic acid, is an anti-inflammatory, antipyretic, analgesic drug widely used in the treatment of arthritis. Numerous gas—liquid chromatographic (GLC) [1—4] and gas chromatographic—mass spectrometric [5—7] assays with varying sensitivities have been reported. The most sensitive of the GLC assay techniques is the procedure reported by Kaiser and Martin [4]. Their determination is sensitive to 0.1 μg/ml ibuprofen in serum; however, their procedure is both complicated and time-consuming. Other GLC assays [1—3], which employ flame-ionization detection, offer more rapid sample preparation but have limited sensitivity and require large serum volumes. High-performance liquid chromatographic (HPLC) techniques [8—13] have been reported for the determination of ibuprofen. These methods have employed chloroform [8], hexane [9] or dichloromethane [10, 13] extractions. Detection limits have been reported as 1 μg/ml [9], 0.5 μg/ml [8, 10, 12] and 0.2 μg/ml [11, 13]. There is no reported evaluation of the extraction conditions for ibuprofen.

We have examined the extraction of ibuprofen from heparinized plasma including the solvent systems used by others [8—10, 13] and have devised a system to achieve quantitative recovery. A rapid, selective and sensitive HPLC method had been developed using ibufenac (4-isobutylphenylacetic acid) as the internal standard. The method has been used for determination of ibuprofen in plasma over a concentration range of 0.2—60 μg/ml. The detection

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limit was found to be 0.04 μg/ml corresponding with 4 ng ibuprofen injected into the column.

MATERIALS AND METHODS

Reagents and standard solutions

Ibuprofen, D,L-2-(4-isobutylphenyl)propanoic acid, generously supplied by Dr. Paul O'Connell of Upjohn (Kalamazoo, MI, U.S.A.), was prepared in a standard solution of 200 mg/l in methanol. The internal standard ibufenac, 4-isobutylphenylacetic acid (supplied by Dr. O'Connell), was also prepared in a standard solution of 200 mg/l in methanol. Isooctane, 2-propanol, toluene, chloroform and petroleum ether were purchased as analytical-grade reagents from Fisher Scientific (Cleveland, OH, U.S.A.). Acetonitrile (UV grade) was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Methanol and acetonitrile were filtered before use; all other reagents were used without purification.

Chromatographic conditions

The liquid chromatograph comprised a Model M6000A pump, a U6K syringe loading injection valve, and an RCM-100 radial compression unit purchased from Waters Assoc. (Milford, MA, U.S.A.). Detection was accomplished by a Model LC-75 variable-wavelength detector purchased from Perkin-Elmer (Norwalk, CT, U.S.A.) and operated at a detection wavelength of 220 nm. A Linear Instruments (Irvine, CA, U.S.A.) Model 585 chart recorder was used for recording the detector output signal. Peak height and area measurements and subsequent sample concentration computations were performed by a Model 3354 laboratory automation system from Hewlett-Packard (Avondale, PA, U.S.A.). A 10 × 0.5 cm Radial-Pak C_{18} (10 μm nominal particle diameter) reversed-phase cartridge (Waters Assoc.) was used for the chromatographic separation. The chromatographic mobile phase was prepared by combining 500 ml of water, 500 ml of acetonitrile, and 1 ml of concentrated phosphoric acid (85%). The eluent was pumped at a flow-rate of 3.0 ml/min.

Sample preparation

Heparinized plasma specimen (1 ml) was combined with 0.25 ml of 1 *M* hydrochloric acid in a capped 15-ml glass test tube. To this were added 50 μl of the ibufenac internal standard solution and 5 ml of an isooctane—2-propanol mixture (85:15, v/v). The capped tubes were mixed at moderate speed for 5 min on a Rotamixer. The tubes then were centrifuged for 5 min at 1240 *g* and the organic layer transferred to a 10 × 75 mm glass tube by pipette. These tubes were placed in a 45°C water bath and the solvent evaporated under a gentle stream of air. The sample residues were reconstituted in 100 μl of methanol, the tubes vortexed, and 10 μl of the sample were injected into the liquid chromatograph.

