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plex occurs in the presence of 3 *M* sodium chloride. To confirm by gel chromatography this dissociative effect of sodium chloride and to determine which process in the two conversions (renin to intermediate molecular weight renin, or the intermediate form to high molecular weight renin) is most affected by the presence of sodium chloride, the hog kidney extract was incubated in 4 *M* sodium chloride at 37°C for 30 min and analyzed by HPLC. Most of the renin after the incubation was converted to intermediate molecular weight renin but not to the high molecular weight form (Fig. 1E). Similarly, high molecular weight renin was dissociated to the intermediate molecular weight form in the presence of 4 *M* sodium chloride at 37°C but no further dissociation occurred.

Fig. 2 shows the effect of pH on the stability of the renin—RBP complex. At pH values below 4, both high molecular weight and intermediate molecular weight renins were quite unstable and converted to renin. About 20% of these higher molecular weight forms of renin escaped the conversion during 2-min acidification at pH 3; however, 15-min acidification of the high molecular weight renin resulted in its complete conversion to normal renin.

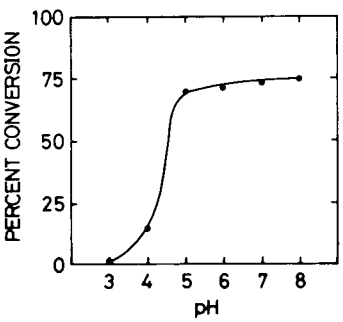

Fig. 2. Effect of pH on the interaction between renin and RBP. The percentage values indicate the proportion of the renin—RBP complex (high molecular weight renin) in the total renin activity.

DISCUSSION

Introduction of the gel TSK G3000SW in HPLC has made it possible to separate proteins according to their size and shape within 40 min [9]. We examined possible applications of this gel to studies on interconversions among various forms of renin. As a result of the interaction with renin-binding protein, active renin has been shown to exist in three forms: renin, and intermediate molecular weight and high molecular weight renins [8]. Although these three forms were distinguished by conventional gel filtration on Sephadex G-100 or Ultrogel AcA 44, the separation was unsatisfactory. The newly developed SW-type gel, however, gave satisfactory resolution. In addition, it drastically reduced elution time, opening up exciting possibilities for elucidating, in more detail, the mechanism and factors which contribute to the formation of the renin—RBP complex. We have shown that at 37°C the interaction can occur spontaneously at a reasonable speed even in the absence of sulfhydryl reagents. This fact, together with our previous observation [10, 11] that the binding capacity of RBP is specific for renin, strongly suggests that RBP may play an important role in

blood pressure regulation. These conclusions are in substantial agreement with those reached by Boyd [1] who used different approaches to study the protein-bound form of renin. Also, our previous finding [8] of the presence of an intermediate molecular weight form of renin, was confirmed using the newly developed gel, TSK-Gel G3000SW.

The present series of experiments has further established the experimental conditions under which the intermediate molecular weight renin is a major form among other interconvertible forms of renin; an intermediate molecular form becomes dominant at low temperatures or at high sodium chloride concentrations, suggesting that the conversion from the intermediate molecular weight renin to the high molecular weight form is a temperature- and ionic strength-sensitive process.

Attempts to demonstrate the presence of the three forms of renin by a single run have so far been unsuccessful. This is mainly due to the nature of the equilibria among them. At 37°C or in the presence of sulfhydryl reagents, the equilibria are shifted entirely to the higher molecular weight forms of renin, making the detection of low molecular weight renin difficult; in the kidney extracts freshly prepared at 4°C or in the samples acidified or treated with sodium chloride, the presence of low molecular weight renin can be demonstrated, but one or both of the higher molecular weight forms of renin are unstable under these conditions. Further studies are required to clarify the nature of equilibria and the mechanism of the interconversions among the various forms of renin.

ACKNOWLEDGEMENT

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CHROMBIO. 953

DETERMINATION OF SERUM UNCONJUGATED ESTRONE, ESTRADIOL-17β AND ESTRIOL DURING PREGNANCY BY SELECTED ION MONITORING

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(First received December 10th, 1980; revised manuscript received April 28th, 1981)

SUMMARY

A simple, rapid and highly specific method by selected ion monitoring (SIM), using 9α,11α-[$^2H_2$]estrone, [2,4-$^2H_2$]estradiol-17β and 2,4-[$^2H_2$]estriol as internal standards, was developed for the determination of serum estrogens during pregnancy. Serum samples were submitted to a simple extraction procedure and were analysed after formation of the trifluoroacetic anhydride derivative. The inter-assay coefficients of variation for estrone, estradiol-17β and estriol were 3.73%, 3.42% and 3.49%, respectively. The results obtained by SIM were compared with analysis performed using radioimmunoassay.

INTRODUCTION

Quantification of estrogens has made considerable progress since the introduction of radioimmunoassays (RIA). These immunological methods are limited, however, by the nonspecificity of antibodies and are complicated by the need for using radioisotopes.

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The purpose of this investigation was to develop a simple, rapid and highly specific assay procedure for the three classical estrogens (estrone, estradiol-17β and estriol) using the technique of selected ion monitoring (SIM).

EXPERIMENTAL

Materials

Blood was collected between 9.00 a.m. and 12 noon from a group of 120 healthy pregnant women visiting Showa University Hospital. Serum was obtained by centrifugation and stored below −10°C until analyzed.

Reagents

Methanol, *n*-hexane, ethyl acetate and trifluoroacetic anhydride from freshly opened bottles were used without any additional purification. Ethanol was redistilled before use.

Synthesis of deuterium-labelled estrogens

[2,4-$^2H_2$]Estriol was prepared by the deuterium exchange reaction according to the method reported by Tökès and Throop [1]. A solution of estriol (100 mg) in deuterated methanol ($CH_3O^2H_2$) (10 ml) and 10% deuteriosulfuric acid in deuterium oxide (4 drops) was refluxed for 2 days. Most of the solvent was removed by distillation and water was added. The precipitates were collected by filtration and dried. Recrystallization from methanol gave the deuterated estriol (72 mg). Analysis by mass spectrum showed that the product consisted of 2,4-d_2-estriol (88.2%), d_1-estriol (10.1%) and d_0-estriol (1.7%).

[2,4-$^2H_2$]Estradiol-17β (50 mg) was prepared in the same manner as described above for estriol. 2,4-d_2-Estradiol-17β (32 mg, 80%) containing d_1-estradiol-17β (16%) and d_0-estradiol-17β (4%) was obtained.

[9α,11α-$^2H_2$]Estrone was prepared by the method of Tsuda et al. [2]. A solution of 9(11)-dehydroestrone (1.0 g) in dimethoxyethane (50 ml) was shaken vigorously in deuterium atmosphere with 5% palladium charcoal (0.4 g). The absorption was stopped after 10 min. The catalyst was removed by filtration and the filtrate was concentrated. 9α,11α-d_2-Estrone (0.9 g, 27%) containing d_0-estrone (2.9%), d_1-estrone (15.2%), d_3-estrone (38.0%), d_4-estrone (13.4%) and d_5-estrone (3.5%) was obtained.

Analysis by nuclear magnetic resonance (NMR) spectrometry showed that two deuteriums were induced into positions 2 and 4. Analysis of the synthesized deuterated estrone by NMR was not performed. However, under the procedure used in this experiment, deuterium should be induced into positions 9 and 11.

Gas chromatography—mass spectrometry

The instrument used in the present study was a Shimadzu LKB-9000 equipped with a multiple-ion detector and was operated under the following conditions. The column was a 1-m glass coil with 1% OV-1 coated on Chromosorb W 50—80 mesh, and was operated at 210°C, with a helium flow-rate of 25 ml/min. The temperature of the separator and ionization source were kept at 270°C and 290°C, respectively. The ionization energy and trap current were

70 eV and 50 μA, respectively. The retention times of estrone trifluoroacetate (TFA), estradiol-17β-TFA and estriol-TFA were 3 min 3 sec, 4 min 30 sec and 1 min 30 sec, respectively. The multiple-ion detector was focused on the molecular ions of TFA derivatives of unlabelled and labelled compounds. Monitor masses were as follows: m/e = 366, 368 for estrone; m/e = 464, 466 for estradiol-17β; and m/e = 576, 578 for estriol. Mass spectra of estrone-TFA, estradiol-17β-TFA, and estriol-TFA are demonstrated in Fig. 1.

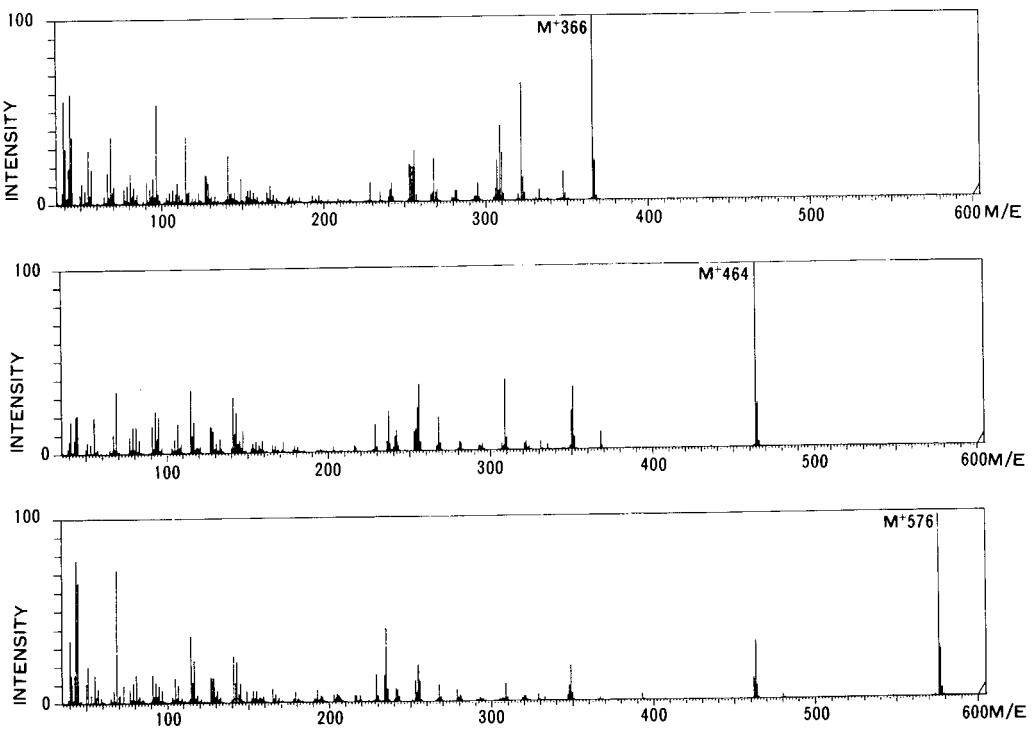


Fig. 1. Mass spectra in the analysis of trifluoroacetates of estrone, estradiol-17β and estriol. Upper spectrum: estrone, $M^+ = 366$; centre spectrum, estradiol-17β, $M^+ = 464$; lower spectrum, estriol, $M^+ = 576$. The mass spectra were measured on a Jeol JMS-D300.

Procedure

The extraction and derivative formation were carried out as summarized in Fig. 2. From a standard solution containing 10 ng/ml deuterium-labelled estrogens in ethanol, 2 ml (20 ng) were added to 0.5 ml of the serum and the serum was allowed to stand for 30 min at room temperature prior to the extraction procedure [3]. After the addition of 2 ml of methanol and stirring, the precipitates were removed by centrifugation. The extracts were washed twice with 2 ml of *n*-hexane to remove lipid and were evaporated to dryness. After the addition of 1 ml of water, estrogens were extracted with 2 ml of water-saturated ethyl acetate. The solvent was removed by evaporation under nitrogen gas. The residue was dissolved in 4 μl of ethyl acetate, and 4 μl of trifluoroacetic anhydride were added.

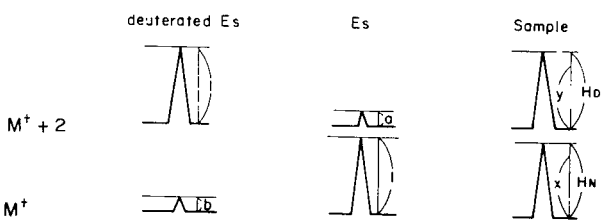
Serum 0.5ml
↓ add deuterium-labelled estrogens as internal standards
Extract with 2 ml methanol
↓
Centrifuge 3000rpm 5 min
↓
Methanol
↓ Removal of lipid with n-Hexane 2 ml×2; discard
Evaporate under N_2 gas stream
↓
H_2O 1 ml
↓
Ethyl acetate 2 ml
↓
Take supernatant
↓
Evaporate under N_2 gas stream
↓
TFA derivative formation
↓
GC-MS

Fig. 2. Extraction and derivatization of estrone, estradiol-17β and estriol to be determined by GC—MS.

Calculation of the content of estrogens

SIM responses were measured as peak heights, and the concentrations of the three classical estrogens were calculated with the aid of the formula given in Fig. 3.

Formula



a ; ratio of deuterated Es containing Es
= 0.035, 0.040 and 0.053 for E_1, E_2 and E_3
b ; ratio of Es containing deuterated Es
= 0.092, 0.016 and 0.061 for E_1, E_2 and E_3
X ; amount of Es in Sample

A ; ratio of deuterization
= 0.270, 0.800 and 0.882 for E_1, E_2 and E_3
B ; amount of added d_2-Es

$$H_D = y + aH_N$$

$$H_N = x + by$$

$$X = AB\frac{x}{y} \fallingdotseq AB\frac{\frac{H_N}{H_D} - b}{1 - a\frac{H_N}{H_D}}$$

Fig. 3. Formula for calculation of the content of estrogens (Es) [estrone (E_1), estradiol-17β (E_2) and estriol (E_3)]. Hormone concentrations were calculated by multiple-ion detection responses as peak heights in mm.

RESULTS

Evaluation of the method

Known amounts of estrone, estradiol-17β and estriol were added to 0.5 ml of healthy male serum. The extraction and derivative formation were carried out as shown in Fig. 2. As seen from Table I, the values for percentage recovery and coefficient of variation (C.V.) were satisfactory.

TABLE I

RESULTS OF THE EVALUATION OF THE METHOD

Estrogen added to male serum (ng)		*n*	Recovered ng/ml (mean ± S.D.)	Percentage recovery (mean ± S.D.)	C.V. (%)
Estrone	10	6	9.65 ± 0.36	96.47 ± 3.57	3.73
Estradiol-17β	20	6	19.32 ± 0.66	96.58 ± 3.29	3.42
Estriol	20	6	19.58 ± 0.68	97.88 ± 3.41	3.49

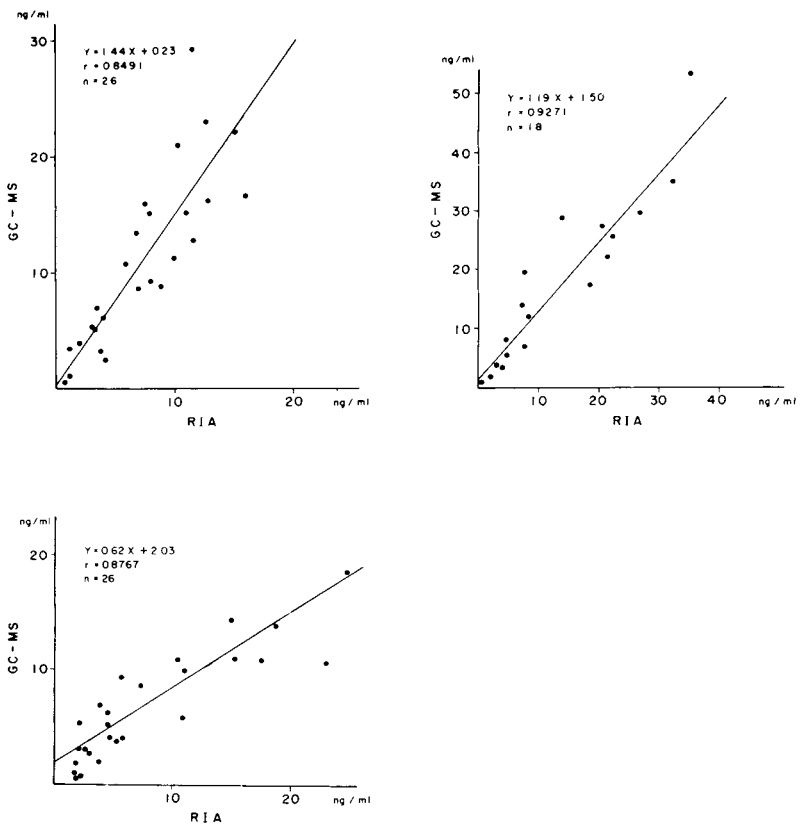


Fig. 4. Correlation between estrone (top left), estradiol-17β (top right) and estriol (bottom left) concentrations obtained by GC—MS and RIA.

Correlation with radioimmunoassay

The radioimmunoassay of the three classical estrogens was carried out according to methods currently used in this laboratory [4]. Anti-estriol-16,17-dihemisuccinate—bovine serum albumin (BSA) was used as an antibody. The cross-reactivity of this anti-serum with various steroids was studied [5]. Specimens were extracted with dichloromethane and separation of all three estrogens was performed by column chromatography using Sephadex LH-20.

Serum samples were quantitated by two different methods — RIA and gas chromatography—mass spectrometry (GC—MS). The correlation between these methods is shown in Fig. 4. The values for the three classical estrogens obtained by GC—MS correlated well with those obtained using RIA.

Clinical results

Normal pregnant women (n = 120) were studied. The levels of the three estrogens increased with progressing gestation (Table II). The mean ± S.D. levels of three estrogens from the 36th to the 40th week were as follows: estrone, 19.8 ± 8.5 ng/ml; estradiol-17β, 31.7 ± 13.4 ng/ml; estriol, 16.5 ± 3.1 ng/ml.

TABLE II

SERUM LEVELS OF UNCONJUGATED ESTROGENS IN NORMAL PREGNANT WOMEN DETERMINED BY GC—MS

Values are expressed as mean ± S.D. Number of samples in parentheses.

Weeks of gestation	Estrone (ng/ml)	Estradiol-17β (ng/ml)	Estriol (ng/ml)
4—7	2.0 ± 1.2 (6)	2.1 ± 1.5 (7)	1.8 ± 0.9 (8)
8—11	2.7 ± 1.0 (11)	3.7 ± 1.6 (6)	1.9 ± 0.9 (12)
12—15	4.5 ± 2.3 (7)	9.8 ± 4.5 (10)	3.9 ± 1.1 (6)
16—19	6.0 ± 1.9 (12)	9.9 ± 4.5 (11)	5.0 ± 2.3 (11)
20—23	7.5 ± 2.9 (16)	12.7 ± 5.6 (14)	5.7 ± 2.1 (11)
24—27	10.4 ± 4.7 (16)	16.7 ± 4.2 (12)	7.7 ± 1.5 (8)
28—31	12.2 ± 2.8 (14)	17.8 ± 6.7 (18)	10.1 ± 4.0 (21)
32—35	13.3 ± 6.7 (20)	22.3 ± 8.5 (21)	10.5 ± 5.3 (21)
36—40	19.8 ± 8.5 (18)	31.7 ± 13.4 (21)	16.5 ± 3.1 (22)

DISCUSSION

The labelled compounds used in the present study were found to be stable in all procedures including extraction, derivative formation and GC—MS measurement. Although the isotopic purity of 9,11-deuterated estrone was

only 27%, the ratio of d_2-estrone to d_0-estrone in the standard labelled estrone was high. Thus this compound could be used as a standard with satisfactory results. 2,4-Di-deuterated estrogens used for estradiol-17β and estriol were of high isotopic purity and could be used as good standard compounds.

Recently, there have been several reports concerning determination of estrogen by GC—MS. Björkhem et al. [6] and Breuer and Siekmann [7] prepared di-deuterated estrogens ([2,4-$^2H_2$]estriol, and [6,7-$^2H_2$]estradiol, respectively), as used in this study. Zamecnik et al. [8] determined estradiol-17β with tetra-deuterated estradiol-17β. These investigators prepared trimethylsilyl ether derivatives. Knuppen et al. [9] determined estradiol as its heptafluorobutylate derivative with [$^2H_2$]estradiol. Derivative formation of estrogens by polyfluorocarboxylic acid seems to be suitable for steroid measurement by GC or GC—MS, since derivatives could be prepared easily with high volatility, and molecular ions were intensive at high masses. In our present study, TFA derivatives were used because of the reason mentioned above.

We believe that the present study is the first to demonstrate the determination of serum unconjugated estrone, estradiol-17β and estriol during pregnancy by the technique of SIM from the same samples.

It is tedious and time-consuming to perform and draw a standard curve, especially when a limited number of samples are determined as applied for RIA. The hormone concentrations in this paper were calculated with the aid of the described formula without using standard curves. This approach has been used to measure testosterone and progesterone by Dehennin et al. [10]. The estimation of peak heights from SIM enables immediate determination of hormone concentration. The amount of estrogens introduced into the GC—MS instrument ranged between 100 and 500 pg in this study. Such amounts were sufficient for accurate determination. This is consistent with the sensitivity of GC—MS.

The mean levels of plasma unconjugated estrone estimated from the 36th week of pregnancy until term by different investigators using chemical methods ranged from approximately 8 to 15 ng/ml [11,12], while values from the radioimmunological method ranged from 4.9 to 25.0 ng/ml [13,14]. The mean serum levels published for unconjugated estradiol-17β determined by chemical methods ranged from approximately 8 to 25 ng/ml [11,12], and for radioimmunological methods ranged from 18 to 25 ng/ml [15,16]. The mean levels of estrone and estradiol-17β measured by the present method seem to be higher than those reported by the others. The mean levels of estriol were also higher than those noted with the chemical method [11]. However, the mean levels of estriol in our study seem to be lower than concentrations reported using radioimmunological methods [17,18].

In this study, conjugated estrogens were not determined, but values of steroid sulfates [19] have to be considered when levels of unconjugated steroids are evaluated.

The possibility of hydrolysis of estrone sulfate in the preparation of the sample for GC—MS can not be completely ruled out, since estrone sulfate is known to be labile and its concentration in blood is relatively high. However, addition of 100 ng of estrone sulfate to male serum resulted in a 0.13% higher value than that in a sample without addition (our recent observation).

With this method, it is possible to complete approximately 30 samples during one working day.

In conclusion, the assay described here is a simple, rapid and specific method for measuring the three classical estrogens.

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CHROMBIO. 969

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY IN SUBREGIONS OF RAT BRAIN

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SUMMARY

A quantitative gas chromatographic—mass spectrometric method has been developed for the determination of glutamic acid decarboxylase (GAD) activity in subregions of rat brain. The five subregions analyzed, weighing approximately 2.51 mg each, were globus pallidus, entopeduncular nucleus, ventromedial thalamus, and substantia nigra medial and lateral. The activity of the GAD enzyme has been determined indirectly by measurement of γ-aminobutyric acid (GABA) using γ-[2,2-$^2H_2$]aminobutyric acid as the internal standard. Both compounds were quantitatively converted to trimethylsilyl-GABA and trimethylsilyl-[$^2H_2$]GABA in 90 min with hexamethylchlorosilane, trimethylchlorosilane, pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide silylating agents. Using selective ion monitoring and electron impact ionization at 70 eV, the limit of detection was 15 ng GABA per mg tissue. This method is compared with a fluorimetric procedure.

INTRODUCTION

γ-Aminobutyric acid (GABA) is widely accepted as being a principal inhibitory neurotransmitter in the central nervous system of vertebrates [1]. However, GABA is distributed in glial cells as well as within neurons, while glutamic acid decarboxylase (GAD) the enzyme which decarboxylates glutamic acid (GA) to GABA, is found only within the neurons [1]. Therefore GAD activity is used as a marker of GABAergic neurons. GABA projections from neostriatum to globus pallidus (GP), entopeduncular nucleus (EP), and substantia nigra (SN) [2, 3] as well as from substantia nigra to ventromedial thalamus (VM) [4] have been reported. Decreased GAD activity has been found in the striatum and SN of post-mortem brains of humans afflicted with Parkinson's disease and Huntington's chorea [5, 6].

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A number of methods have been developed for the indirect measurement of GAD activity via measurement of GABA formation. Roberts and Simonsen [7] reported a radioenzymatic measurement of GAD activity by measurement of $^{14}CO_2$ from the decarboxylation of ^{14}C-labeled GA. The topographical distribution of GAD has been reported by Fonnum et al. [2] using a radiometric assay procedure. Lowe et al. [8] reported a fluorimetric procedure for the determination of GAD activity in neural tissue by measurement of GABA formation. Holdiness et al. [9] reported a modification of this fluorimetric method for the measurement of GAD activity in subregions of rat brain. Values reported for GAD activity were 7.91 ± 1.47 (GP), 6.87 ± 2.07 (EP), 3.83 ± 0.69 (VM), 13.80 ± 2.14 (substantia nigra medial, SN_M) and 8.23 ± 2.26 (substantia nigra lateral SN_L) μg GABA per hour per mg protein.

Recently, mass spectrometric methods have become available for measurement of GABA. Bertilsson and Costa [10] reported a gas chromatographic—mass spectrometric (GC—MS) assay method using γ-[2,2-$^2H_2$]aminobutyric acid as the internal standard with pentafluoropropionic anhydride and hexafluoroisopropanol as the derivatizing agents. Cattabeni et al. [11] developed a GC—MS procedure for GABA by forming the trimethylsilyl (TMS) derivative and used 5-aminovaleric acid as the internal standard. GABA concentration in the rat cerebellum was measured but not GAD activity.

To date only one mass fragmentographic method has been developed for the measurement of GAD activity. Cattabeni et al. [12] described a procedure in which rat cerebellum tissue homogenate was incubated with [$^2H_5$]GA and measured the formation of [$^2H_5$]GABA. It was not determined whether any isotopic discrimination occurred.

In this paper is described a GC—MS procedure for the indirect measurement of GAD activity in rat brain. In this method each tissue sample homogenate is divided into two parts. One part is inactivated with trichloroacetic acid (TCA) to serve as a blank. This gives the amount of GABA that originally existed in the tissue at the time of sampling. The other half of the sample is incubated in a substrate-buffer solution in which GABA is formed enzymatically by GAD. From the difference in GABA content of the two samples, the net GABA formation is derived and GAD activity can be calculated. Samples are collected from the discrete nuclei of GP, EP, VM, SN_M, and SN_L by a micropunch procedure [13] and a deuterated internal standard is used for quantitation.

EXPERIMENTAL

Materials

The chemicals used in this study were sodium-L-glutamate (Pflatz and Bauer, Stamford, CT, U.S.A.), trichloroacetic acid (Sigma, St. Louis, MO, U.S.A.), GABA (Aldrich, Milwaukee, WI, U.S.A.), pyridoxal 5-phosphate and Triton X-100 (Eastman Chemical Co., Rochester, NY, U.S.A.). The internal standard was synthesized by the method of Bertilsson and Costa [10]. Sylon HTP [hexamethylchlorosilane—trimethylchlorosilane—pyridine (3:1:9)] from Supelco (Bellefonte, PA, U.S.A.) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.) were the derivatizing agents used

and were prepared by mixing 1000 μl Sylon HTP with 300 μl BSTFA.

Instrumentation

A Finnigan 4000 GCMS quadrupole mass analyzer spectrometer with a Model 6000 automated data system has been used in all experiments. GC analysis was performed at 140°C on a 1.8 m × 2 mm I.D. glass column with 3% OV-17 on Gas-Chrom Q (100—120 mesh), with helium flow-rate of 25 ml/min, injection port 175°C, jet separator 200°C, ion source 250°C and electron impact ionization potential of 70 eV. The ions monitored were *m/e* 304.1 and *m/e* 306.2 for GABA and [$^2H_2$]GABA, respectively. Methane was the reagent gas for all chemical ionization experiments. A Beckman Cary 118 ultraviolet—visible spectrophotometer has been used for the protein assay [14] at a wavelength of 725 nm and a Heat Systems Model W-220F sonicator for homogenization of tissue.

Sample collection

The sample collection procedure has been previously described [9]. This procedure minimizes the rapid onset of GABA formation observed in post-mortem tissue [15,16]. From each 1 mm thick brain tissue slice, two tissue punches were taken. These punches were from symmetrical locations in the left and right hemispheres. Each pair of punches was transferred immediately to a 12-ml polypropylene Eppendorf tube containing 100 μl of internal standard. The internal standard solution was prepared by addition of 2.0 mg [$^2H_2$]GABA to 25 ml of a solution consisting of 0.5 *M* potassium chloride, 0.010 *M* EDTA, 0.5% Triton X-100 and 0.4 *M* sodium phosphate buffer, pH 6.4. The tissue punches were homogenized for 10 sec under low power sonication. It has been found that sonication up to 30 sec has no effect on the enzyme's activity. After sonication, 50 μl were transferred to an identical tube containing 20 μl of 10% TCA to denature the GAD enzyme and precipitate protein. This second tube served as the blank and its concentration of GABA was subtracted from the original sample. The samples were frozen in dry ice until all had been collected and could be incubated together; however, the samples were never kept in dry ice for over 30 min. The substrate-buffer was prepared as previously described [9] and 50 μl of this solution were added to both the sample and blank tubes before they were incubated for 2 h at 38°C. Inactivation of the enzyme was completed by addition of 20 μl of 10% TCA to the original sample tube. The tubes were centrifuged at 950 *g* for 20 min and 80 μl of each solution were transferred to 1.5-ml micro test tubes and evaporated to dryness under vacuum. The material was derivatized with 50 μl of the derivatization agent at room temperature for 90 min. All samples were run immediately using 1—2 μl injections. Retention time of GABA and its deuterated analogue was 1.10 min under the conditions stated. The precipitated tissue was analyzed for protein as described by Lowry et al. [14] and activity of GAD is reported as μg GABA per hour per mg protein.

RESULTS AND DISCUSSION

A mass spectrum of the TMS derivative and deuterium labeled derivative

is presented in Fig. 1. The base peak in both spectra is *m/e* 174. The ions monitored $(M - 15)^+$ by electron impact at 70 eV were *m/e* 304.1 (GABA) and *m/e* 306.2 ([$^2H_2$]GABA). The relative abundancies of *m/e* 304.1 and *m/e* 306.2 were 22% and 20%, respectively. From mass spectra analysis, [$^2H_2$]-GABA gave a small contribution to *m/e* 304.1 of 0.22% relative abundance while GABA had an ion of 12.83% relative abundance at *m/e* 306.2. The signal intensities of the above mentioned ions were 2.5 times stronger at 70 eV than at 30 eV.

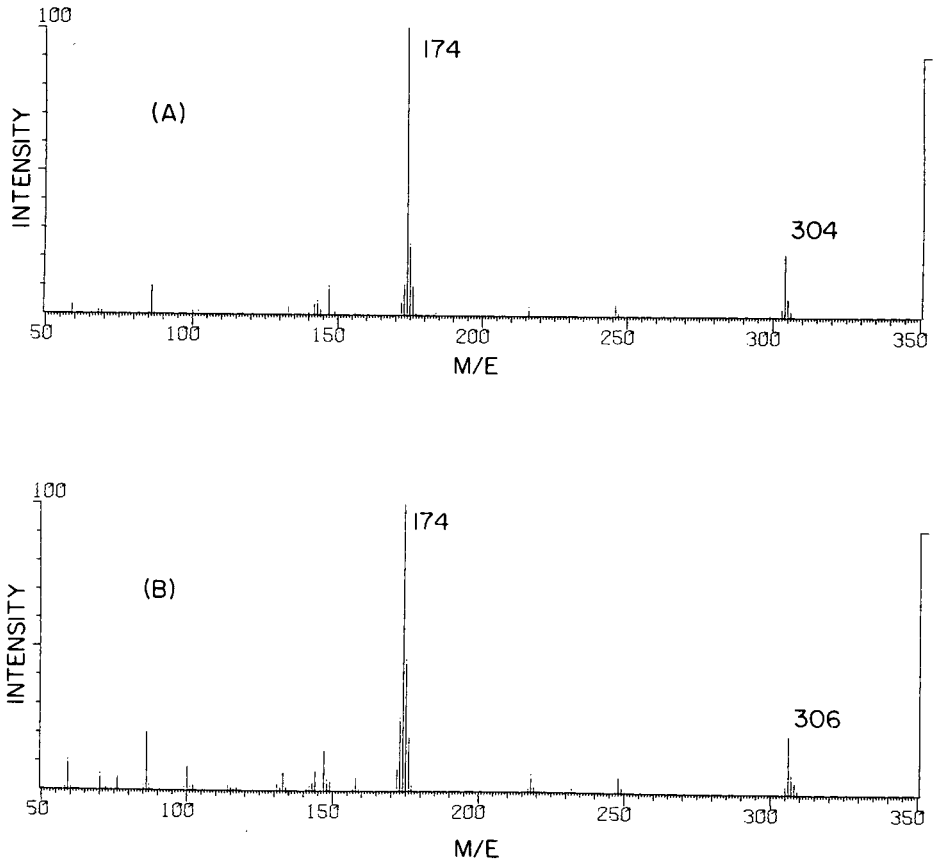

Fig. 1. Mass spectra of the trimethylsilyl derivatives of (A) GABA and (B) [$^2H_2$]GABA at 70 eV.

To test whether greater sensitivity could be achieved, spectra of pure standards of each compound were obtained using chemical ionization with methane as reagent gas. In both samples the base peak was *m/e* 174. The $(MH)^+$ ions of GABA (*m/e* 320) and [$^2H_2$]GABA (*m/e* 322) were both present at 7% relative abundance. At 70 eV the $(M - 15)^+$ relative abundancies of *m/e* 304.1 (GABA) and *m/e* 306.2 ([$^2H_2$]GABA) were 75% and 73%, respectively. It was found that the chemical ionization signal intensities of these two ions were 4.25 times less than the signal intensities at 70 eV by electron impact. Electron impact at 70 eV was therefore used in the GABA measurement.

The high sensitivity of the present method enables quantitative analysis of GABA down to 15 ng GABA per mg tissue with a 2:1 signal-to-noise ratio. The minimum detectable amount with a 2:1 signal-to-noise ratio of pure standards was 0.9 ng GABA (*m/e* 304.1) and 1.2 ng [$^2H_2$]GABA (*m/e* 306.2). The recovery of GABA for this GC—MS procedure was 95% ± 4.2% and the recovery of GABA for the fluorimetric procedure was 94% ± 6.3%.

A calibration curve was constructed in which known amounts of GABA (0—10 μg) were added to tubes containing a fixed amount of [$^2H_2$]GABA (2 μg) and the mixture carried through the incubation and derivatizing procedure. A least-squares fit (r = 0.997) of these data gave a linear relationship between peak area ratio and GABA concentration from which the following equation resulted:

$$\mu g\ GABA = 0.987 \frac{\text{Area GABA}}{\text{Area } [^2H_2]\text{GABA}} + 0.007\ \mu g\ GABA \quad (1)$$

Eqn. 1 was used to calculate the GABA concentration of the tissue samples.

The tissue punch placement is presented in Fig. 2. All tissue slices are 1 mm thick and the left and right hemispheric punches are combined for each nucleus. The first punch is the GP at AP 7.0 and the other locations are AP 6.0 (EP and VM) and AP 3.0 (SN_M and SN_L) based upon the atlas of Pellegrino and Cushman [17]. An average weight of representative tissue samples from combined left and right brain punches (n=10) is 2.51 ± 0.16 mg tissue (wet weight) with an average protein content of 0.105 mg protein per mg tissue.

After incubation and derivatization, the concentration of GABA was measured and the corresponding blank subtracted from each sample to obtain the net GABA formation. Overall 1—3 fold GABA increases were observed for samples in this procedure. By the fluorimetric method, 1—3 fold GABA increases over the blank GABA concentration were also noted.

In Fig. 3, a mass fragmentogram from one of the tissue samples is presented. The chromatographic retention time of 1.10 min of the endogenous compound is identical to that of its deuterated analogue. The average GAD activity values (± S.D.) found by this method in each brain region (n = 5) are 7.75 ± 0.93 (GP), 6.70 ± 1.02 (EP), 3.68 ± 0.70 (VM), 13.66 ± 1.06 (SN_M), and 8.12 ± 0.95 (SN_L). These values agree closely with the fluorimetric data obtained in this laboratory for GAD activity in these subregions of rat brain.

Previous studies have been made by radioisotopic methods to determine GAD activity in rat brain tissue. Nagy et al. [3] recorded activities of 9.07 μg GABA per hour per mg protein for middle GP and activities for whole dissected regions of 22.66 (GP), 12.57 (EP) and 31.93 (SN). Tappaz et al. [18] reported activities of 47.48 (GP), 23.28 (nucleus ventralis thalamus), 49.85 (SN-compacta), and 114.3 (SN-reticulata) while Walaas and Fonnum [1] reported dry weight activities of 38.72 μg GABA per hour per mg tissue (GP) and 20.01 (EP). However, it has been observed that the measurement of the evolution of $^{14}CO_2$ is not a true estimate of GAD activity in crude homogenates because of alternative routes of CO_2 production from glutamate [19—21]. Wilson et al. [20] measured the production of $^{14}CO_2$ and [^{14}C]GABA in cultured neural cells from [^{14}C]glutamate and reported GAD activities by [^{14}C]GABA forma-

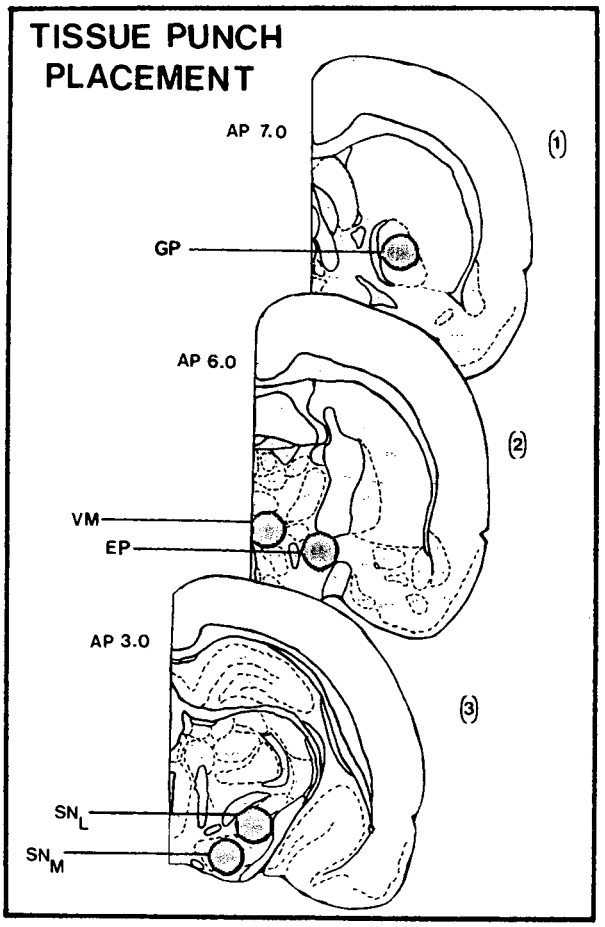

Fig. 2. Tissue punch placement. The first punch was from a 1 mm thick slice containing the globus pallidus (GP). The second tissue slice contained the entopeduncular nucleus (EP) and ventromedial thalamus (VM). The third slice contained the substantia nigra medial (SN_M) and lateral (SN_L) punches. The numbers on the left refer to the anterior—posterior (AP) axis coordinates in the brain atlas of Pellegrino and Cushman [17]. Punch diameter, 1.35 mm.

tion 10% less than those measured by $^{14}CO_2$ production. Drummond and Phillips [19] obtained similar results from non-neural tissue and indicated that the increased $^{14}CO_2$ formation from alternative pathways in crude homogenates may be due to the coupled reactions of glutamic acid dehydrogenase and α-ketoglutarate dehydrogenase. By measurement of a characteristic ion of GABA and comparison of its retention time to that of a deuterated internal standard, this assay is more specific than the measurement of $^{14}CO_2$ production from [^{14}C]glutamate in crude homogenates.