Recovery experiments

Samples containing ibuprofen at 20 μg/ml were prepared by adding 100 μl of the 200 mg/l ibuprofen standard methanol solution to empty 15-ml glass

tubes. The methanol was evaporated at 45°C under a gentle air stream and 1 ml of heparinized drug-free plasma and 0.25 ml of 1 *M* hydrochloric acid were added to each tube. The capped tubes were placed on a Rotamixer for 5 min to allow dissolution of the drug in the acidified plasma. Extraction of each experimental sample group was performed with 5 ml of one of the following solvents: chloroform, toluene, hexane, dichloromethane, petroleum ether and isooctane—2-propanol (85:15, v/v). All tubes were mixed at moderate speed on the Rotamixer for 5 min and then centrifuged at 1240 *g* for 5 min. The organic layers were transferred by pipette to 10 × 75 mm glass tubes and the solvents evaporated at 45°C under a gentle air stream. A standard for 100% recovery was prepared by adding 100 μl (equal to 20 μg of the ibuprofen) standard solution in methanol and drying off the methanol in an identical manner to that of the extraction solvents. All extraction sample residues and the ibuprofen standard solution residues were reconstituted in 100 μl of a 200 mg/l solution of ibufenac in methanol. In this recovery analysis, ibufenac served as an external standard. Ibuprofen:ibufenac peak height and area ratios obtained with experimental specimens were compared with the ratios obtained with the quantitative recovery standard.

RESULTS AND DISCUSSION

The results of the extraction efficiency and recovery experiments are shown in Table I. The isooctane—isopropanol (85:15, v/v) extraction solvent gave complete recovery of the drug from the heparinized plasma samples. Recovery of ibuprofen from plasma with the other organic extraction solvents generally was less than 70%. Chloroform (the solvent employed by Pitre and Grandi [8]) gave recoveries of 65.0 ± 3.0%. Although we used hydrochloric acid rather than phosphoric acid to acidify the plasma, hexane (used by Shimek et al. [9]) gave less than 50% recovery. Dichloromethane (used by Kearns and Wilson [10] and Lockwood and Wagner [13]) gave recoveries of 68.6 ± 7.9%.

TABLE I

EXTRACTION OF IBUPROFEN FROM PLASMA

The fraction of ibuprofen extracted from plasma into the named solvents was evaluated as described in the text using an external standard method. The data are expressed as mean ± S.D. for six individual samples.

Solvent	Percentage ibuprofen recovered
Hexane	48.2 ± 1.4
Petroleum ether	55.3 ± 1.2
Chloroform	65.0 ± 3.0
Dichloromethane	68.6 ± 7.9
Toluene	69.5 ± 2.7
Isooctane—2-propanol	102.6 ± 4.7

A chromatogram of a processed drug-free plasma specimen without added internal standard is shown in Fig. 1. The chromatogram of a processed plasma sample containing 1 μg/ml ibuprofen and added internal standard is shown in

Fig. 2. The detection limit for ibuprofen in plasma (at a signal-to-noise ratio greater than 5:1) was found to be 0.04 μg/ml, corresponding with 4 ng ibuprofen injected into the chromatograph. There was complete separation of the chromatographic peaks of the internal standard and ibuprofen without interference from endogenous compounds in plasma samples from normal volunteers and uremic patients. The separation time is 3.3 min but a chromatographic peak of an endogenous sample constituent occurs at 4.3 min. Subsequent injections can be made at intervals of 6 min. Digoxin, clonidine, quinidine, procainamide, theophylline and propranolol were shown not to interfere with the assay. There is good stability of the instrument signal baseline at maximum detector sensitivity. Accuracy and precision of ten replicate