The highest GAD activity found in the brain is in the SN [1] and such was the case for the subregions tested in this study. The activities in the GP, EP, and SN are in agreement with the pattern of activities found by Nagy et al. [3] and Walaas and Fonnum [1].

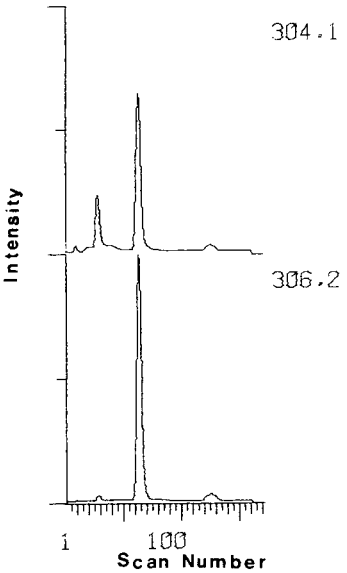

Fig. 3. Mass fragmentogram of *m/e* 304.1 of GABA and *m/e* 306.2 of [$^2H_2$]GABA from tissue sample.

A comparison of regional GAD activities was made with our data and the previously cited literature. Due to the different ways activity values are reported (i.e. per mg tissue, mg protein, dry weight, wet weight), ratios of activities between regions were used for comparison. Although a direct comparison of these ratios is not ideal, the ratios should be relatively consistent from method to method. The ratio of GP to EP obtained by this GC—MS method is 1.16, which is comparable with the results of Nagy et al. [3] (GP:EP = 1.80) and Walaas and Fonnum [1] (GP:EP = 1.93) while the GP to VM ratio of 2.11 obtained by this assay procedure is comparable with that of Tappaz et al. [18] (GP:VM = 2.03). Other possible discrepancies among these activity values could be due to extraneous $^{14}CO_2$ production from alternative pathways, variations in anterior—posterior axis locations of the brain regions, differences in protein content among regions, comparison of whole dissected regions as opposed to micropunches and differences in the strains of rats tested.

CONCLUSION

The GC—MS method described in this paper is useful for determination of GAD activity in subregions of rat brain. Similar activity values are obtained by the GC—MS method and by the fluorescence method. The regional GAD activity ratios of GP to EP and GP to VM obtained in this study are comparable with the ratios of values in the literature.

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CAPILLARY GAS CHROMATOGRAPHY OF PYRIMIDINES AND PURINES: N,O-PERALKYL AND TRIFLUOROACETYL-N,O-ALKYL DERIVATIVES

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SUMMARY

Preparation and capillary gas chromatographic properties of volatile derivatives of eighteen pyrimidine and purine nucleic acid bases are described. N,O-peralkylation using methylsulfinyl carbanion, methyl or ethyl iodide reagent, and alkylation preceded by N-trifluoroacetylation produced derivatives having minimal adsorption and tailing compared with trimethylsilyl derivatives. Relative retention times and linearity of flame ionization or nitrogen—phosphorus detector response were measured using polar (Superox-FA) and apolar (SE-30) liquid phases. Application of gas chromatography—mass spectrometry to derivatives of DNA hydrolysates using mass chromatography is demonstrated.

INTRODUCTION

The nucleic acid bases play major roles in the chemical and biological characteristics of nucleic acids. The detection and quantification of chemically or biologically modified bases are of importance in a number of areas, including studies of carcinogenesis and the structure and function of transfer RNA. Analytical approaches based on gas chromatography (GC) [1,2] are often appropriate due to favorable sensitivity, structural selectivity, and the complementary use of gas chromatography—mass spectrometry (GC—MS) [3,4].

The most common procedure for analysis of bases using GC requires conversion of the free base to the corresponding trimethylsilyl (TMS) derivative [2], and detailed studies aimed at defining optimum conditions for these reactions have been reported [5—7]. Procedures for methylation of bases using sodium methoxide—methyl iodide [8], diazomethane or on-column methods [9,10], methylsulfinyl carbanion—methyl iodide [11], or other techniques [12] have been reported, some of which were used for assays of 5-fluorouracil [10, 11,

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13]. Most GC analyses have been made using short packed glass columns, while limited use of glass capillary columns have been reported more recently [4,14—17].

Increased demands for improved detection limits and selectivity in analysis of nucleic acid hydrolysates prompted us to use glass capillary columns for chromatographing the TMS derivatives of a variety of bases. Initial attempts using commercially prepared, wall-coated and support-coated open tubular glass columns produced poor results because of high levels of sample adsorption by the column. Several column deactivation techniques reported in the literature [18—20] were used to prepare in our laboratory columns of exceptionally low activity. Although these columns were an improvement over those available commercially, they still exhibit substantial reactivity toward the derivatized bases. Commercially prepared fused silica columns have now been tested and show the least adsorption. These columns are, however, still unable to satisfactorily elute very small quantities of TMS derivatives of purine bases. Fig. 1 shows a chromatogram of the TMS derivatives of approximately 100 ng each of the common nucleic acid bases, separated on a 12-m fused silica column coated with SE-30. The quality of this chromatogram is high, based on our experience with this particular separation, yet it shows tailing and baseline humps which are typically observed at the base of peaks from cytosine, adenine and guanine derivatives.

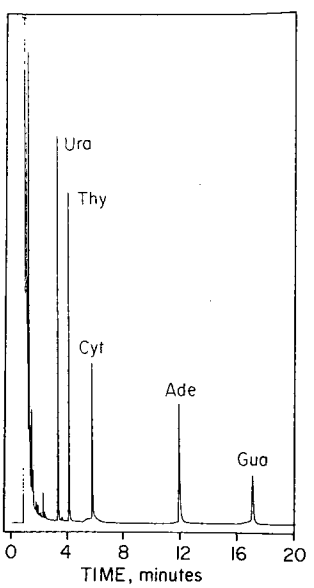


Fig. 1. Chromatogram of ca. 100 ng each of TMS derivatives of uracil (Ura), thymine (Thy), cytosine (Cyt), adenine (Ade), and guanine (Gua), using 12-m SE-30 fused silica column and flame ionization detection.

Another possible limitation of the use of TMS derivatives for analysis of DNA hydrolysates is inadequate resolution of cytosine from 5-methylcytosine [21], the principal modified base in DNA.

Since we were unable to find capillary columns (either commercial or home made; glass or fused silica) which satisfactorily elute small quantities of silylated bases, we have developed a procedure for preparation of N,O-peralkyl or trifluoroacetyl-alkyl derivatives. It will be shown that these derivatives exhibit excellent chromatographic properties on both polar and apolar capillary columns, and provide linear detector response over the range 0.2—20 ng per injection. Flame ionization, nitrogen—phosphorus and MS detection were used.

A study of the mass spectra of these derivatives is reported in a separate publication [22].

EXPERIMENTAL

Materials

Purine and pyrimidine bases were obtained from commercial sources: adenine, 5-fluorouracil, N^6,N^6-dimethyladenine, and uracil (Calbiochem-Behring, La Jolla, CA, U.S.A.); guanine, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 1-methyladenine, N^6-methyladenine, 5-methylcytosine, orotic acid, 2-thiouracil, and thymine (Sigma, St. Louis, MO, U.S.A.); cytosine, N^2,N^2-dimethylguanine, 5-fluorocytosine, N^2-methylguanine, and 7-methylguanine (Vega Biochemicals, Tucson, AZ, U.S.A.). Analytical grade dimethyl sulfoxide, cyclohexane, hydrochloric acid, formic acid, sodium hydride (50% oil suspension), ethyl acetate, and trifluoroacetic anhydride were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.); ethyl iodide, methylene chloride, diethyl ether, methanol, and sulfuric acid were obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.). Methylsulfinyl carbanion solution, 0.5 *M*, was prepared as reported previously [23]; the sodium hydride oil suspension was extracted several times with anhydrous diethyl ether just prior to use. The reagent was filtered and 0.4-ml portions were stored at −18°C.

N,O-Perethyl derivatives for group I compounds (1—12)

Up to 100 μg of purine or pyrimidine base, dissolved in 100 μl of dry dimethyl sulfoxide, were mixed with 20 μl of 0.5 *M* methylsulfinyl carbanion reagent; after 10 sec, 40 μl of ethyl iodide were added and the solution allowed to react for 1 h. Then, 3 ml of water were added and the reaction mixture extracted twice with 1.5 ml of cyclohexane—methylene chloride (9:1); the combined extracts were evaporated to dryness at 50°C under a stream of nitrogen.

N-Trifluoroacetyl-N,O-ethyl derivatives for group II compounds (13—18)

Stock solutions of these bases were made up in 0.06 *N* hydrochloric acid in methanol; aliquots containing up to 100 μg of product were dried in vacuo prior to derivatization. The residue was mixed with 100 μl of ethyl acetate and 70 μl of trifluoroacetic anhydride and heated for 3 min at 90°C. The reaction mixture was dried at 50°C under a nitrogen stream. The acylated base was subsequently ethylated, as described above. The reaction was terminated using 0.05 *N* sulfuric acid instead of water. The dry residues were in each case dissolved in ethyl acetate, and 20 ng or less of each derivative injected onto the capillary column.

Calf thymus DNA (Sigma) was hydrolyzed by heating with 88% formic acid for 2 h at 150°C in a sealed glass tube. Separate samples containing 10 μg of hydrolyzed DNA were dried and derivatized using both procedures described above. Aliquots representing 2% of the total hydrolysate were injected into the gas chromatograph, using the falling needle injector.

Gas—liquid chromatography

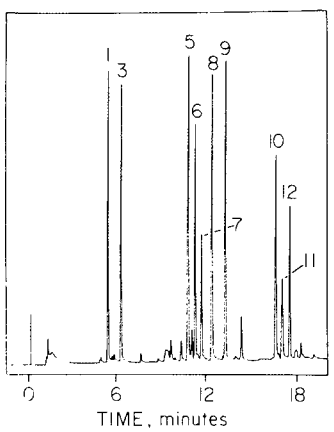
A Varian Model 3700 gas chromatograph, equipped for operation with capillary columns and with flame ionization and nitrogen—phosphorus-selective detectors was used for most of the chromatographic analyses. A fused silica column (12 m × 0.22 mm I.D.) coated with SE-30 was supplied by Hewlett-Packard (Avondale, PA, U.S.A.) and a whisker-type [24] glass column (10 m × 0.25 mm I.D.) coated with Superox-FA (*m*-nitroterephthalic acid-modified Superox-4), was obtained from Alltech Assoc. (Deerfield, IL, U.S.A.). Operating conditions were as follows: injector and detector temperatures 260 and 280°C, respectively; helium was used as a carrier gas at a linear velocity, $\vec{u}_0$ of 20 cm/sec. The thermionic detector was operated with air and hydrogen flow-rates of 175 and 4.5 ml/min, respectively; the bead current was set to obtain a background current of 10^{-10} A. Samples were injected through an inlet splitter (1:60 ratio) or with an all-glass falling needle injector (Alltech Assoc.). The fused silica columns were coupled to this device by means of a 1/4 to 1/16 in. reducing union, with the column end just below the resting point of the needle. A small glass insert (1.0 mm O.D., 0.5 mm I.D.) was used to center the column inside the injector.

Gas chromatography—mass spectrometry

Mass spectra were obtained with an LKB 9000S instrument interfaced to a DEC PDP-11/40 computer. The samples were introduced through the gas chromatographic inlet, fitted with a 1.8 m × 2.0 mm I.D. silanized glass column, packed with 1% OV-17 on 100—200 mesh Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.); helium was used as a carrier gas at a flow-rate of 30 ml/min. Injector, separator and ion source temperatures were 250, 270, and 290°C, respectively. Electron-impact mass spectra were recorded at an electron energy of 70 eV. For the analysis of DNA hydrolysates, an SE-30 coated fused silica column (12 m × 0.22 mm I.D.) was used. The separator was provided with make-up gas (helium, flow-rate 26 ml/min) [25] and samples were introduced with the falling needle injector. Data were acquired in the repetitive scanning mode (50—350 mass units) at a rate of 4 sec per scan.

RESULTS AND DISCUSSION

As a means of derivatization for GC, peralkylation provides a useful means of maximizing favorable chromatographic characteristics by complete replacement of active hydrogens. The use of a strong base such as methylsulfinyl carbanion assures hydrogen replacement for a wide structural variety of bases. This reagent has found use for a range of compound classes, including carbohydrates [26], peptides [27], and nucleosides [28]. When applied to bases in the present study, those listed in group I (see Fig. 2) formed satisfactory



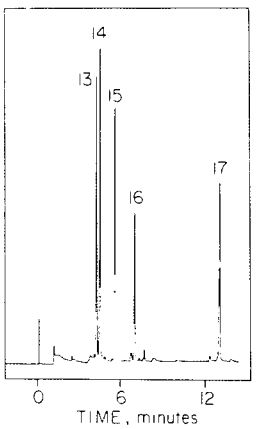


Fig. 2. N,O-Perethyl derivatives (group I) of uracil (1), thymine (3), 5-hydroxymethyluracil (5), N^6,N^6-dimethyladenine (6), orotic acid (7), N^6-methyladenine (8), adenine (9), N^2,N^2-dimethylguanine (10), 1-methyladenine (11), and N^2-methylguanine (12). Fused silica column, coated with SE-30; oven temperature 130°C, linearly programmed at 5°C/min, nitrogen-selective detection.

Fig. 3. N-Trifluoroacetyl-ethyl derivatives (group II) of 5-fluorocytosine (13), cytosine (14), 5-methylcytosine (15), 5-hydroxymethylcytosine (16), and guanine (17). Fused silica column, coated with SE-30; oven temperature 170°C, linearly programmed at 5°C/min, nitrogen-selective detection.

derivatives, while those in group II (see Fig. 3) required N-acylation prior to alkylation. Of the bases studied, adenine is unique among the members of group I because it cannot be derivatized using the group II procedure. The other members of group I remain unreacted during the acetylation step. It has been suggested that methylation of free amino groups may produce quaternized methyl iodide salts [23], a problem which is circumvented by acylating the amino group prior to alkylation. Although methylation can also be used, ethyl derivatives are preferred in order to distinguish bases which contain natural methyl groups. In addition, mass spectra of the ethyl derivatives [22] offer more detail for structural characterization and more variety for selection of masses for selected ion monitoring.

Typical chromatograms from group I and group II compounds obtained using a 12-m apolar fused silica capillary column are given in Figs. 2 and 3. It is evident that this procedure yields principally a single product for each component base and that these derivatives do not undergo significant reversible adsorption under these chromatographic conditions. This is in contrast to trimethylsilyl derivatives of the same compounds (cf. Fig. 1) which, except for uracil and thymine, show tailing and adsorption on the same column under similar conditions. The superior chromatographic properties of the peralkyl derivatives are probably because alkylation replaces both hydrogens on the exocyclic amino groups while silylation replaces only one under usual reaction conditions.

Diazomethane with the catalyst boron trifluoride etherate [29] was also examined as an alternative alkylation procedure to derivatize N^4-trifluoroacetyl-

cytosine. This method was rejected because it gave many structurally different products and because reagents such as diazoethane are not readily available.

Fig. 3 shows that the trifluoroacetyl-ethyl derivative of the modified base 5-methylcytosine elutes much later than cytosine. This is in contrast to the trimethylsilyl derivatives where 5-methylcytosine follows very closely after cytosine on the same apolar column. This feature should prove advantageous in the analysis of DNA hydrolysates where 5-methylcytosine may be present at levels below 1% relative to cytosine.

Since methylation of the base is the most common form of biological modification occurring in nucleic acids, the ethyl derivatives are generally more useful than their methyl analogues. The ability of the present analytical system to unambiguously deal with the problem may be illustrated with the series N^6,N^6-dimethyladenine (6), N^6-methyladenine (8), and adenine (9), in which ethyl groups are substituted for methyl groups on the exocyclic nitrogen. Fig. 2 shows that this relatively short, apolar capillary column easily separates these three substances.

Methylene unit (MU) values [30] of these derivatives were measured by co-chromatography with even-numbered *n*-alkanes on capillary columns coated with polar (Superox-FA) or apolar (SE-30) liquid phases. These data are given in Table I and represent the average of three replicate measurements, with a standard deviation of approximately 0.02 MU. In general, the SE-30 column elutes the alkylated bases between the temperatures of 130 and 220°C within

TABLE I

RELATIVE RETENTION TIME DATA FOR N,O-PERETHYL AND N-TRIFLUOROACETYL-ETHYL DERIVATIVES OF PYRIMIDINE AND PURINE BASES

Parent base	Mol. wt.	No. of C_2H_5 groups	No. of CF_3CO groups	MU values	
				SE-30	Superox-FA
Group I					
Uracil (1)	168	2	–	14.34	23.53
5-Fluorouracil (2)	186	2	–	14.40	24.00
Thymine (3)	182	2	–	14.75	23.22
2-Thiouracil (4)	184	2	–	14.80	22.92
5-Hydroxymethyluracil (5)	226	3	–	17.01	24.99
N^6,N^6-Dimethyladenine (6)	191	1	–	17.25	26.94
Orotic acid (7)	240	3	–	17.46	25.34
N^6-Methyladenine (8)	205	2	–	17.78	26.92
Adenine (9)	219	3	–	18.24	26.82
N^2,N^2-Dimethylguanine (10)	235	2	–	19.88	30.73
1-Methyladenine (11)	205	2	–	20.08	34.74
N^2-Methylguanine (12)	249	3	–	20.31	30.71
Group II					
5-Fluorocytosine (13)	281	2	1	16.97	27.33
Cytosine (14)	263	2	1	17.13	27.17
5-Methylcytosine (15)	277	2	1	17.94	28.29
5-Hydroxymethylcytosine (16)	321	3	1	18.96	28.81
Guanine (17)	331	3	1	22.33	35.72
7-Methylguanine (18)	317	2	1	22.30	37.75

20 min, while the Superox-FA column elutes the same compounds 8—13 MU higher. The change in MU values for all of these bases studied on these two liquid phases is very large and is useful in the identification of unknowns. For most analyses we prefer to use the SE-30 column because of the lower elution temperatures. As is evident from Table I, the reversal of elution order between the two columns may be useful for qualitative identification of bases from their relative retention times.

The number of functional groups in each case was determined from mass spectrometric data and is presented in Table I. These data also show that alkylation occurs only on nitrogen atoms except for those bases which contain free hydroxyl (5, 16) or carboxylic acid (7) groups. Acylation attaches one trifluoroacetyl group to the exocyclic nitrogen in the cytosine and guanine series of bases. Attempts to substitute a trifluoroacetyl group at the exocyclic nitrogen of adenine were unsuccessful. A description of structural and MS fragmentation properties of these derivatives will be published elsewhere [22].

Determination of the relative amounts of modified and unmodified forms of base is often an objective in the analysis of nucleic acid hydrolysates. The quantitative performance of the present derivatization procedure was tested using N^6-methyladenine and 5-methylcytosine where these compounds are present over the range from 1—10% of adenine and cytosine in DNA. Adenine and cytosine were present at 1 μg prior to derivatization. These experiments were performed using the SE-30 capillary column, the nitrogen—phosphorus detector, and the falling needle injector system. The GC oven temperature was isothermal at 170°C and the retention times approximately 7 min. Peak height ratios of N^6-methyladenine to adenine and of 5-methylcytosine to cytosine were plotted against the respective quantities of these substances present in the original samples. Linear calibration curves were obtained for both cases: $y = 0.004x + 0.001$, $r = 0.998$ and $y = 0.010x - 0.004$, $r = 0.998$, respectively, when plotted as peak height ratios vs. weight ratios. These results demonstrate satisfactory performance of the entire analytical procedure over the range studied. In particular, sample adsorption by the column is not significant even in the sub-nanogram range. We have been unable to satisfactorily elute trimethylsilyl derivatives of these bases at low levels using the same capillary column.