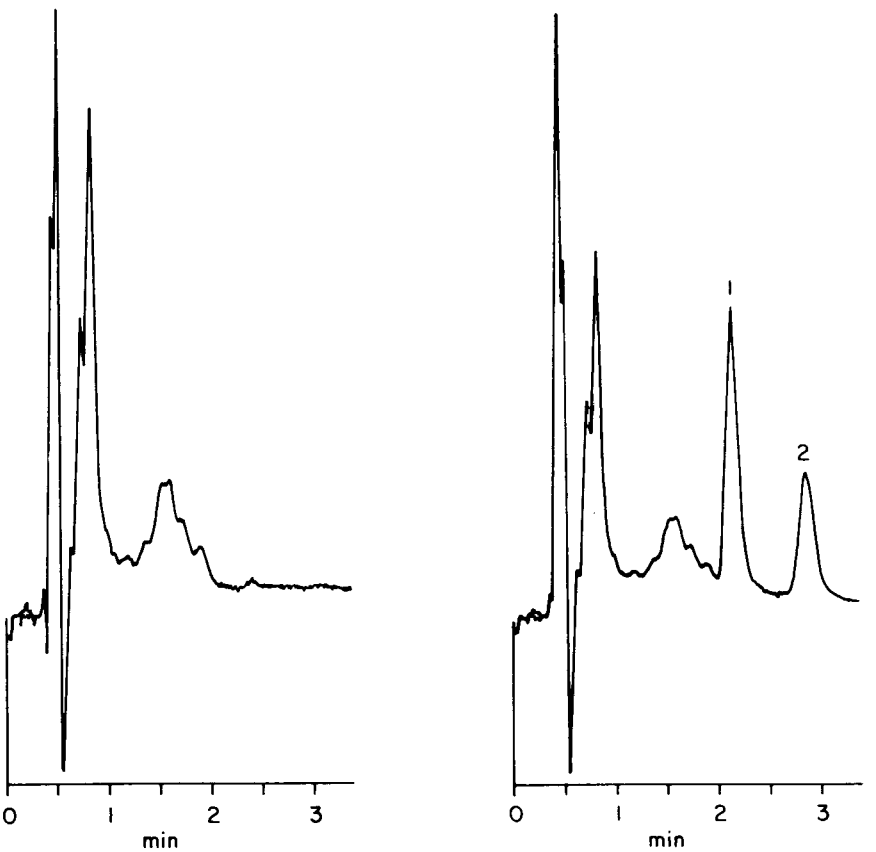

Fig. 1. Chromatogram obtained upon extraction of a drug-free plasma specimen without added internal standard according to the outlined experimental procedure. The column was a 10 × 0.5 cm I.D. radially compressed cartridge of Radial-Pak C_{18} (10 μm nominal particle diameter). The chromatographic eluent was aqueous 20 m*M* phosphoric acid—acetonitrile (50:50) and was pumped at a flow-rate of 3.0 ml/min. The absorbance detector was operated at 220 nm. The full scale of the ordinate is 0.04 absorbance units.

Fig. 2. Chromatogram obtained upon preparation of a plasma containing 1 μg/ml ibuprofen and internal standard according to the described method. The chromatographic conditions were as described in Fig. 1. The internal standard ibufenac (1) was eluted at 2.3 min; ibuprofen (2) was eluted at 3.0 min.

injections of a single prepared serum specimen containing 2 μg/ml ibuprofen was 2.04 ± 0.05 μg/ml.

A series of standard curves of ibuprofen:internal standard peak height ratios were established over sample ibuprofen concentration ranges of 0.2—60 μg/ml. A low-concentration standard curve (0.2—2 μg/ml) of ibuprofen:internal standard peak height ratios versus sample ibuprofen concentration was linear (r^2 = 0.999) with an intercept value of about 5% of the peak height ratio obtained for the standard solution containing 0.2 μg/ml. A 5—60 μg/ml standard curve was linear (r^2 = 0.999) and passed through the origin. A plasma sample (containing 0.5 μg/ml ibuprofen) determined as an experimental sample in six replicate trials against the low-concentration standard curve was 0.52 ± 0.01 μg/ml.