When using the falling needle injector, the entire sample less the solvent is placed on the column, a factor of potential importance for quantitative measurements [31]. This system is particularly useful where only a small amount of nucleic acid is available for analysis. The nitrogen—phosphorus detector typically has a much lower detection limit than the flame ionization detector because of its selective ionization of compounds containing nitrogen or phosphorus, and because of its inherently higher sensitivity, The detection limit for both N^6-methyladenine and 5-methylcytosine is about 10^{-10} g (signal-to-noise ratio > 10). Similar measurements show that the sensitivity of the nitrogen—phosphorus detector is about 30 times greater than that of the flame ionization detector. The trifluoroacetyl derivatives are potentially useful for high sensitivity measurements using an electron-capture detector or negative ion chemical ionization MS.

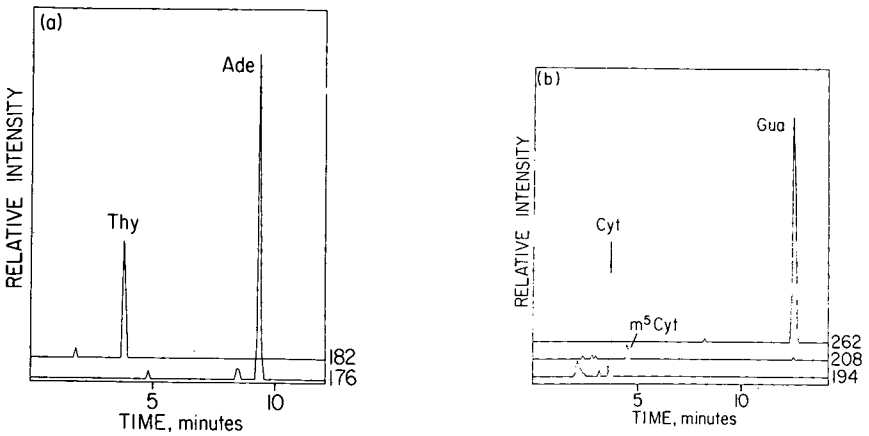


Fig. 4. Selected ion recordings (mass chromatograms) of a calf thymus DNA hydrolysate, analyzed on a fused silica column, coated with SE-30. Oven temperature 100°C, linearly programmed at 4°C/min. (a) N,O-Perethyl derivatives, *m/z* 182 thymine (3), *m/z* 176 adenine (9); (b) N-trifluoroacetyl-ethyl derivatives, *m/z* 262 guanine (17), *m/z* 208 5-methylcytosine (15), *m/z* 194 cytosine (14).

These derivatives are also potentially useful for analysis of nucleic acid hydrolysates by GC—MS. Fig. 4 shows a selected ion recording of a derivatized hydrolysate of calf thymus DNA, with data acquired in the repetitive scanning (mass chromatography) mode. The quantity represented corresponds to 200 ng of DNA, or approximately 80 ng of free bases. The ions chosen for display were selected from mass spectra of each derivative [22]. Although use of a calibration curve is preferable, a crude estimate of quantities can be calculated after correction for percentage of total ion current carried by each selected ion. In this fashion the 5-methylcytosine content is estimated as 11% of cytosine compared to literature reports of 5—9% [32], while guanine/cytosine and thymine/adenine ratios are approximately 0.80 and 0.97, respectively, suggesting a uniform and high yield in hydrolysis and derivatization. Further experiments based on internal calibration techniques and the more sensitive and accurate method of selected ion monitoring are in progress.

ACKNOWLEDGEMENTS

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SIMULTANEOUS EXTRACTION AND SEPARATION OF TRACE AMINES OF BIOLOGICAL INTEREST

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SUMMARY

A method is described for the simultaneous extraction and separation of the trace amines 2-phenylethylamine, *m*-tyramine, *p*-tyramine, *p*-octopamine, normetanephrine, and 3-methoxytyramine. The method involves acetylation in aqueous solution, specific hydrolysis of phenolic acetate groups, derivatization with trifluoroacetic anhydride and analysis on a gas chromatograph equipped with an electron-capture detector. Analyses utilizing both packed glass columns and glass capillary columns are described.

The method possesses the potential for quantitative as well as qualitative analysis, with one or more of the following amines employed as internal standards: benzylamine, 3-phenylpropylamine, tranylcypromine, and 2-(4-chlorophenyl)ethylamine.

INTRODUCTION

Neurochemical research in recent years has focused on a group of amines normally present in urine, but detectable in only trace quantities in the central nervous system (CNS). The functional role of these trace amines in the CNS has not been unequivocally determined. However, a number of these substances have been implicated in several nervous disorders. Migraine [1], epilepsy [2], Parkinsonism [3], schizophrenia [4], and depression [5] have been cited as neurological disorders in which the *para*-isomer of tyramine (*p*-TA) may be involved. A decrease in the urinary excretion of the acid metabolite of *para*-octopamine (*p*-OA) has been exhibited in depressed patients [5]. The CNS symptoms of hepatic failure (tremor, coma) may be explained in terms of *p*-OA accumulation in central adrenergic nerves [6]. A role for the trace amine 2-phenylethylamine (PEA) in the aetiology of depression, mania, and schizophrenia has been suggested [7—9].

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In addition, clinical interest in the involvement of catecholamines in psychiatric and neurological disease states has led to a research interest in the O-methylated metabolites of noradrenaline and dopamine, normetanephrine (NME) and 3-methoxytyramine (3-MT), respectively. The latter has been suggested as a reliable indicator of dopaminergic neuron activity in brain, as formation of this metabolite arises from the action of catechol-O-methyl transferase on dopamine released into the synaptic cleft [10, 11].

Methods of analysis developed for qualitative and quantitative determination of the trace amines in biological fluids and brain tissue include thin-layer chromatography [12], colorimetric determination [13], electrophoresis [14], high-performance liquid chromatography [15, 16], gas chromatography (GC) [17—19], spectrophotofluorometric methods [20—25], radioenzymatic procedures [26—29], combined GC—mass spectrometry [30—33], and integrated ion-current mass spectrometry [34].

Although the three latter procedures represent the most widely used methods for trace amine analysis at present, disadvantages are inherent in these techniques. Such disadvantages include complexity of methodology, the requirement for expensive instrumentation (mass spectrometric methods) and an inability to differentiate *m*-tyramine (*m*-TA) and *p*-TA (radioenzymatic procedures), which are both present in brain and body fluids [35, 36]. In addition, radioenzymatic techniques are not used presently for the simultaneous quantitative determination of a spectrum of trace amines in the picogram range.

A GC procedure reported in the literature [18] documents the separation of *p*-TA, octopamine (OA), 3-MT and NME with application to urine. This procedure, however, was not developed to provide sufficient sensitivity for analysis of these amines in brain tissue and no mention is made of the ability of the procedure to separate *p*-TA from the structurally related *meta*-isomer. In our laboratory, we have recently developed an analytical technique for the quantitation of *m*- and *p*-TA in urine [37] using GC with a packed column. We have now initiated efforts for simultaneous extraction and separation of additional trace amines, and the results of these efforts with packed and capillary columns are described in this paper.

EXPERIMENTAL

Chemicals and reagents

The following were used: trifluoroacetic anhydride (TFAA), benzylamine (BZA), *p*-tyramine (*p*-TA) hydrochloride, *p*-octopamine (*p*-OA) hydrochloride, cyclohexane (spectrophotometric grade) (Aldrich, Milwaukee, WI, U.S.A.); pentafluoroproprionic anhydride (PFPA) (Pierce, Rockford, IL, U.S.A.); tranylcypromine (TCP) hydrochloride, normetanephrine (NME) hydrochloride, 3-methoxytyramine (3-MT) hydrochloride, 2-phenylethylamine (PEA) hydrochloride (Sigma, St. Louis, MO, U.S.A.); *m*-tyramine (*m*-TA) hydrochloride (Vega Biochemicals, Tucson, AZ, U.S.A.); 3-phenylpropylamine (PPA) and 2-(4-chlorophenyl)ethylamine (CPEA) were obtained as the free bases (Aldrich), and the hydrochloride salts were synthesized in our laboratories; sodium bicarbonate (reagent grade) (Amachem, Portland, OR, U.S.A.); ethyl acetate

(glass distilled) (Caledon Laboratories, Georgetown, Canada); ammonium hydroxide (reagent grade) (J.T. Baker, Phillipsburg, NJ, U.S.A.); and acetic anhydride (analytical grade) (BDH Chemicals, Toronto, Canada).

Extraction and derivatization

All agitations and centrifugations of immiscible liquid systems, unless specified, were performed with an Evapo-Mix Shaker (Buchler Instruments) and a GLC-1 Centrifuge (Sorvall), respectively. Solutions each containing 500 ng to 2 μg of the four amines, *p*-TA, *p*-OA, NME, and 3-MT and 500 ng of the internal standard CPEA were prepared in 0.5 *M* hydrochloric acid (2.5 ml). Two amines, *m*-TA and PEA and three potential internal standards, BZA, TCP and PPA, were added for GC analysis utilizing a glass capillary column. Solid sodium bicarbonate was added to basify each solution and acetic anhydride (300 μl) was added with shaking. Additional amounts of sodium bicarbonate were added intermittently with vortexing until all effervescence had ceased [17]. The aqueous phase was extracted with ethyl acetate (4 ml for 3 min), followed by agitation of the isolated organic phase with ammonium hydroxide (10 *N*, 400 μl) for 40 min and subsequent neutralization with 8 *M* hydrochloric acid (300 μl). After the ethyl acetate layer was evaporated to dryness under nitrogen, ethyl acetate (25 μl) and TFAA (75 μl) were added to the residue. Derivatization was allowed to proceed for 30 min at room temperature. Cyclohexane (300 μl) and saturated aqueous sodium tetraborate (3.0 ml) were added to each tube followed by a 15-sec vortex. The organic layer was isolated after a brief centrifugation.

In addition to the above extraction and derivatization procedure, modifications to this procedure were undertaken for analysis of the amines on the SP 2100 GC capillary column. One modification involved omitting the hydrolysis step (agitation of the organic layer with ammonium hydroxide) and derivatizing the acetylated compounds directly with TFAA. For the second modification, PFPA was substituted for TFAA as the derivatizing agent with the hydrolysis step either included or omitted. For PFPA derivatization, ethyl acetate (25 μl) and PFPA (75 μl) were added to each residue, and reaction proceeded at 60°C for 30 min.

Gas—liquid chromatography

GC analyses were performed on a Hewlett-Packard Model 5835A gas chromatograph equipped with a 15 mCi ^{63}Ni source linear electron-capture detector. The following glass columns (1.8 m × 4 mm I.D.), packed with stationary phase in our laboratory, were used: 3% OV-1 on Gas-Chrom Q (100—120 mesh) and 3% OV-17 on Gas-Chrom Q (100—120 mesh) (Serva, Heidelberg, G.F.R.); 10% OV-1 on Chromosorb W HP (100—120 mesh) (Terochem Laboratories, Edmonton, Canada); 5% OV-7 on Chromosorb 750 (100—120 mesh) (Chromatographic Specialties, Brockville, Canada); 3% OV-3 on Chromosorb W HP (80—100 mesh) (Pierce). The carrier gas was argon—methane (90:10) maintained at a flow-rate of 30 or 40 ml/min. The column temperature ranged between 150 and 195°C. The temperature of both the injection port and detector was 250°C. For capillary analysis, a Grade AA WCOT SP 2100 glass capillary column (10 m) (Supelco, Bellefonte, PA, U.S.A.) was

used. Helium at 7 p.s.i. was used as carrier gas, with argon—methane (90:10) at a flow-rate of 35 ml/min used as make-up gas to the detector. The initial column temperature of 80°C was maintained for 0.6 min and increased to temperatures between 120 and 170°C at rates of 10–30°C/min. The temperature of both the injection port and detector remained at 250°C. Injection volumes of 1 μl and 2 μl were used for analysis on capillary and packed columns, respectively.

RESULTS AND DISCUSSION

Initial efforts in the development of a sensitive, specific GC technique for the simultaneous analysis of trace amines was directed towards resolution of *p*-TA, *p*-OA, NME and 3-MT on glass columns packed with various stationary phases. Two potential internal standards, TCP and/or CPEA, were initially included in the assay.

A lack of sensitivity and poor resolution characterized the results obtained from two of the stationary phases, namely 5% OV-7 and 10% OV-1. Good separability of the four amines and internal standards was achieved with the 3% OV-1 stationary phase. However, this column proved unsuitable as metanephrine, a 3-O-methylated metabolite of epinephrine, interfered with NME regardless of the GC operating conditions employed. The 3% OV-3 column proved to be a suitable column for the spearation of NME, 3-MT, and the two internal standards, CPEA and TCP, but *p*-TA could not be resolved from its metabolite, *p*-OA. The packed column which provided the best separation was the 3% OV-17 phase. The derivatives of *p*-OA, *p*-TA, 3-MT, NME, and the internal standard CPEA gave symmetrical peaks on GC analysis with retention times of 1.8, 2.4, 5.5, 9.1, and 3.6 min, respectively. The temperature program employed was as follows. An initial temperature of 155°C was maintained for

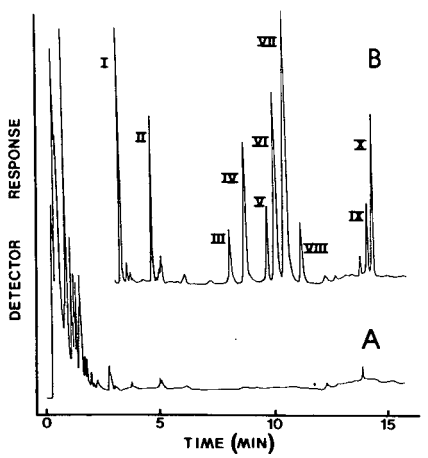


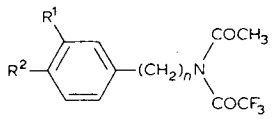
Fig. 1. GC traces of (A) hydrochloric acid blank; (B) standard prepared with 500 ng of each of the amines. Peaks are derivatives of: I, benzylamine; II, 2-phenylethylamine; III, 3-phenylpropylamine; IV, *m*-tyramine; V, tranylcypromine; VI, *p*-tyramine; VII, 2-(4-chlorophenyl)-ethylamine; VIII, *p*-octopamine; IX, normetanephrine; X, 3-methoxytyramine on an SP-2100 glass capillary column.

4.7 min followed by an increase of 5°C/min to a final temperature of 195°C. Although resolution of the amines was achieved, sensitivity of the GC assay required improvement. On both the 3% OV-3 and 3% OV-17 packed phases, the limit of sensitivity for all four amines was 170 pg on column. By extrapolating the amount to a volume of 4 ml of extracted rat brain supernatant, this corresponds to a whole brain concentration of ca. 25 ng/g, which exceeds whole brain concentrations reported for trace amines.

In an attempt to improve the sensitivity of the assay, an SP-2100 wall-coated open-tubular glass capillary column was substituted for the packed column system. Two amines, *m*-TA and PEA and three additional internal standards, BZA, PPA and TCP were included in the assay. Sharp, symmetrical peaks resulted for all amines and internal standards using the following temperature program: an initial temperature of 80°C was maintained for 0.6 min followed by a temperature increase of 30°C/min to 120°C. At a run time of 11.6 min, the column temperature was increased at 160°C at a similar rate. Retention times of the derivatized amines PEA, *m*-TA, *p*-TA, *p*-OA, NME, and 3-MT were 4.9, 8.8, 10.1, 11.2, 13.7, and 14.1 min, respectively (Fig. 1). The four internal standards BZA, PPA, TCP and CPEA chromatographed at 3.6, 7.8, 9.6, and 10.5 min, respectively. Compounds found not to interfere with the assay were *ortho*-tyramine, *meta*-octopamine, metanephrine, synephrine, phenylethanolamine, noradrenaline, dopamine, tryptamine, and 5-hydroxytryptamine. Sensitivity was substantially increased for all amines, as 3-pg amounts could now be detected on column. This on-column value corresponds to less than 1 ng/g of amine in rat whole brain. Calibration curves were also constructed by adding varying amounts (1—1000 ng) of each of the amines to 0.5 *N* hydrochloric acid, carrying the samples through the procedure described above, and comparing the peak heights to those of the internal standards. A linear relationship (r^2, coefficient of determination, $\geqslant$ 0.9903) was found between peak height ratios (amine to internal standard) and the varying quantities of the amines.

The structures of the acetylated and trifluoroacetylated derivatives of the internal standards and *p*-TA have been described elsewhere [37—39]. Derivatized PEA, *m*-TA, *p*-OA, NME and 3-MT have now been studied using combined GC—mass spectrometry with both electron-impact (EI) and chemical ionization (CI) sources [40]. The structures are shown in Fig. 2.

The amphoteric character of phenolic amines predisposes these substances to incomplete extraction from aqueous media. Acetylation in aqueous solution resulted in alleviation of this problem, with formation of lipophilic compounds possessing N-acetylated and phenolic O-acetylated functions [37, 38]. Alcoholic hydroxyl groups were not acetylated. Extraction of the derivative into ethyl acetate was quantitative (> 95% recovery). The subsequent hydrolysis step freed the phenolic OH moieties for perfluoroacylation with TFAA (see Fig. 2). The N-acetyl group was not hydrolyzed under these conditions, so the resultant compound was still favourably soluble in ethyl acetate; in addition, there was still a free hydrogen on the nitrogen, and in the case of some substances, a side-chain alcoholic hydroxyl hydrogen atom, which could be replaced by TFAA (Fig. 2). Reaction of the N-acetylated, phenolic O-acetylated derivatives directly with TFAA (omitting the hydrolysis step)



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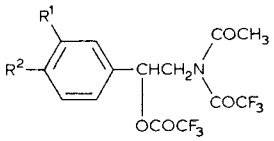

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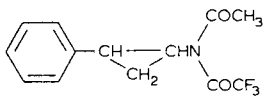

III

Product	R^1	R^2	n	Derivative of:
I a	H	H	1	Benzylamine
I b	H	H	2	2-Phenylethylamine
I c	H	H	3	3-Phenylpropylamine
I d	H	Cl	2	2-(4-Chlorophenyl)ethylamine
I e	CF_3COO	H	2	*m*-Tyramine
I f	H	CF_3COO	2	*p*-Tyramine
I g	CH_3O	CF_3COO	2	3-Methoxytyramine
II a	H	CF_3COO	—	*p*-Octopamine
II b	CH_3O	CF_3COO	—	Normetanephrine
III	—	—	—	Tranylcypromine

Fig. 2. Proposed structures for the derivatives of some amines of biological interest as formed by acetylation with acetic anhydride, hydrolysis with ammonium hydroxide, and perfluoroacylation with trifluoroacetic anhydride.

proved to be unsatisfactory as the resultant compounds (N-acetyl, N-TFAA, phenolic O-acetyl; and alcoholic O-TFAA in derivatized *p*-octopamine and normetanephrine) had extended retention times and decreased sensitivity as compared to the derivatives formed in the assay including the hydrolysis step. Furthermore, the derivative formed from tryptamine (another amine present in urine and brain) had the same retention time as the derivative for *p*-TA. Derivatization of the amines with PFPA did not improve the situation, and this procedure was subsequently abandoned as a derivatization method.

Although one of the packed GC columns (3% OV-17) appeared to successfully separate some of the specific amines, an increased sensitivity was required. A new GC technique with the glass capillary column, SP-2100, has been described and shown to provide not only specificity for analysis of the amines but also an on-column sensitivity which approaches that of the radioenzymatic or mass spectrometric procedures. The major advantage of this GC method for analysis of trace amines is the simultaneous extraction and separation achieved. We have previously applied the basic procedure to the quantitation of *p*-TA in rat whole brain after extracting this amine from brain homogenate by using liquid ion exchange and back extraction with 0.5 *N* hydrochloric acid, and a preliminary report of this procedure for *p*-TA has been published [38]. With the modifications and refinements made since that time and noted in the present report, separation of six amines and four potential internal standards in a single injection is now possible. Utilization of this new procedure for simultaneous analysis of PEA, *m*-TA, *p*-TA, *p*-OA, NME and 3-MT in biological samples is presently being investigated.

ACKNOWLEDGEMENTS

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MODIFICATION OF THE SIMULTANEOUS DETERMINATION OF ALDITOL ACETATES OF NEUTRAL AND AMINOSUGARS BY GAS—LIQUID CHROMATOGRAPHY

APPLICATION TO THE FRACTIONATION OF SIALOGLYCOPROTEINS FROM BONE

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SUMMARY

A modification of a gas—liquid chromatographic method is described that allows better simultaneous separations of the neutral and aminosugar alditol acetate derivatives as single peaks. Using 3% SP-2340 on 100—200 mesh Supelcoport, retention times were relatively short and baseline separation between glucose and galactose was achieved. The method is particularly suitable for monitoring the fractionation of complex mixtures of glycoproteins and glycosaminoglycans, and its application is illustrated in the fractionation of bone matrix extracts subjected to ion-exchange chromatography. A convenient procedure allowing the separation and estimation of sialic acid in the same aliquot is also described and evaluated.

INTRODUCTION

The advantages and limitations of methods used for cleavage of the glycoconjugates of glycoproteins and the estimation of the various volatile carbohydrate derivatives by gas—liquid chromatography (GLC) have been extensively reviewed [1—4]. A number of the currently described GLC methods yield multiple peaks for each component making quantitation complex. A procedure resulting in single peaks for each monosaccharide [3] avoids this problem by employing a resin-catalyzed hydrolysis of neutral sugars and aminosugars, followed by nitrous acid deamination of the resin bound hexosamine to neutral 2,5-anhydrohexoses and subsequent chromatography of all the corresponding neutral alditol acetates. However, in this procedure, separation between the

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alditol acetates of glucose and galactose is not complete and this often presents a problem during purification procedures of glycoproteins due to exogenous sources of glucose-containing contaminants. A method giving single aldonitrile derivatives has also been reported [5], but retention times are relatively long and these derivatives have appreciable water solubility.

In this communication we report on an improved GLC method, with relatively short retention times and better separations of the alditol acetates of the constituent monosaccharides, including a baseline separation between glucose and galactose.

The application of this method is illustrated by the analysis of isolated glycoproteins and glycosaminoglycans and has proved particularly useful in monitoring the fractionation of glycoprotein-rich extracts from bone by ion-exchange chromatography. GLC methodology had not been previously applied to glycoproteins from bone [6—8] and it would appear that this method is also suited to the analysis of small samples of bone, such as are available from bone biopsy and reconstructive surgery for arthritic conditions. The procedure has also been modified so that an initial separation and estimation of the bound sialic acid is conveniently performed in the same aliquot prior to analysis of the remaining monosaccharides. This method could therefore be applied to the study of both glycoproteins and proteoglycans in pathological processes of bone or in the growth and development of bony tissue.

MATERIALS AND METHODS

Reagents

Acetic acid, acetic anhydride, methanol, hydrochloric acid, and sodium borohydrate were certified Fisher grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Pyridine, solution grade, was obtained from Pierce (Rockford, IL, U.S.A.). The resins AG 50W-X8 (H^+), 200—400 mesh, and AG 1-X2 (Cl^-) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). AG 1-X2 (HCO_3^-) was prepared by passing a 2 mol/l $NaHCO_3$ solution through a column of AG 1-X2 (Cl^-) and washing thoroughly with deionized water. DEAE-Sephadex, A-25 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), converted to the acetate form using 0.5 mol/l sodium acetate and equilibrated with 0.05 mol/l Tris acetate buffer, pH 8.0.

Biological materials

Chondroitin sulfate A was a standard obtained from Drs. Mathews and Cifonelli, University of Chicago (see also Table II). Fetuin was obtained from Sigma (St. Louis, MO, U.S.A.) (Type IV, lot No. F3004). Solubilized bone matrix (SBM) enriched in glycoproteins was obtained from rat bone by a method previously described [9], Briefly, decalcified bone matrix was extracted with 4 mol/l guanidinium chloride and the extract dialyzed against water. The retentate was then extracted with isotonic (0.154 mol/l) sodium chloride, which was again dialyzed against water. The retentate from this step was soluble in salt solutions of relatively low ionic strength and was termed SBM.

Hydrolysis of neutral and aminosugars of the glycoconjugates

The solubilized glycoproteins were hydrolyzed by means of a resin-catalyzed hydrolysis procedure [3,10,11]. Either weight aliquots of bone matrix, or the dried content of each combined fraction was dissolved in 1 ml of 0.02 mol/l hydrochloric acid and transferred into a 16 × 125 mm glass culture tube (Kimax) containing 1 ml of the AG 50W-X8 (H^+) resin in 0.02 mol/l hydrochloric acid. The tubes were sealed with PTFE-lined screw caps and the contents hydrolyzed at 100°C for 24 h.

Deamination and isolation of carbohydrates

The samples were removed from the hydrolysis oven, allowed to cool, and centrifuged to remove condensate from septa and walls of the hydrolysis tubes. The septa were carefully removed and 50 μl of an internal standard solution containing 50 μg myoinositol were added, followed by the addition of 10 μl of a freshly prepared 5.5 mol/l solution of $NaNO_2$. The tubes were sealed again and they were subjected to intermittent vortexing at room temperature for 60 min for completion of the deamination. Following deamination, 500 μl of 40% w/v suspension of AG 50W-X8 (H^+) [3] were added to the deaminated mixture to remove excess Na^+ ions and convert $NaNO_2$ to HNO_2. After shaking the tubes for approximately 30 min, contents were transferred to a set of two separate columns, the upper column containing AG 50W-X8 (H^+) [3] and draining into a lower one packed with AG 1-X2 (HCO_3^-) resin. The columns were prepared from disposable pipettes (Oxford Macroset) plugged with glass wool, and packed to a height of 4 cm with the resin. Hydrolysis tubes were then rinsed five times with 5 ml distilled water and the washings were transferred to the columns. The eluates were collected in 30-ml tubes and evaporated to a small volume in a rotary evaporator at 30°C. The concentrated solutions were then brought to dryness in a centrifugal bio-dryer.

Reduction and derivatization

The contents of each tube were dissolved in 100 μl of water and 100 μl of 0.22 mol/l $NaBH_4$ were added to reduce the free sugars, and the reaction was allowed to proceed to completion in 1 h at room temperature. Following reduction, excess $NaBH_4$ was decomposed with glacial acetic acid. Borate was removed as volatile trimethyl borate by the addition of four 200-μl portions of methanol—hydrochloric acid (1000:1) and concentrated to dryness in the bio-dryer after each addition. The samples were acetylated for 30 min at 100°C with 100 μl of acetic anhydride and 100 μl of pyridine. They were then cooled, and 2—5 μl aliquots were injected into the gas chromatograph.

Preparation of standards

A series of standard solutions containing 5—100 μg of each of the neutral and aminosugars and 50 μg of the internal standard was prepared. A calibration curve of the ratio of areas vs. the ratio of weights was plotted for each sugar.

The results were calculated using the following relationship:

$$\text{Percentage sugar} = \left[\frac{\text{peak area of sugar derivative}}{\text{peak area of internal standard}} - IC\right] \left[\frac{\text{internal standard weight}}{S}\right] \times \frac{100}{\text{sample weight}}$$

where S is the slope and IC is the intercept of the calibration curve.

Sialic acid hydrolysis and preliminary fractionation

Sialic acid was released from the solubilized sialoglycoproteins under mild acid conditions using 5 ml of 0.1 mol/l sulfuric acid for 1 h at 90°C in 10 × 100 mm screw cap tubes. One of two procedures was then followed. When direct analysis was carried out, 0.2—0.5 ml aliquots from 1 ml of the glycoprotein-containing fraction were subjected to the mild acid hydrolysis procedure and portions of the hydrolysate were analyzed colorimetrically for sialic acid [12]. When a preliminary separation of sialic acid and other anionic components (glycosaminoglycans) was carried out, the entire sample, or a portion thereof, that had been subjected to mild acid hydrolysis, was passed through a 0.7 × 6 cm Dowex 1-X8 column, and after washing the columns with 8 ml of water to recover the desialylated glycoproteins, the bound sialic acid was eluted with 8 ml of 1 mol/l acetic acid—sodium acetate buffer, pH 4.6, as previously described [13].

Apparatus

A Varian Model 2800 gas chromatograph equipped with dual flame ionization detectors, temperature programmer and coiled glass columns (158.4 cm × 4 mm I.D.) was employed. Two different column packing materials were used. Column packing No. 1 was composed of 0.75% HIEFF - IBP, 0.25% EGSS - X, and 0.1% 144-B on 60—80 mesh Gas-Chrom Q, as had been previously suggested [3]. Column packing No. 2 consisted of 3% SP-2340 on 100—120 mesh Supelcoport. Chromatography was conducted with temperature programming beginning at 150°C with a program rate of 2°C/min to a final temperature of 220°C. The injection port temperature was 220°C and the detector temperature was 310°C. The carrier gas was nitrogen at a flow-rate of 40 ml/min. Hydrogen and air flow-rates to each detector were 30 ml/min and 300 ml/min, respectively. Peak areas were measured with a Gould 110 Recorder equipped with an electronic chart integrator.

Fractionation of solubilized bone matrix enriched in glycoproteins

For the separation of solubilized glycoproteins a column of DEAE-Sephadex A-25, 20 cm × 2 cm, prepared as described above, was used. A sample containing 10—50 mg of solubilized rat bone matrix was dissolved in 3—5 ml of the Tris acetate buffer and was carefully layered on top of the column bed. After the sample had drained into the bed, the gel surface and the column wall were washed with 5 ml of the buffer solution. The column was eluted first isocratically with 100 ml of Tris acetate buffer and then switched to gradient

elution. The following gradients were used sequentially: 0—0.13 mol/l, 0.13—0.8 mol/l and 0.8—2 mol/l sodium chloride, all made up in Tris acetate buffer, pH 8.0. Total volume of each gradient was automatically controlled by means of a solenoid valve operated through a thermistor sensing circuit. At the end of the gradient elution the column was washed with 100 ml of 2 mol/l sodium chloride in the same buffer solution.

Fractions containing 5 ml each were collected on an LKB fraction collector. Single column fractions were pooled into another set of combined fractions representing a volume eluted under an individual peak or band with a maximal absorbance at 280 nm (see Fig. 2). Carbohydrate-containing material as reducing sugar equivalent [14] was also determined in the eluted fractions. These fractions were then dialyzed against double-distilled deionized water until free of chlorine. The dialysants were then concentrated to a smaller volume in a desiccator under vacuum and lyophilized. The entire amount or a portion of each combined fraction was used for GLC analysis for the constituent neutral and aminosugars as described above, and spectrophotometric analysis for sialic acid [12].

General methods

UV absorption of eluted fractions from DEAE-Sephadex was determined routinely at 230 and 280 nm using a Shimadzu Spectronic 200 UV spectrophotometer. Hydroxyproline was determined in hydrolysates (6 mol/l hydrochloric acid for 24 h at 100°C) of dialyzed and lyophilized fractions with a modification of a colorimetric method [15] or by amino acid analysis.

RESULTS

Chromatographic resolution of alditol acetates

The retention times of the alditol acetate derivatives of neutral and aminosugars are shown in Table I. The resolution of a mixture containing neutral and aminosugars on two different columns is shown in Fig. 1A and B. Preliminary work was carried out on column No. 3 [3]. However, a column packed with SP 2340, packing No. 2, was found to be superior. On this column, base-

TABLE I

RETENTION TIMES FOR ALDITOL ACETATE DERIVATIVES OF NEUTRAL AND AMINOSUGARS

Sugar	Retention time (min)
L-Fucose	12.5
D-Arabinose	17.9
D-Glucosamine	22.3
D-Xylose	23.3
D-Galactosamine	28.7
D-Mannose	30.3
D-Galactose	32.6
D-Glucose	35.7
Myoinositol	39.2

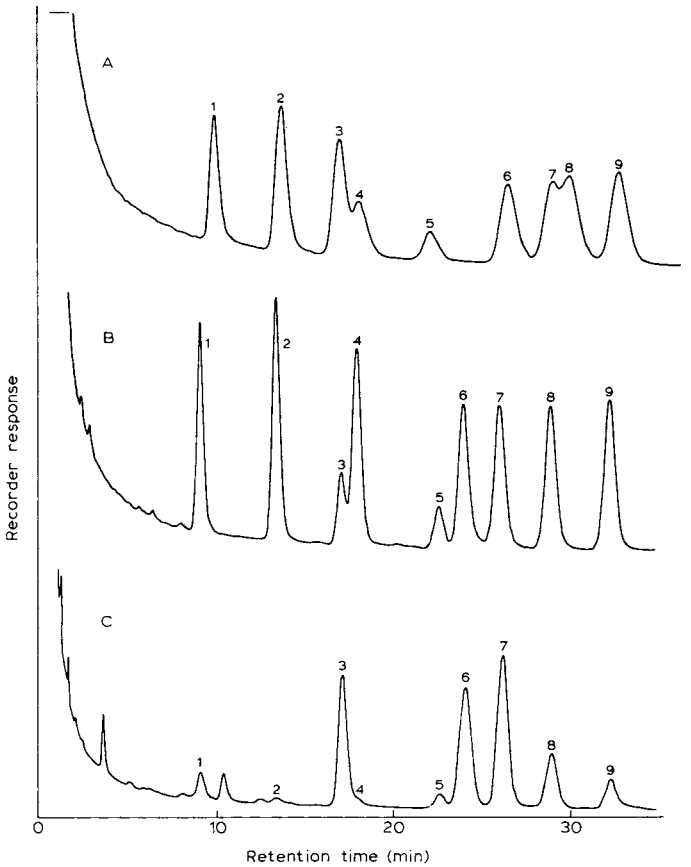

Fig. 1. Separation of sugar alditol acetate derivatives on two different GLC columns. (A) Column packed with 0.75% HIEFF-IBP, 0.25% EGSS-X and 0.1% 144B on 60—80 mesh Gas-Chrom Q. The alditol acetate derivatives of the neutral sugars and aminosugars shown above are numbered as follows: (1) fucose, (2) arabinose, (3) xylose, (4) glucosamine, (5) galactosamine, (6) mannose, (7) galactose, (8) glucose, (9) inositol. Peaks 4 and 5 probably represent the respective 2,5-anhydrohexositol acetate derivatives. (B) Column packed with SP-2340 on 100—200 mesh Supelcoport. The alditol acetate peaks are numbered as follows: (1) fucose, (2) arabinose, (3) glucosamine, (4) xylose, (5) galactosamine, (6) mannose, (7) galactose, (8) glucose, (9) inositol. (C) Gas chromatogram of solubilized bone matrix, utilizing the second column packing described above (Fig. 1B) and in the text. The chromatogram was obtained from solubilized bone matrix fractionated on DEAE-Sephadex, using a steep linear sodium chloride gradient, pH 7.0. Under these conditions most of the glycoprotein coelutes with the soluble collagen at the beginning of the gradient and the chromatogram shown is from this fraction. Note the baseline separation between glucose and galactose.

line separation of glucose and galactose was achieved. Also, adequate separation of xylitol and 2,5-anhydromannitol peaks formed from xylose and glucosamine was obtained. Fig. 1C illustrates that satisfactory baseline separations of the sugars are obtained also from fractions containing complex mixtures of glycoproteins and collagen from bone matrix.

Separation of sialic acid and its simultaneous estimation

A procedure for separation of sialic acid that permits its estimation as well as the estimation of neutral and aminosugars in the same sample was evaluated in the following manner (Table II). Weighed samples of fetuin were subjected to mild acid hydrolysis and the hydrolyzed samples were applied to a Dowex 1-X8 column which retained the sialic acid, as described in Materials and Methods. The remaining asialoglycoprotein was recovered in the water eluate from the Dowex 1-X8 column and was subjected to resin hydrolysis, deamination, reduction and GLC analysis. The sialic acid was then eluted with acetic acid—sodium acetate buffer and estimated colorimetrically, as described in Materials and Methods. Recovery of the sialic acid from the Dowex 1-X8 column was between 92—98% in terms of the sialic acid released after mild acid hydrolysis from fetuin or the bone matrix fractions. Recoveries from the Dowex 1-X8 column for the neutral and aminosugars were quantitative and agree well with published values for these constituents obtained by colorimetric methods after strong acid hydrolysis of fetuin.

TABLE II

FRACTIONATION OF CHONDROITIN SULFATE (CS), FETUIN AND SOLUBILIZED BONE MATRIX (SBM) ON DOWEX 1-X8 BEFORE AND AFTER MILD ACID HYDROLYSIS

Chondroitin sulfate A (Drs. M.B. Mathews and J.A. Cifonelli, University of Chicago) had a standard hexosamine content of 24.94% by the Elson Morgan colorimetric method [19]. Fetuin was a Sigma product (see Materials and Methods) with a sialic acid content of 6.2%. Previous estimates of the content of fetuin monosaccharides by colorimetric methods [2] were, mannose, 3.0%, glucosamine, 4.9%, galactosamine, 0.6%, galactose, 4.6%.

Sugar in eluate	Sugar content of water eluate (percentage of dry weight of fetuin, CS, and SBM)					
	Material applied to Dowex 1-X8					
	Fetuin* (2 mg)	Fetuin** (2 mg)	CS* (1 mg)	CS** (1 mg)	SBM* (5 mg)	SBM** (5 mg)
L-Fucose	—	—	—	—	(t)§	(t)
Xylose	—	—	(c)***	—	—	—
Mannose	3.3	3.1	—	—	1.1	1.1
Glucosamine	6.1	5.8	(c)	—	2.8	2.8
Galactosamine	0.4	0.3	26.6	—	1.8	—
Galactose	4.9	4.7	(c)	—	1.6	1.6

*Samples analyzed directly, without prior desialylation.

**Samples passed through a column of Dowex 1-X8 (acetate form) after desialylation procedure. Sialic acid recoveries from the Dowex 1-X8 column were between 92 and 98% (see text for details).

***(c) Constituent carbohydrate was present in low concentrations and was not quantitated.

§ (t) Constituent carbohydrate was present in trace amounts.

The behaviour of chondroitin sulfate A (chondroitin 4-sulfate), the major glycosaminoglycan in bone, on the Dowex 1-X8 column was also evaluated. This compound is entirely retained by the resin. Direct analysis gave values for galactosamine that were comparable to those obtained by a colorimetric

method following strong acid hydrolysis for the glycosaminoglycan standard (Table II). When whole solubilized bone matrix was applied to the Dowex 1-X8 column, all of the galactosamine-containing material was retained, but recovery of the other neutral sugars and glucosamine was complete in the water eluate from the column (Table II). This suggests that the preliminary separation on Dowex 1-X8 of glycosaminoglycan and free sialic acid from the desialylated glycoproteins can be used reliably, in conjunction with the modified GLC method for the analysis of complex mixtures of glycoconjugates, utilizing the same sample.