The assay procedure was used in pharmacokinetic studies with normal volunteers and uremic patients on dosage regimens of 400 mg ibuprofen twice daily. Fig. 3 shows an ibuprofen plasma concentration—time curve for a normal volunteer following a morning 400-mg oral dose of ibuprofen. Full results of these studies will be reported elsewhere.

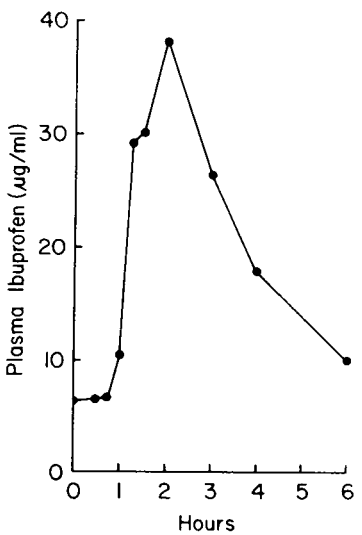

Fig. 3. Plasma ibuprofen concentration—time curve following oral ingestion of 400 mg ibuprofen. A normal male volunteer took 400 mg ibuprofen at 12-h intervals for five days. On the fifth day, a zero-time plasma sample was obtained before the morning dose of ibuprofen.

ACKNOWLEDGEMENTS

This work was supported by research funds from the Veterans Administration Medical Research Service. We thank S. Ingalls, P. Minkler and E.S. Ricanati, M.D., for suggestions and help, and Ms. D.A. Collins and S.L. Evans for preparation of the manuscript.

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Journal of Chromatography, 311 (1984) 449—455
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2270

Note

New method for the determination of yohimbine in biological fluids by high-performance liquid chromatography with amperometric detection

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(First received February 28th, 1984; revised manuscript received July 25th, 1984)

Yohimbine is an alkaloid, isolated from *Corinanthe yohimbe* in 1896 by Spiegel [1], and known for its aphrodisiac properties. It is an α_2-adreno-blocker used particularly in animal psychopharmacology experiments because it easily penetrates the central nervous system and produces a complex pattern of responses [2]. Recently, its α_2-adrenergic properties have been utilized in several cases of orthostatic hypotension in humans [3], and it has been suggested for some forms of obesity, an antilipolytic role of the α_2-adrenergic receptors of human adipocytes having been reported [4].

Although yohimbine has been known and used for a long time, no method for its measurement in biological fluids has been described. Only one quantitative method using a colorimetric reaction has been reported [5, 6]. More recently two methods have been described using fluorimetric [7] and spectrophotometric [8] analysis. All these methods are only suitable for measurement in pure solution, or in tablets or mixtures of alkaloids, however. A semiquantitative method using thin-layer chromatographic separation has been developed for the analysis of *Rauwolfia* alkaloids [9] and a study of heteroyohimbine alkaloids based on gas—liquid chromatography has been reported [10].

The present report describes a method for the analysis of yohimbine in human and rat plasma by reversed-phase high-performance liquid chromatography (HPLC) with amperometric detection.

MATERIALS AND METHODS

Chemicals and drugs

Yohimbine hydrochloride (Fig. 1) was from Sigma, St. Louis, MO, U.S.A. and the internal standard (Fig. 1), 4-amino-3,5-dichloro-α-(*tert.*-butylamino-

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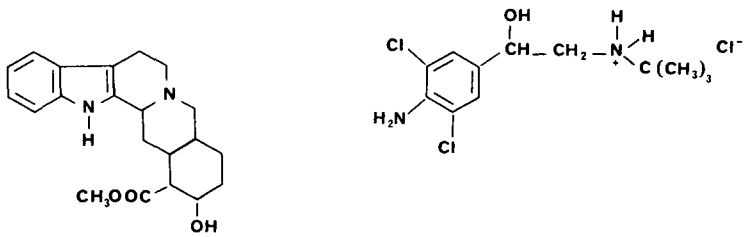
methyl)benzyl alcohol hydrochloride, was obtained from Boehringer Ingelheim, France.

Standard solutions of yohimbine were prepared in methanol at concentrations of 1 μg/ml and 10 μg/ml.

Standard solutions of internal standard were also prepared in methanol at a concentration of 10 μg/ml.

All reagents used were of analytical grade: methanol (Carlo Erba, Italy), chloroform (U.C.B., Belgium), dichloromethane (J.T. Baker, The Netherlands), isopropanol (Carlo Erba), ammonium phosphate monobasic (Sigma) and sodium hydroxide (Prolabo, France).



YOHIMBINE INTERNAL STANDARD

Fig. 1. Chemical structures of yohimbine and internal standard.

Chromatographic conditions

Apparatus. The chromatographic system consisted of a Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) fitted with an additional pulse damper (Touzard et Matignon, France), a Rheodyne sample valve equipped with a 50-μl loop. The column was a Waters μBondapak C_{18} (30 cm × 3.9 mm I.D., 10-μm non-spherical particle size).

A Metrohm electrochemical detection system, composed of a 641 VA detector, a 656 electrochemical detector equipped with a glassy carbon electrode and a silver/silver chloride reference electrode, was used to oxidize the compounds at a potential of 1.15 V. The sensitivity was set at 50 nA full scale.

All chromatograms were recorded on a Servotrace recorder (Sefram, France) at a chart speed of 5 mm/min.

The mobile phase. The mobile phase consisted of distilled water—methanol (52:48) containing ammonium phosphate (0.01 *M*) and was thoroughly degassed and filtered through a 0.2-μm filter disc (Millipore, Bedford, MA, U.S.A.) before use. The flow-rate was kept constant at 1 ml/min, corresponding to a pressure of about 90 bars (1300 p.s.i.).

Extraction of samples

To 1 ml of rat plasma are added 50 μl of a solution containing 10 μg/ml internal standard, 0.5 ml of 0.1 mol/l sodium hydroxide and 6 ml of chloroform—dichloromethane—isopropanol (6:1:1, v/v). The mixture is shaken for 20 min using an alternating agitator (Realis type 44-40, France). The solution is then centrifuged for 10 min at 900 *g* and the supernatant is

discarded. The lower organic phase is transferred to a clean tube, then evaporated to dryness using a vortex evaporator (Buchler Instruments Division, Fort Lee, NJ, U.S.A.).

The residue is dissolved in 100 μl of the mobile phase, 25 μl of which are injected into the chromatograph.

Calibration curve

The calibration curve is obtained by adding yohimbine at concentrations of 25, 50, 100, 200, 300, 400, 600 ng/ml to rat control plasma. These standards are then extracted under the same experimental conditions. The peak heights are measured and the ratios of peak height of yohimbine to peak height of internal standard are plotted against concentration.