Application of the modified GLC method to the fractionation of glycoproteins from bone

Fractionation of solubilized bone matrix on DEAE-Sephadex, utilizing a three-step linear salt gradient resulted in a significant separation of glycoproteins from collagenous proteins (Fig. 2). All of the hydroxyproline-containing material eluted in fraction 1 (F1) while the carbohydrate-containing material eluted largely in fraction 3 (F3) (see also Table III) that had been processed without previous desialylation or passage through the Dowex 1-X8 column. In these fractions, the sialic acid was estimated directly after mild acid hydrolysis in an aliquot of each fraction. Fraction 3 contained significant amounts of galactosamine. Compounds containing this aminosugar could be removed (after desialylation) by a passage of fraction 3 through a Dowex 1-X8 column, as described above for the unfractionated bone matrix, without a significant change in the estimates of the other sugars recovered in the water eluate from the column (data not shown). Retained high molecular weight material on Dowex 1-X8 could be eluted with strong acid and was shown to contain chondroitin sulfate by cellulose acetate electrophoresis [16,17].

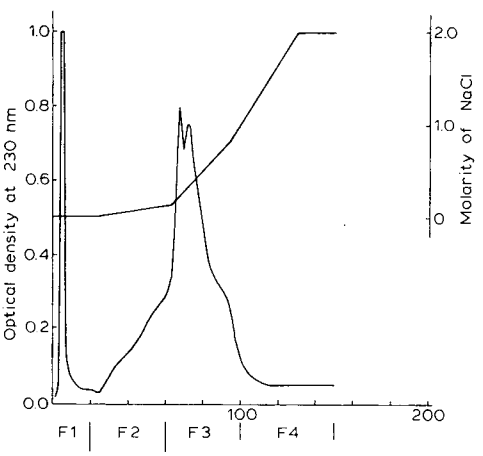

Fig. 2. Separation of solubilized bone matrix on DEAE-Sephadex, using a three-step linear sodium chloride gradient, in Tris acetate buffer, 0.05 mol/l, pH 8.0. The left-hand vertical axis shows the elution pattern as monitored by UV absorbance at 230 nm and the right-hand vertical axis indicates the molarity of the eluting sodium chloride gradient. Aminosugar, neutral sugar, and sialic acid contents are given in Table III.

TABLE III

SUGAR CONTENT IN FRACTIONATED SOLUBILIZED BONE MATRIX

A sample of solubilized bone matrix (30 mg) was fractionated on DEAE-Sephadex column and the fractions were processed and analyzed directly for neutral sugars and aminosugars, as described in Materials and Methods. For sialic acid analysis, 0.2-ml aliquots out of 1-ml final volume for each fraction were subjected to mild acid hydrolysis and the sialic acid was estimated [12] directly.

Components identified	Sugar fractions (μg)				Total sugar (% dry weight)
	F1	F2	F3	F4	
Mannose	1.7	13.5	280.0	1.7	0.98
Glucosamine	—	22.2	840.0	—	2.80
Galactosamine	—	—	455.0	58.0	1.80
Galactose	1.7	—	425.3	6.8	1.60
Sialic acid	—	—	810.0	—	2.7

The reproducibility of this method was also examined, using five replicate (from the same batch) 5-mg samples of solubilized bone matrix material. Values obtained were within a range of 5% or better. Reproducibility and recoveries of the method with known standards of neutral and aminosugars is 100 ± 3%.

DISCUSSION

A practical problem in the alditol acetate method that yields single derivatives for each monosaccharide [3] was that the separation of certain pairs of sugars (the derivatives of glucose and galactose and the derivatives of xylose and glucosamine) was incomplete. A method giving single aldonitrile acetate derivatives has also been reported [5], but this procedure does not resolve the problem of partial separations between xylose and glucosamine, and furthermore, retention times are comparatively long (e.g. 54 min for glucosamine and 70 and 75 min for glucose and galactose, respectively), which limits the application of this method for multiple sample analysis. Aldonitrile acetates of aminosugars also have appreciable water solubility, thus making derivative clean-up procedures difficult and may exhibit erratic chromatographic properties [18]. In the modification of the alditol acetate method [3] that we describe, utilizing column packing No. 2 (see Materials and Methods), separation between glucose and galactose is complete, the separation between xylose and glucosamine is improved, and the retention times are comparatively short (Table I).

The baseline separation of the glucose and galactose alditol acetate derivatives is critical during fractionation procedures of glycoconjugates extracted from tissues, since exogenous sources such as dialysis bags and Sephadex columns were shown to make variable contributions to detectable glucose (data not shown). In addition, deamination of mannosamine also yields glucose [3,19].

The preliminary separation of glycosaminoglycan (or proteoglycan) and the hydrolyzed sialic acid on the Dowex 1-X8 column adds considerable flexibility to fractionation and allows quantitative estimates of the neutral and amino-sugars of the asialoglycoproteins and the sialic acid to be carried out in the same aliquot.

ACKNOWLEDGEMENT

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CHROMBIO. 974

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHOLIPIDS WITH UV DETECTION: OPTIMIZATION OF SEPARATIONS ON SILICA

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SUMMARY

Chromatography of phospholipids was performed on silica columns with detection by absorbance at 205 nm using mixtures of hexane—isopropanol—water in which the role of water and isopropanol in elution was investigated. One system was developed which provided adequate separation of most major phospholipid species. However, lipids with several ionizable groups were not well separated and gave multiple broad peaks. A second system was developed utilizing sulfuric acid for ion suppression. The behavior of phospholipids in this system was found to be dependent on the presence of quaternary ammonium, amino, or hydroxyl groups. Except for plasmalogen, phospholipids were recovered intact. This system was optimized to provide baseline resolution of essentially all phospholipid species commonly found in mammalian tissues.

INTRODUCTION

Development of systems for high-performance liquid chromatography (HPLC) of phospholipids has been rather slow considering the wealth of data available on thin-layer chromatography (TLC) of these compounds. However, almost all TLC solvent systems for phospholipids are incompatible with the most commonly used detection system of ultraviolet (UV) absorbance because of the presence of chloroform or acetic acid. Therefore, direct adaptation of TLC systems to HPLC of phospholipids is somewhat difficult. Chloroform based solvent systems have been used with a moving wire detector for chromatography of phospholipids [1, 2]. Phospholipids from actively metabolizing cells have been analyzed in a chloroform-based system by counting incorporated ^{32}P in the eluent [3]. Photometric detection of amino-con-

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taining phospholipids has been accomplished after derivatization of these groups [4].

Phospholipids do not have a specific absorbance, but may be detected by monitoring the absorbance of double bonds in their fatty acid moieties. Two systems have been developed using solvents that are transparent near 200 nm. Jungawala et al. [5] used an acetonitrile—methanol—water system on a silica column to achieve a rapid and complete separation of phosphatidylcholine and sphingomyelin. However, other phospholipids were not resolved. Several classes of phospholipids were separated on silica by Hax and Geurts van Kessel [6] using gradient elution with hexane—isopropanol—water.

This last system, combining non-destructive UV detection with the separation of several lipid classes appeared to be the most promising for our needs. We therefore sought to optimize this system and expand its usefulness to minor species of phospholipids. We report here our investigations on the effects of solvent composition and especially pH on phospholipid separation and describe a system which appears capable of giving baseline resolution of most phospholipid species.

EXPERIMENTAL

The chromatograph used was a Varian Model 5020 equipped with three solvent inlet valves, a temperature-controlled column-mounting block, and an air-actuated Valco loop injector using the 50-μl loop. A variable-wavelength (Vari-Chrom) detector was operated at 205 nm with an 8-nm bandpass. Columns were 30 cm × 4 mm I.D. packed with 5 μm silica. Packed columns were obtained from Varian (Palo Alto, CA, U.S.A.) (Micro-Pak Si-5) or from Alltech (Arlington Heights, IL, U.S.A.) (Spherisorb S-5W). A 4 cm × 4 mm I.D. guard column packed with Vydac adsorbent was used throughout. All separations were performed at 28°C.

Solvents were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Sulfuric acid (Ultrex grade) was from J.T. Baker (Phillipsburg, NJ, U.S.A.). Cholesterol (Chol), phosphatidic acid (PhA), phosphatidylglycerol (PhG), diphosphatidylglycerol (DPhG) and polyphosphoinositides (DPI, TPI) were from Sigma (St. Louis, MO, U.S.A.). Bovine liver phosphatidylinositol (PhI) and plasmalogen were from Applied Science Labs. (State College, PA, U.S.A.). Other lipids phosphatidylserine (PhS), phosphatidylethanolamine (PhE), lysophosphatidylethanolamine (ly-PhE), phosphatidylcholine (PhC), sphingomyelin (SM), and cerebrosides (Cer.) were from PL Biochemicals (Milwaukee, WI, U.S.A.).

Tissue lipids were extracted by homogenizing approximately 1 g fresh blotted tissue with 3 ml methanol followed by the addition of 3 ml of chloroform. After centrifugation the pellet was re-extracted with 4 ml chloroform—methanol—hydrochloric acid (100:100:1). The combined extracts were adjusted to a chloroform—methanol ratio of 2:1 and partitioned with 0.2 volumes of 0.1 *N* hydrochloric acid. The lower phase was collected and the upper phase re-extracted with chloroform—methanol (85:15). The combined lower phases were run over a silica Sep-Pak (Waters Assoc., Milford, MA, U.S.A.) to remove non-lipid contaminants. This was washed with 5 ml methanol. Lipid extracts

were evaporated with dry nitrogen and re-dissolved in methylene chloride—methanol (2:1). Myelin from rat cerebra was collected from a five-step Ficoll gradient [7].

Solvent mixtures for HPLC were prepared by stirring in a closed container for several minutes with a PTFE stirring bar, and then degassed by application of a vacuum. Corrected retention time $t' = t_x - t_0$, where t_0 is the elution time for an unretained compound. Capacity factor $k' = t'/t_0$. Resolution $R(A/B) = t'_A - t'_B / \frac{1}{2}(w_A + w_B)$ where w is the peak width at baseline.

Lipids were identified by use of standards and by collection of peaks followed by TLC. Peaks were collected in 0.1—0.2 ml pyridine, evaporated with nitrogen, and partitioned with 2 ml methylene chloride—methanol (2:1) and 0.4 ml 50 m*M* hydrochloric acid. TLC was performed on 0.25-mm silica gel HL plates from Analtech (Newark, DE, U.S.A.) with chloroform—methanol—ammonia (65:25:5) or butanol—acetic acid—water (6:1:1). Phospholipids were detected with the spray reagent described by Vaskovsky et al. [8].

RESULTS AND DISCUSSION

In our initial work we experimented with the system described by Hax and Geurts van Kessel [6]. We found, as described by these authors, that water was the important solvent component in eluting phospholipids. Attempts to achieve elution by increasing isopropanol and decreasing water failed. We also found that decreasing the gradient rate did not improve resolution because peak broadening surpassed the increase in retention times. In order to sharpen peaks with a slower gradient we tried adding ammonium chloride as a counter ion. This was somewhat successful in particular for PhS and PhI. Retention times were only slightly affected, while peak broadening was reduced. In the remaining experiments on this system, 0.1 *M* NH_4Cl was substituted for water. The ammonium ion could also be provided by ammonium sulfate, avoiding the corrosive effects of chloride ion.

Effect of hexane—isopropanol ratio in a neutral system

In Table I, we present the results of experiments in which the hexane—isopropanol ratio was varied in solvents with a fixed aqueous content. For all the lipids tested increasing solvent isopropanol content did not accelerate elution as would be predicted for a simple adsorption mechanism. Rather, at a given solvent aqueous content, phospholipids eluted more rapidly and as sharper peaks as the isopropanol was decreased. This effect was least marked for PhG and PhI in which a phosphodiester is the only ionizable group, and greatest for the choline-containing phospholipids. The retention of PhS and DPI, both with two ionizable groups in addition to the phosphodiester, increased to a greater extent with increasing isopropanol than that of PhE with only one group. The data suggest that isopropanol may act by enhancing the interaction of ionized groups with the silica surface.

These findings were used to produce the chromatogram shown in Fig. 1. A small amount of tetrahydrofuran was added to the B solvent to offset a decreasing baseline. The aqueous component increased from 3 to 10% while

TABLE I

VARIATION OF CAPACITY FACTOR k' WITH HEXANE—ISOPROPANOL RATIO IN A NEUTRAL SYSTEM

(A) Column, Micro-Pak Si-5, flow-rate, 1.2 ml/min; solvent, 3.5% 0.1 M NH_4Cl, hexane as indicated, isopropanol to 100%. (B) Conditions as above except 7% 0.1 M NH_4Cl.

	Hexane (%)	k'_{PhG}	k'_{PhE}	k'_{PhI}	k'_{PhS}
A	30	4.8	7.1	8.4	30
	42	4.8	5.9	8.7	19
	48.25	4.6	5.4	8.2	15.7
	54	4.0	4.3	7.3	11.2

	Hexane (%)	k'_{PhC}	k'_{SM}	k'_{DPI}
B	38.4	11.7, 12.6	14.6, 17.7	16.8
	43.2	7.3, 8.0	9.0, 11.4	15.0
	46	3.6, 4.0	4.4, 5.6	6.8
	48	2.3 (single)	3.0, 3.4	4.1

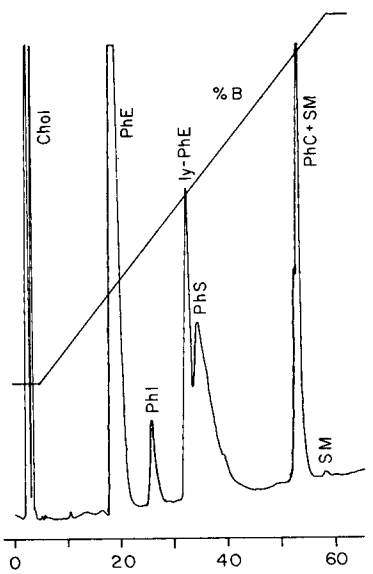


Fig. 1. HPLC of lipid extract from rat brain — neutral system. Column, Micro-Pak Si-5; solvents: A, hexane—isopropanol (50:50); B, hexane—isopropanol—tetrahydrofuran—0.1 M NH_4Cl (40:45:5:10); flow-rate, 1.0 ml/min, 30% B for 0 to 5 min increasing to 100% B at 60 min.

hexane and isopropanol remain relatively constant. This system is similar to that reported by Hax and Geurts van Kessel [6] except for the higher solvent hexane content and the use of ammonium chloride. The order of elution of compounds is also similar to that reported for LiChrosorb SI-60 [6] except for PhA.

By use of standards or by collection of the eluent followed by TLC, we

found PhA to elute as several peaks in a broad region between 35 and 45 min. Similar behavior was found for DPhG and for the polyphosphoinositides. This, coupled with the fact that these are minor components of tissue phospholipids, makes these compounds virtually undetectable by UV absorbance in chromatograms such as Fig. 1. This system might be improved somewhat by further increasing the solvent hexane content while decreasing water and isopropanol.

Use of sulfuric acid for ion suppression

While a neutral system provided adequate resolution of the major phospholipid species, it proved only marginal for PhS and unusable for other species with several ionizable groups. The broad multiple peaks obtained for these compounds were judged to arise from the presence of multiple ionized forms, since carboxyl and secondary phosphate ionizations have pK's in the same region as the solvent pH. We therefore tried to achieve suppression of these ionizations. Sulfuric acid was chosen for its UV transparency, acid strength, and lack of interference with assays for phosphate on collected lipids.

With the addition of small amounts of sulfuric acid, the elution of neutral or acidic phospholipids is greatly enhanced. Phospholipids may be divided into three types according to their behavior. First are choline-containing phospholipids which retain the behavior noted in Table I. Water is essential for elution of these species and isopropanol retards them. Second are the phospholipids containing only phosphate and hydroxyl groups which behave in a normal adsorption mode: isopropanol accelerates elution and water in small quantities, affects peak shape more than retention time. Phosphate apparently does not participate greatly in adsorption, and elution order is roughly according to the number of hydroxyl groups. Geometry of these hydroxyl groups also plays a role, as TPI with two hydroxyls fixed axial and equatorial on the inositol ring elutes before PhG. The third group with intermediate properties consists of amino-containing phospholipids. The amino group appears more effective in adsorption than the hydroxyl; this is also seen in the greater retention of cerebroside over monogalactosyldiglyceride. For these lipids, both water and isopropanol, acting in opposite fashion, affect retention.

Effect of solvent water content on elution and resolution

Because of the effect of water on retention of amino-containing phospholipids, resolution and even elution order depend upon the ratio of isopropanol to water. This is illustrated in Table II which shows the effects of a series of different solvents used to establish our final system. With a high ratio of isopropanol to water PhI elutes between PhS and PhE. As water is reduced, the separation of PhI from PhS is improved while that between PhI and PhE is impaired. This occurs primarily because of the greater effect of water on PhE and PhS relative to PhI. The effect of sulfuric acid concentration is seen with the two solvents containing 2% water. While more sulfuric acid is required with solvents of higher water content to achieve ion suppression, once this level is achieved additional sulfuric acid may sharpen peaks but has little effect on retention time.

TABLE II

DEVELOPMENT OF GRADIENT SYSTEM

Sample, lipid extract from rat liver; column, Micro-Pak Si-5; solvent A, hexane—isopropanol—sulfuric acid (97:3:0.02), flow-rate, 1.5 ml/min, 2% B 0 to 5 min increasing linearly to 50% B at 50 min.

Solvent B hexane—isopropanol—water—sulfuric acid	Corrected retention time (min)			Resolution	
	PhS	PhI	PhE	PhI/PhS	PhE/PhI
48:48:4:0.5	38.0	39.1	45.0	0.50	2.13
49:48:3:0.5	37.0	38.6	44.6	0.87	2.73
49:49:2:0.4	33.8	36.1	40.2	1.52	2.34
49:49:2:0.2	33.4	35.6	40.0	1.16	1.57

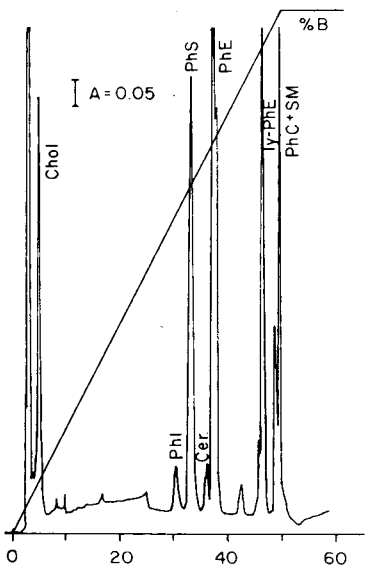


Fig. 2. HPLC of lipid extract from rat brain — acid system with high water. Column, Micro-Pak Si-5; solvents, hexane—isopropanol—water—sulfuric acid, A (95:5:0:0.02), B (60:37.5:2.5:0.1); flow-rate, 1.2 ml/min, 0 to 100% B at 50 min. Note elution of PhI before PhS.

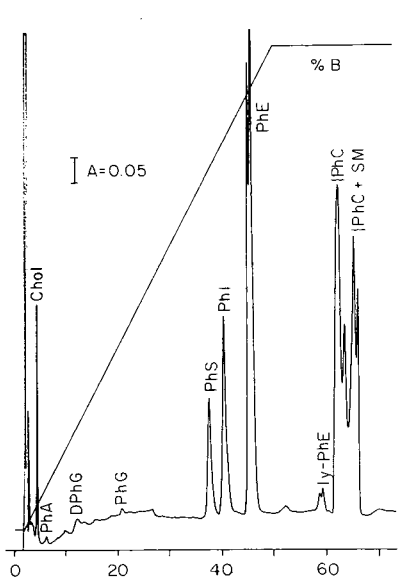
At even higher ratios of water to isopropanol PhS will actually elute after PhI. This is shown in Fig. 2 which depicts separation of rat brain lipids using a system where the amount of water is near saturation for this isopropanol content. This results in several solvent artifacts in the first half of the chromatogram. This sytem was not found to be stable, and the resolution between PhI and PhS degraded within a few weeks on irregular silica columns and could not be achieved at all on Spherisorb. In addition, the exact conditions for this separation varied widely from column to column, resulting in a large expenditure of time for optimization.

In contrast, a system with low water content gives essentially the same separations on various silica columns including Spherisorb. This system may

be rapidly optimized for a new column using the principles discussed above for a particular application. The A solvent, 3% isopropanol in hexane with 0.02% sulfuric acid was chosen for consistent resolution of PhA from the solvent front. The peak shape of PhA may be improved by addition of B solvent. Then a fresh lipid sample may be chromatographed using a slow gradient with a series of B solvents containing varying amounts of water and sulfuric acid in approximately 1:1 hexane—isopropanol as described in Table II.

Behavior of phospholipid species

Applications of this system to lipids from several tissues are shown in Figs. 3—5. The resolution of PhS, PhI, and PhE is best seen in a lipid sample from a tissue such as liver which is deficient in glycolipids (Fig. 3). In whole brain (Fig. 4) and especially in myelin (Fig. 5) cerebrosides and sulfatides (Cer.) overlap the retention times of PhI and PhE. Most of the SM is found in the last peak of these chromatograms but a portion elutes as a broader pre-peak along with PhC. Complete separation of these compounds may be achieved by stopping the gradient with 90% B at 45 min. This however increases the analysis time by about 10 min.



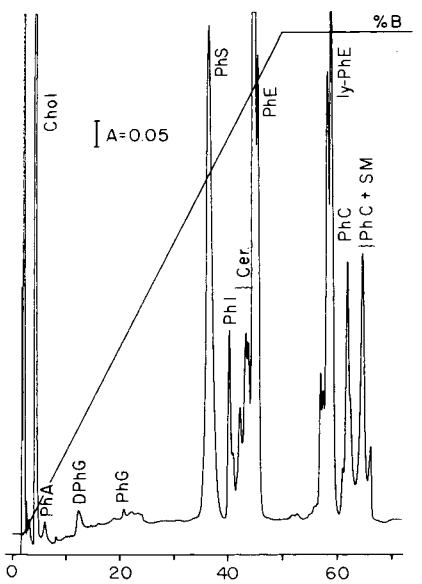


Fig. 3. HPLC of lipid extract from rat liver — acid system with low water. Column, Micro-Pak Si-5; solvents, hexane—isopropanol—water—sulfuric acid, A (97:3:0:0.02), B (75:24:0.9:0.1); flow-rate, 1.5 ml/min, 4% B for 2 min then increasing linearly to 100% B at 50 min.

Fig. 4. HPLC of lipid extract from rat brain — acid system with low water. Conditions as in Fig. 3.

The multiple peaks seen for PhE and PhC are probably related to fatty acid species, since their relative abundances are consistently related to the tissue of origin. A different phenomenon is seen for DPhG. Part of this lipid is not retained by the column and elutes with the solvent. This portion, which may

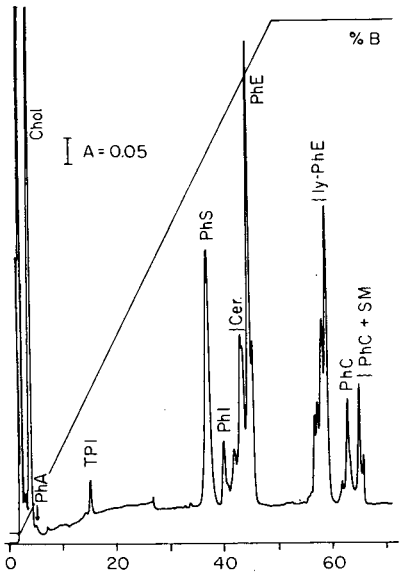


Fig. 5. HPLC of lipid extract from isolated rat cerebral myelin. Conditions as in Fig. 3.

be most of this species, increases with the total load of lipid and the volume and polarity of the sample solvent. This problem is most pronounced when DPhG is present as a free acid.

Stability of phospholipids

The large amounts of ly-PhE seen in samples from brain (Figs. 4 and 5) are also seen in heart but not liver (Fig. 3). This tissue specificity [9], as well as the high UV absorbance indicate that this is 1-lyso PhE derived from plasmalogen. While some plasmalogen is degraded by our extraction procedure (see Fig. 1), most of the degradation of the acid sensitive vinyl ether bond occurs on the column. This degradation may be complete, since commercially prepared plasmalogen, which gave no detectable ly-PhE on TLC, ran as 80—90% ly-PhE on the HPLC system. Except for plasmalogen all other phospholipids are recovered intact. Peaks collected in a small amount of pyridine may be re-chromatographed by HPLC or TLC.

Column regeneration

The HPLC system now in use (Figs. 3—5) appears stable after two months. However, several precautions are taken for column condition. The two most important are the use of a guard column which is changed regularly and a specific regeneration scheme used between runs. Pure isopropanol is connected to the third solvent inlet and a 30-min program is run. This consists of 20 column volumes of 50% isopropanol and solvent A, changing to pure A over 20 volumes, and a final 20 volumes with the starting solvent composition. This scheme is also used at the start of the day after 20 volumes of B solvent. The column is conditioned overnight by 100 ml of isopropanol and 100 ml of hexane. Eventually column performance does degrade for phospholipids and probably other ionic compounds, but not for the aromatic com-

TABLE III

EFFECT OF COLUMN CONDITION ON CAPACITY AND RESOLUTION

Sample and conditions as in Fig. 3.

Column	Corrected retention time (min)			Resolution PhI/PhS
	PhE	PhS	PhI	
New, 2 months	44.4	36.1	39.0	2.06
Old, 6 months	41.4	33.2	36.5	0.96
Reconditioned	41.8	33.1	36.5	1.32

pounds found in text mixtures. Resolution between PhI and PhS is lost first due mainly to peak broadening. Column performance may be partially restored by a reconditioning process. The column in isopropanol is purged by pumping 100 ml each of 0.1% sulfuric acid in water, isopropanol, methylene chloride, and hexane. Results of this operation are shown in Table III. While the column is not restored to its original condition, complete resolution of PhS and PhI is regained. This is mainly due to a sharpening of these peaks rather than a change in retention times.

Low-wavelength UV absorbance offers a sensitive means for detection of most phospholipids. The chromatograms shown in Figs. 3—5 were run at low detector sensitivity and represent lipids from 5 to 10 mg wet weight of tissue. However, the absorbance recorded arises primarily from double bonds in fatty acid moieties [5], and molar extinction coefficients will vary with fatty acid composition which may change with tissue type and metabolic state. Further, since most unsaturated fatty acids are found at the 2-position of glycerol, 2-lysophospholipids may be virtually transparent at 205 nm. Direct quantitation might be more reliable with a moving wire detector [1, 2] or by post-column reactions of phosphate [10]. If quantitation of phospholipids is routinely performed by phosphate assays on isolated lipids HPLC offers an easier and more complete method for collection than scraping TLC plates.

Only plasmalogen appears to be adversely affected by HPLC in acid solvents. This might be an advantage in certain circumstances since the degradation appears to be complete. For other lipids collection from the HPLC eluent avoids the oxidation of fatty acids which may occur on TLC plates with drying. This method may prove excellent for the preparative isolation of individual phospholipids. In our hands HPLC provides better and more consistent resolution of minor phospholipid species than two-dimensional TLC. These may be isolated by HPLC more easily than by TLC since the former will tolerate a much greater sample load without loss of resolution.

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NOTE ADDED IN PROOF

The lack of retention of DPhG (most notable in Fig. 3) may be avoided simply by dissolving the phospholipid sample in the starting solvent. With this procedure, DPhG is eluted before PhA.

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DEMONSTRATION OF THE FORMATION OF RENIN AND RENIN-BINDING PROTEIN COMPLEX USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatograph equipped with the newly developed gel, TSK G3000SW, was used to study the interaction between renin and renin-binding protein (RBP). Previously, the interaction could only be demonstrated after overnight gel chromatography in the presence of a non-physiological sulfhydryl reagent. However, this new high-speed gel chromatography provided a clear separation of renin and renin—RBP complex within 40 min. It also demonstrated that the renin—RBP complex was formed at 37°C in the absence of sulfhydryl reagent. These results indicate that the binding protein may play an important role in blood pressure regulation.

INTRODUCTION

Since the report of Boyd in 1974 [1] that a high molecular weight renin in hog kidney extract was a combination of renin and a carrier protein in a complex which is reversible under some conditions, and because of similar findings by Leckie and McConnell [2] for rabbit kidney, it has been hypothesized that kidneys contain renin-binding protein (RBP) which can be separated from and then recombined with renin. More recently, higher molecular weight forms of renin have been described in blood samples taken from patients suffering from Wilms' tumor of the kidney [3] or from stroke-prone spontaneously hypertensive rats in a malignant phase [4]. Since these observations suggest that the plasma level of higher molecular weight forms of renin would be a useful diagnostic aid, RBPs in the kidney and in the circulation have been extensively studied.

However, concerning the molecular weight of this renin-binding protein and the mechanism of the association, there have been large discrepancies among

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the results reported from different laboratories: Boyd [1] and Yamamoto et al. [5] have suggested molecular weights of over 50,000, while Leckie and McConnell reported a value of only 13,000 for their RBP preparation; Boyd [1] and Leckie and McConnell [2] have shown that warming a mixture of renin and RBP solution to 37°C led to the complex formation, while Kawamura et al. [6], using dog kidney, have reported that warming did not stimulate the slow association they observed at a low temperature.

On the other hand, Funakawa et al. [7] and Murakami et al. [8] have found that modification of sulfhydryl group(s) in RBP or renin seemed necessary to produce rapid association. Differences in kidney sources used by the above-mentioned investigators might account for some of these discrepancies, but, at least concerning the effect of temperature, we rather suspected that the low temperatures to which samples were exposed overnight during the analysis by gel filtration might affect the equilibrium between renin and renin—RBP complex.

To minimize the analysis time we employed in the present study high-performance liquid chromatography (HPLC) with a recently developed rigid and porous gel which provided rapid separation of proteins at high flow-rates [9]. This procedure gave good resolution and high reproducibility in separating the protein-bound forms of renin and demonstrated that the interaction between renin and RBP was in fact temperature-dependent and that at 37°C it proceeded within minutes.

EXPERIMENTAL

Preparation of hog kidney extracts

The hog kidneys obtained from a slaughterhouse were stripped of the capsule and bisected. The cortices were separated from the medulla and homogenized in 4 volumes of ice-cold 0.01 *M* sodium pyrophosphate buffer (pH 6.5) containing 0.1 *M* NaCl. The homogenate (20 ml) was centrifuged at 100,000 *g* for 1 h using a Beckman preparative ultracentrifuge L5 and the resulting supernatant was used as hog kidney extract.

Detection of renin—RBP complex

High-performance liquid chromatographic (HPLC) gel filtration was used to detect renin—RBP complex on the basis of molecular weight difference between renin and the renin—RBP complex. Analysis was conducted on a Toyo Soda Model HLC-803 instrument fitted with two coupled G3000SW columns (60 × 0.75 cm) using 0.01 *M* sodium pyrophosphate (pH 6.5) containing 0.1 *M* sodium chloride and 0.02% sodium azide as solvent. The G3000SW columns are packed with a rigid, hydrophilic, porous, spherical silica gel with a particle size of 10 ± 2 μm. The system was run at 100 kg/cm^2, 1 ml/min flow-rate, at room temperature. The column effluent was monitored with an UV detector at 280 nm. Samples (50 μl) containing 0.1 m*M* bovine serum albumin (BSA, M_r = 68,000) and 0.1 m*M* egg albumin (EA, M_r = 45,000) as internal molecular weight standards were injected through a loop injector. Fractions (0.25 ml) were collected every 15 sec for 45 min and aliquots were then assayed for renin activity.

Preparation of samples for HPLC

The effect of temperature on the interaction between renin and RBP was examined using hog kidney extracts incubated for fixed periods at 37°C.

When studying the effect of sulfhydryl reagents and sodium chloride on the interaction, hog kidney extracts were treated with 10 m*M* sodium tetrathionate, 10 m*M* dithiothreitol or 4 *M* sodium chloride at 37°C for 45 min. The precipitate formed during the incubation was removed by centrifugation before injecting on to the HPLC column.

To examine the pH-dependency of the renin—RBP complex formation, 2 g of hog kidney cortices were homogenized in 8 ml of 0.5 *M* Na_2HPO_4—citric acid buffer (pH 3—8) of constant ionic strength and centrifuged as described above; the supernatant was used as hog kidney extract.

RESULTS

Effect of temperature on the formation of renin—RBP complex

In an effort to solve the controversial problem whether incubation at 37°C stimulates the binding of RBP to renin or inactivates RBP, we incubated hog kidney extracts at 37°C for fixed periods of time and subjected them to the high-speed gel chromatography. As shown in Fig. 1, a single run on the column was completed in 40 min with a clear separation of the various forms of renin. This result indicated that the procedure could provide a better analysis of the reversible interaction between renin and RBP than conventional gel filtration which is considered not suitable for the analysis of systems in temperature-dependent equilibrium, since it usually requires overnight elution and therefore is carried out at low temperatures to preserve the activity of these biologically active materials. Fig. 1A and B illustrate the stimulatory effect of heat on the renin—RBP complex formation. Incubation of the extract for 1 min at 37°C produced an appreciable amount of higher molecular weight forms of renin (Fig. 1A). After 45 min incubation, most of the renin originally present in the extract was converted to higher molecular weight forms of renin (Fig. 1B). Their molecular weights were 70,000* ± 3,000 and 52,000 ± 2,000 (Fig. 1B), which correspond, respectively, to the high molecular weight and intermediate molecular weight renin, as reported in our previous paper [8]. This conversion was hardly affected by the presence of dithiothreitol, a protective reagent for sulfhydryl groups, eliminating the possibility that during the incubation at 37°C thiol groups in renin and/or RBP were oxidized. Fig. 1C shows that the high molecular weight renin formed by the warming was unstable at 4°C, and was dissociated into its components, intermediate molecular weight renin and RBP, by the cold treatment. Further dissociation of the renin did not occur, indicating that the intermediate form is relatively stable at low temperatures. In control experiments, we observed that the incubation of pure hog renin at 37°C, in the absence of RBP, did not produce higher molecular weight renins.

*This value was higher than that (60,000 ± 2,500) estimated using Ultrogel AcA 44 or Sephadex G-100 [8]. This discrepancy is probably due to the difference in the supporting medium or the elution time on gel filtration.

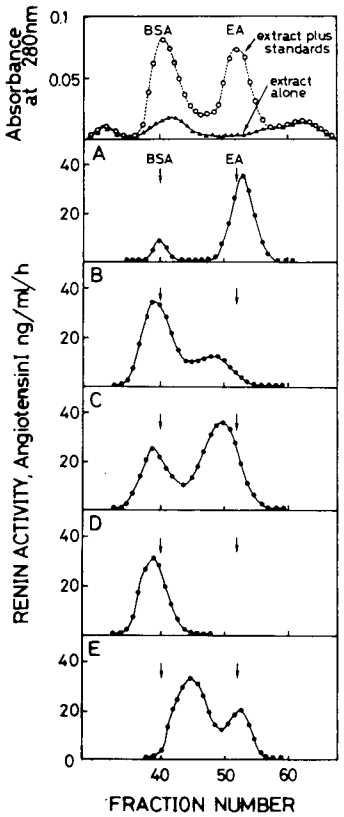

Fig. 1. HPLC separation of renin and renin—RBP complex formed by various treatments of hog kidney extracts. Incubation conditions in hog kidney extract: (A) 37°C for 1 min; (B) 37°C for 45 min; (C) 37°C for 45 min followed by incubation at 4°C for 16 h; (D) 37°C for 15 min with 10 m*M* sodium tetrathionate; (E) 37°C for 45 min with 4 *M* sodium chloride. Typical elution profiles, monitored by measuring absorbance at 280 nm, of the hog kidney extract with and without internal molecular weight standards are shown on the top panel; since nearly the same profile was obtained for the samples analyzed, only renin activity profiles are shown in the other panels. Renin activity was determined by the rate of formation of angiotensin I from hog renin substrate as described in our previous paper [8]. BSA = bovine serum albumin; EA = egg albumin.

Stimulation of the renin—RBP complex formation by the modification of thiol groups

It is well established that at low temperatures the interaction between renin and RBP occurs very slowly; however, the rate of the interaction is markedly enhanced by the addition of thiol blocker. To see if this is the case even at high temperatures, we incubated the extract in the presence of 10 m*M* tetrathionate at 37°C. As shown in Fig. 1D, the addition of tetrathionate to the incubation mixture did indeed facilitate the complex formation; in less than 15 min the complete conversion from renin to high molecular weight renin was attained.

Effect of pH and sodium chloride on the interaction

Boyd [1] has shown that almost complete dissociation of renin—RBP com-

plex occurs in the presence of 3 *M* sodium chloride. To confirm by gel chromatography this dissociative effect of sodium chloride and to determine which process in the two conversions (renin to intermediate molecular weight renin, or the intermediate form to high molecular weight renin) is most affected by the presence of sodium chloride, the hog kidney extract was incubated in 4 *M* sodium chloride at 37°C for 30 min and analyzed by HPLC. Most of the renin after the incubation was converted to intermediate molecular weight renin but not to the high molecular weight form (Fig. 1E). Similarly, high molecular weight renin was dissociated to the intermediate molecular weight form in the presence of 4 *M* sodium chloride at 37°C but no further dissociation occurred.

Fig. 2 shows the effect of pH on the stability of the renin—RBP complex. At pH values below 4, both high molecular weight and intermediate molecular weight renins were quite unstable and converted to renin. About 20% of these higher molecular weight forms of renin escaped the conversion during 2-min acidification at pH 3; however, 15-min acidification of the high molecular weight renin resulted in its complete conversion to normal renin.

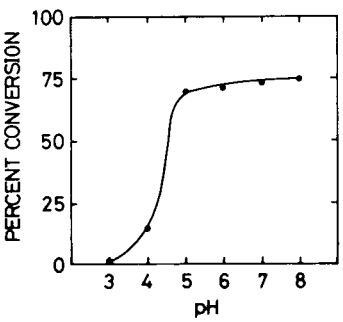

Fig. 2. Effect of pH on the interaction between renin and RBP. The percentage values indicate the proportion of the renin—RBP complex (high molecular weight renin) in the total renin activity.

DISCUSSION

Introduction of the gel TSK G3000SW in HPLC has made it possible to separate proteins according to their size and shape within 40 min [9]. We examined possible applications of this gel to studies on interconversions among various forms of renin. As a result of the interaction with renin-binding protein, active renin has been shown to exist in three forms: renin, and intermediate molecular weight and high molecular weight renins [8]. Although these three forms were distinguished by conventional gel filtration on Sephadex G-100 or Ultrogel AcA 44, the separation was unsatisfactory. The newly developed SW-type gel, however, gave satisfactory resolution. In addition, it drastically reduced elution time, opening up exciting possibilities for elucidating, in more detail, the mechanism and factors which contribute to the formation of the renin—RBP complex. We have shown that at 37°C the interaction can occur spontaneously at a reasonable speed even in the absence of sulfhydryl reagents. This fact, together with our previous observation [10, 11] that the binding capacity of RBP is specific for renin, strongly suggests that RBP may play an important role in

blood pressure regulation. These conclusions are in substantial agreement with those reached by Boyd [1] who used different approaches to study the protein-bound form of renin. Also, our previous finding [8] of the presence of an intermediate molecular weight form of renin, was confirmed using the newly developed gel, TSK-Gel G3000SW.

The present series of experiments has further established the experimental conditions under which the intermediate molecular weight renin is a major form among other interconvertible forms of renin; an intermediate molecular form becomes dominant at low temperatures or at high sodium chloride concentrations, suggesting that the conversion from the intermediate molecular weight renin to the high molecular weight form is a temperature- and ionic strength-sensitive process.