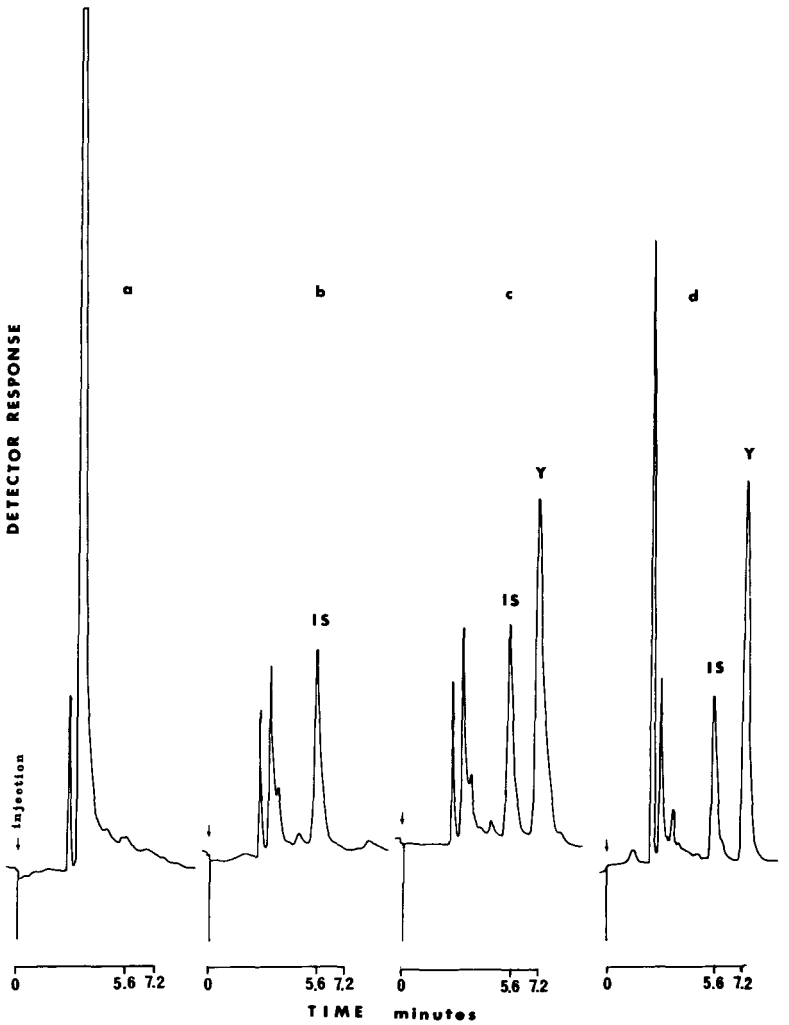


Fig. 2. Typical chromatograms obtained from rat plasma after injection of: (a) blank plasma control; (b) plasma control spiked with internal standard (IS); (c) plasma control spiked with IS and 600 ng/ml yohimbine (Y); (d) plasma sample obtained 2 h after 0.2 mg/kg oral dose of yohimbine (Y).

RESULTS

The chromatograms obtained in this study are shown in Fig. 2. Under the experimental conditions used the retention times of internal standard and yohimbine are 5.6 and 7.2 min, respectively (capacity coefficient, k', 1 and 1.6, respectively).

The calibration curve shows good linearity (correlation coefficient = 0.999) in the range 0—600 ng/ml and passes through the origin. The slope of the graph is $2.8271 \cdot 10^{-3}$ $(ng/ml)^{-1}$.

Precision

The reproducibility of the method was checked for three plasma concentrations: 50, 300 and 600 ng/ml. Ten determinations were made the same day at each concentration. The coefficients of variation are shown in Table I.

The "accuracy" of the method was controlled for three plasma concentrations: 50, 300 and 600 ng/ml. Each concentration was assayed daily over a period of five days. The coefficients of variation are shown in Table I.

TABLE I

REPRODUCIBILITY AND ACCURACY OF HPLC ASSAY FOR YOHIMBINE

Yohimbine (ng/ml)	Coefficient of variation (%)	
	Reproducibility	Accuracy
50	4.5	2.4
300	2.5	6.3
600	1.4	5.2

Recovery

We have estimated the recovery by comparing the peak height after an injection of a pure solution of yohimbine and after the injection of extracted plasma containing the same quantity of yohimbine. The percentage extraction is about 86%. Results are shown in Table II.

Sensitivity

Under the conditions described in this paper, the quantitation limit for

TABLE II

RECOVERY OF YOHIMBINE FROM RAT PLASMA

Yohimbine (ng/ml)	Recovery (%)
50	85
300	93
600	82
Mean	86.6

yohimbine was 10 ng/ml for a 1-ml sample, but it could easily be improved down to 1 ng/ml either by taking up the dried residue of the extract with 50 μl of the mobile phase or by increasing the sensitivity of the detector. No interferences between the peaks appeared.

APPLICATION OF THE METHOD

Animal study

To demonstrate the biological applicability of the method, rats were medicated orally with 0.2 mg/kg yohimbine. Blood was drawn from the orbital sinus and placed in tubes containing heparin as the anticoagulant, 2 h after treatment. Plasma was processed as described above. Chromatograms are shown in Fig. 2.

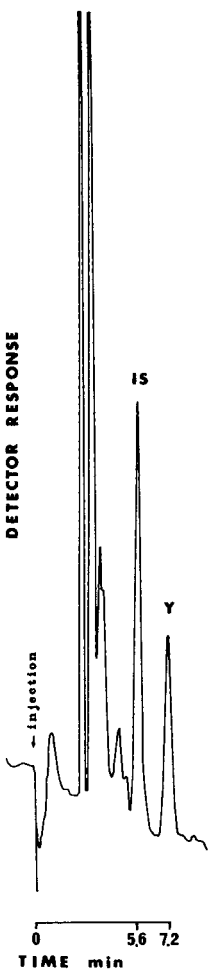


Fig. 3. Chromatogram obtained from human plasma 90 min after an oral dose of 6 mg of yohimbine (sensitivity was set at 10 nA full scale).

Clinical study

The method has been employed to determine the plasma levels of yohimbine in healthy volunteers following an oral dose of 6 mg. Blood was collected into heparinized tubes, 45 min and 90 min after treatment. After centrifugation, plasma was frozen in propylene tubes and kept at −20° C until assay. For this study, the sensitivity of the detector must be set at 10 nA full scale because of the plasma levels. A chromatogram is shown in Fig. 3.

DISCUSSION

Several mobile phases were tried. Different proportions of water and methanol as well as different salts were used. The ratio 52:48 of water—methanol gave the best separation consistent with a sufficiently short retention time. Ammonium phosphate proved to give fewer impurities on the chromatogram.

This method is easy to handle and not expensive: the mobile phase composed of few costly constituents is quickly prepared and efficient separations are obtained with a standard column. The one-step extraction, however, requires some care. Evaporation should be carried out gently without heating or shaking. If no care is taken, some problems with the reproducibility, due to splashing, may occur. No peak with a retention time similar to that of yohimbine was found in blank plasma samples.

Other alkaloids (reserpine, ajmaline, colchicine, emetine, raubasine, quinine) have been tested using the method and no interference with yohimbine was observed. Usually these compounds are retained longer on the column and appear later on the chromatogram. These compounds were tested for their suitability as an internal standard but they did not suit the method because of their too long or too short retention time. Colchicine, however, shows a convenient retention time, consistent with a good resolution; but it forms two crystalline compounds with chloroform, which do not yield the chloroform unless heated between 60 or 70° C for a considerable time.

The compound chosen as internal standard was used in our laboratory for pharmacological experiments and was suitable for the method described above (retention time consistent with the analysis, lack of interference with endogenous peaks, extraction in similar conditions).

The assay proved to be quite sensitive with a low limit of quantitation, 10 ng/ml, compared with the high plasma levels obtained in animal and clinical studies. In fact, the mean concentration of yohimbine in rat plasma 2 h after a 0.2 mg/kg oral dose of yohimbine, was 459 ng/ml. In healthy volunteers the yohimbine plasma levels were 76 ng/ml at 45 min, and 88 ng/ml at 90 min after an oral dose of 6 mg of yohimbine.

The same assays using ultraviolet detection were tried but were not sensitive enough for pharmacokinetic studies in humans. Mainly, the resolution of the chromatogram was poor, the peaks of yohimbine appearing broad because of the detection cell volume.

In summary, the HPLC assay using electrochemical detection shows good reproducibility, sensitivity and selectivity. It has the advantage of being a relatively convenient and simple method, and easily applicable to pharmacokinetic studies in rats and humans.

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Related articles published in *Journal of Chromatography*, Vols. 299, 301 and 302

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Erratum

J. Chromatogr., 307 (1984) 180—184
Page 183, text line 5 from bottom, "15 to 120 ng" should read "15 to 300 ng".

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