Attempts to demonstrate the presence of the three forms of renin by a single run have so far been unsuccessful. This is mainly due to the nature of the equilibria among them. At 37°C or in the presence of sulfhydryl reagents, the equilibria are shifted entirely to the higher molecular weight forms of renin, making the detection of low molecular weight renin difficult; in the kidney extracts freshly prepared at 4°C or in the samples acidified or treated with sodium chloride, the presence of low molecular weight renin can be demonstrated, but one or both of the higher molecular weight forms of renin are unstable under these conditions. Further studies are required to clarify the nature of equilibria and the mechanism of the interconversions among the various forms of renin.

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THE USE OF C_8-OCTYL COLUMNS FOR THE ANALYSIS OF CATECHOLAMINES BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

The chromatographic behavior of norepinephrine (NE), epinephrine, dopamine, 3,4-dihydroxyphenylalanine, and 3,4-dihydroxyphenylacetic acid on octylsilane (C_8) reversed-phase high-performance liquid chromatography columns was observed under various mobile phase conditions including manipulations of pH, pairing ion and methanol concentrations. The optimum isocratic conditions permitting quantitative resolution of these substances in minimum time and with maximum detector response were determined. Employing a pH 3.0—3.2 mobile phase comprising an aqueous buffer solution containing 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and 0.2 m*M* 1-octanesulfonate, admixed with a volume of methanol equal to 4% of the aqueous volume, the performance of the C_8 columns compares favorably to that of the more widely used C_{18} columns. The column eluates were monitored with an amperometric detector utilizing a glassy-carbon flow-cell electrode. The detector response for NE was 1.5—2.0 nA/ng and the baseline noise was as little as 0.002 nA thereby permitting quantitation of 5-pg levels or more in the injected samples. By coupling the liquid chromatographic system to a procedure which eliminates non-catechol contaminants from the neuronal and body fluid specimens by alumina adsorption of the catechols, a sensitive and dependable method was developed and employed for the determination of catechol levels in discrete regions of rat brain, cat spinal cord, and in human plasma.

INTRODUCTION

Ion-exchange [1—6] and reversed-phase [6—12] high-performance liquid chromatographic (HPLC) techniques have been used successfully to resolve the catecholamines and their related metabolites in extracts of tissues and in body fluids. Reversed-phase chromatography appears to be most useful in the analysis of mixtures of such substances which possess both acidic and basic organic functional groups [6]. The most widely used reversed-phase methods

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employ isocratic conditions with the mobile phase flowing over a bonded octadecylsilane solid phase such as found in the μBondapak C_{18} and the Li-Chrosorb RP-18 columns [8—12]. The selectivity and the resolution of these columns are enhanced by the use of a pairing-ion species such as 1-octanesulfonate (OSA) [12,13] and by the admixture of an organic modifier such as methanol in the mobile phase [12]. In such separations, an acidic pH proves optimal and modest pH adjustments produce significant changes in the retention of acidic substances [6,12].

One of the most significant advances in quantitation techniques available in liquid chromatography has been the development of the amperometric detector capable of monitoring column eluates and of detecting picogram quantities of oxidizable or reducible products [14]. Until recently, most amperometric detectors have utilized the carbon-paste flow-cell electrode which requires frequent standardization and repacking and whose performance deteriorates in the presence of admixed organic solvents [12].

Improvements in column and in detector technologies have produced high-performance bonded octylsilane (C_8) and octadecylsilane (C_{18}) solid phases which yield enhanced resolution, efficiency, and stability, and sensitive, low-maintenance, and durable glassy-carbon amperometric electrodes. We have utilized these components in the development of a sensitive and dependable method to determine the basal levels of norepinephrine (NE), epinephrine (EPI), dopamine (DA), 3,4-dihydroxyphenylalanine (DOPA), and 3,4-dihydroxyphenylacetic acid (DOPAC) in selected tissues and body fluids.

The optimum mobile phase conditions giving baseline resolution and maximum detector sensitivity with minimum processing time are described for the C_8 column (Ultrasphere-Octyl) and compared to conditions which yield similar results in the C_{18} column (Ultrasphere-I.P.). These parameters were determined from the systematic study of a range of mobile phase conditions which varied with respect to pairing-ion concentration, pH, and methanol admixture. Tissue samples and body fluids are prepared by a purification procedure utilizing alumina adsorption to eliminate non-catechol substances and to concentrate the products of interest.

MATERIALS AND METHODS

Chemicals

The catecholamine standards, norepinephrine (NE), epinephrine (EPI), and dopamine (DA) were purchased from Sigma (St. Louis, MO, U.S.A.); the 3,4-dihydroxyphenylalanine (DOPA) and the 3,4-dihydroxyphenylacetic acid (DOPAC) were from Calbiochem (San Diego, CA, U.S.A.). The 1-octanesulfonic acid (OSA), sodium salt was purchased from Regis Chemical (Morton Grove, IL, U.S.A.). Glass-distilled methanol from Burdick & Jackson was purchased from Rainin Instrument (Woburn, MA, U.S.A.). All other chemicals were reagent grade from Baker (Phillipsburg, NJ, U.S.A.).

Mobile phase solutions

The aqueous solutions were prepared with glass-distilled deionized water. The buffer, prepared from concentrated solutions of 1.0 *M* NaH_2PO_4 (adjusted

to pH 2.8—2.9 with 1.0 *M* H_3PO_4) and 10% Na_2EDTA, contained 0.1 *M* phosphate and 0.1 m*M* EDTA. The crystalline pairing-ion reagent, 1-octanesulfonate (OSA) was added to the diluted buffer as prepared and the aqueous solution then was filtered (Millipore, 0.22 μm GS-type) and degassed under vacuum prior to the admixture of methanol. The proportion of aqueous buffer to methanol varied from 100:1 to 100:4 for the C_8 column and was as high as 100:8 for the C_{18} column. The pH of the mobile phase was approximately 3.0 and was adjusted with NaOH or H_3PO_4 to achieve optimum resolution of DOPA from NE and EPI.

Chromatography

The HPLC system consisted of the following components purchased from Rainin Instrument: Altex 420 Microprocessor Control System, Altex 100A pumps, Rheodyne 7210 and 7125 injector valves, Altex Ultrasphere-Octyl (C_8) and Ultrasphere-I.P. (C_{18}) columns (5 μm particle size; 250 × 4.6 mm), Kipp & Zonen BD 41 two-channel recorder. The amperometric detector was the Model LC-16 from Bioanalytical Systems (West Lafayette, IN, U.S.A.) and consisted of a TL-5 glassy-carbon electrode and an LC-3 controller. A vintage LC-2A detector also was used in some experiments. For maximum sensitivity the working electrode was operated at +0.9 V vs. a Ag/AgCl reference electrode.

Columns were operated at ambient temperatures and the flow-rate was 1.0 ml/min during experiments and was maintained at 0.1 ml/min when the columns were not in use. The amperometric detectors were left on as long as mobile phase flowed through the columns and flow-cells. This minimized the start-up time. Columns and detectors remained stable for extended periods of use, up to six weeks. At the end of a use period, the columns were washed free of mobile phase components with 400—500 ml of water and then were returned by means of a gradient to 70% methanol for storage. A few of the columns in use for over six months retain good efficiency and resolution despite prolonged exposure to aqueous solutions and acidic samples.

Standards

All stock catechol solutions (1.0 mg/ml free base) were prepared with 0.01 *N* HCl containing 0.1 m*M* EDTA. These solutions were stored in 100-μl aliquots at —20°C until used. Thawed aliquots were diluted with mobile phase solvent to yield working standards containing 10 or 50 ng/ml of each catechol. These working standards were prepared for each experiment and were kept on ice until used. The 20-μl injected standard mixture contained 0.2 or 1.0 ng of each catechol and the detector response was linear with concentrations from 0.1—20.0 ng for each species.

Tissue sample preparation

The catechol substances in tissue and in body fluid specimens were isolated by a modified alumina adsorption procedure employing batch elution to minimize final volumes [15]. Briefly, specimens were homogenized in 15% trichloroacetic acid and then centrifuged. The supernatant fluid was rapidly adjusted to pH 8.4—8.5 in the presence of EDTA and Tris buffer was poured

into screw-cap conical tubes containing 100 mg of activated alumina [16]. After thorough mixing, the fluid was removed and the alumina was washed with several volumes of water. The catechols were then eluted from the alumina with 0.3 ml of 0.8 *N* perchloric acid (PCA). Excess PCA was removed from the eluent by precipitation as the potassium salt formed on the addition of a small volume of concentrated KOH—K_2HPO_4 solution containing EDTA and OSA. An aliquot of methanol was added to the eluent, which then was similar in buffer and methanol composition to the mobile phase and which contained less than 0.1 *N* PCA. The final pH of the sample was 1.5—2.0 and it was kept on ice until injected into the HPLC system. These precautions were taken to safeguard the reversed-phase columns. The elimination of non-catechol substances permitted the operation of the detector at a potential of +0.9 V to yield maximum sensitivity. Tissue recoveries were determined in each experiment and generally ranged from 40% for DOPA and DOPAC to 60% for NE. Results were corrected for recoveries. The experimental procedures followed in the rat brain studies have been described previously [15].

RESULTS AND DISCUSSION

In order to establish the optimum mobile phase conditions required for the baseline resolution of NE, EPI, DOPA, DA and DOPAC on the C_8-Octyl columns, a systematic study of the influence of pH, OSA concentration, and methanol admixture on the retention of these catechol substances was conducted. The relationship of retention to OSA and methanol levels at pH 3.0 is shown in Fig. 1. At all pairing-ion concentrations, the admixture of methanol dramatically curtails the retention of the catechol compounds, compressing the elution profile for standard mixtures. The maximum methanol concentration is determined by the limit of resolution of NE, DOPA, and EPI and is approximately 4% for the C_8-Octyl columns. Increasing the concentration of the pairing-ion reagent increases the retention of the catecholamines and of DOPA but reduces that of DOPAC which lacks the cationic ammonium group. At the lowest OSA concentration examined (0.1 m*M*), quantitative resolution of the five catechols was achieved in the absence of methanol. Under these conditions, the DA and DOPAC peaks were quite broad and the peak heights minimal, the results of band broadening during the course of the extended elution time (approximately 1 h at ambient temperature and 1.0 ml/min flow-rate). The admixture of methanol effectively curtails band broadening and enhances peak height, thereby improving detector response (Fig. 2). The optimum conditions for achieving quantitative resolution, maximum sensitivity, and minimal elution time entail the use of 0.2 m*M* OSA and 4% methanol, at pH 3.0.

The retention of DOPA and of DOPAC is most sensitive to modest pH manipulations in this acidic range. Fig. 3 demonstrates the effect of pH on the retention of these catechol substances in the range 2.8—3.8. The catecholamines are unaffected by variations in this pH range while the DOPA retention is significantly altered below pH 3.4 and that of DOPAC above pH 3.4. Similar findings for DOPA [12] and DOPAC [17] have been reported previously in C_{18} reversed-phase columns. The operating pH of the mobile phase can be

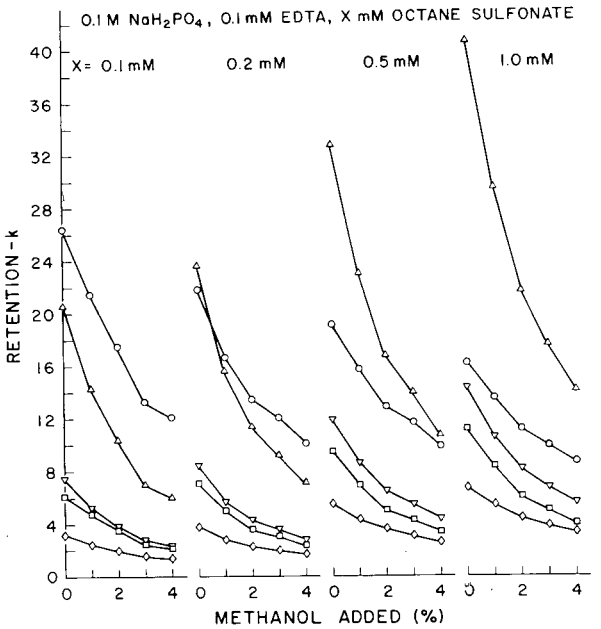

Fig. 1. Effect of OSA concentration and methanol admixture on retention of NE (◇), DOPA (□), EPI (▽), DA (△), DOPAC (○). Column: Ultrasphere-Octyl, 25 × 0.46 cm. Eluent: aqueous buffer, 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and OSA (0.1, 0.2, 0.5, or 1.0 m*M*); methanol, 0—4% volume of aqueous buffer. pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient. Detector: +0.9 V vs. Ag/AgCl reference electrode.

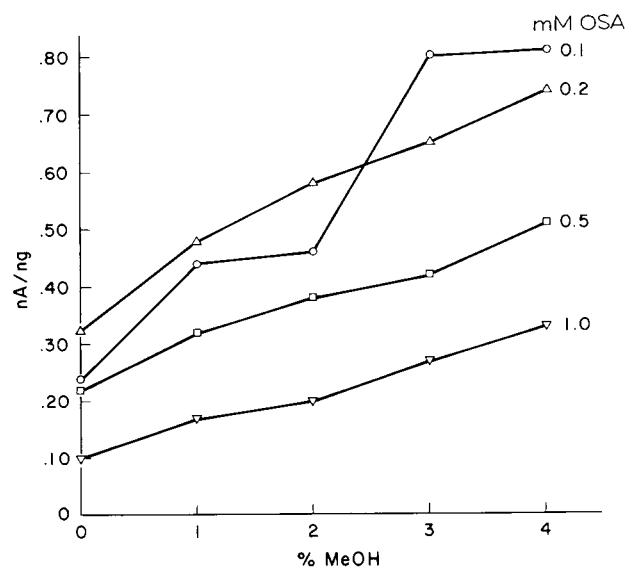

Fig. 2. Effect of OSA concentration and methanol admixture on detector response to DA. Mobile phase: 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and OSA (0.1, 0.2, 0.5, 1.0 m*M*); methanol (0, 1, 2, 3, 4%): buffer. pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient. Detector: +0.9 V vs. Ag/AgCl reference electrode. Column: Ultrasphere-Octyl.

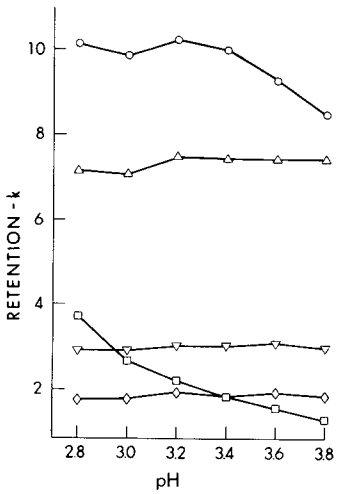

Fig. 3. Effect of pH on retention of NE (◇), DOPA (□), EPI (▽), DA (△), DOPAC (○). Mobile phase: 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, 0.2 m*M* OSA, and 4% methanol. Flow-rate: 1.0 ml/min. Temperature: ambient. pH adjusted with 1.0 *M* NaOH or 1.0 *M* H_3PO_4. Column: Ultrasphere-Octyl, 25 × 0.46 cm. Detector: +0.9 V vs. Ag/AgCl.

adjusted to achieve a fine tuning of any column, as circumstances require, and since the column performance rapidly reflects this influence, such manipulation can be effected shortly prior to use. As shown in Fig. 3 the baseline resolution of DOPA from NE and EPI is achieved under several pH conditions. At pH values below 2.9, DOPA emerges after EPI, while at pH values above 3.4, it emerges ahead of NE in which case it may overlap with minimally-retained solvent front substances. We chose the pH range 3.0—3.2 in which DOPA emerges between NE and EPI since no contaminant peaks were observed in this region in the tissue and body fluid samples examined. In the experiment involving the DOPA-decarboxylase inhibitor, seryltrihydroxybenzylhydrazine (Roche No. Ro 4-4602), a large peak was seen in the chromatograms after the EPI peak position.

Thus, the standard conditions under which the C_8-Octyl columns are operated include a mobile phase containing 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, 0.2 m*M* OSA, and 4% volume of admixed methanol, pH 3.0—3.2, a flow-rate of 1.0 ml/min, ambient temperature, and amperometric detectors set at a working electrode potential of +0.9 V vs. Ag/AgCl reference electrodes. Under these conditions, the detection sensitivity permits the quantitation of as little as 5 pg of NE in the injected (20 μl) sample. The detector response for NE is 1.5—2.0 nA/ng and the baseline noise is less than 0.002 nA (LC-16 detector). The column efficiency as measured by a theoretical plate calculation has shown values of *N* ranging from 7000—12,000 for these 250-cm columns, using the DA peak retention time and width at half-height ratio.

Fig. 4 compares the elution profiles of catechol mixtures separated by the C_8-Octyl column with that achieved by a C_{18} Ultrasphere-I.P. column. The greater retention affinity of the C_{18} column required the admixture of a larger volume of methanol and produced a virtually congruent chromatogram. Owing

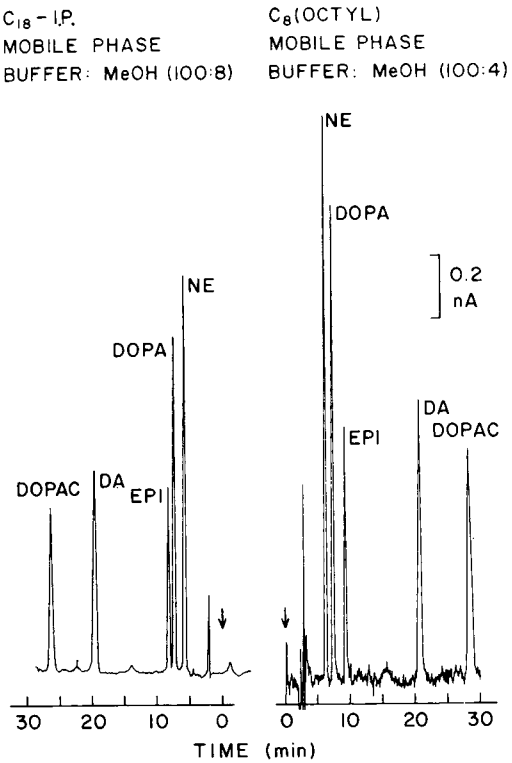

Fig. 4. Chromatograms of NE, DOPA, EPI, DA, DOPAC separations effected by C_8 Ultrasphere-Octyl and C_{18} Ultrasphere-I.P. columns. Mobile phase: pH 3.0; aqueous buffer (0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and 0.2 m*M* OSA)—methanol, 100:8 for C_{18}-I.P. and 100:4 for C_8-Octyl. Detectors: LC-16 for C_{18}-I.P. and LC-2A for C_8-Octyl; both +0.9 V vs. Ag/AgCl. Columns: 25 × 0.46 cm. Flow-rate: 1.0 ml/min. Temperature: ambient. Sample loop: 20 µl. Standard mixture: 1.0 ng/20 µl, each catechol.

to the reversed output polarities of the detectors the injection points are juxtaposed and the elution directions opposite thus giving a mirror-like comparison. The aqueous buffer conditions of the mobile phase are identical. The pH optimum of the C_{18}-I.P. column (pH 3.2—3.25) is slightly higher than that required to position DOPA exactly between NE and EPI in the C_8-Octyl columns.

The equilibration of the C_8-Octyl columns with the mobile phase has been studied and the results are shown in Fig. 5. Early attempts to place columns into use after a few hours of mobile phase equilibration failed since the retention times of the eluted peaks were not constant. In this experiment, the columns were pre-equilibrated with mobile phase containing a water—methanol mixture similar to that in the complete mobile phase (100:4). At a given time, the columns were set into mobile phase and chromatograms were obtained of the separation of a standard mixture of the catechols every hour for 5 h. When the elution times of the peaks were found to be changing after this length of time, the columns were allowed to equilibrate overnight and the separation was repeated the next day. By then, the retention times remained constant. Fig. 5

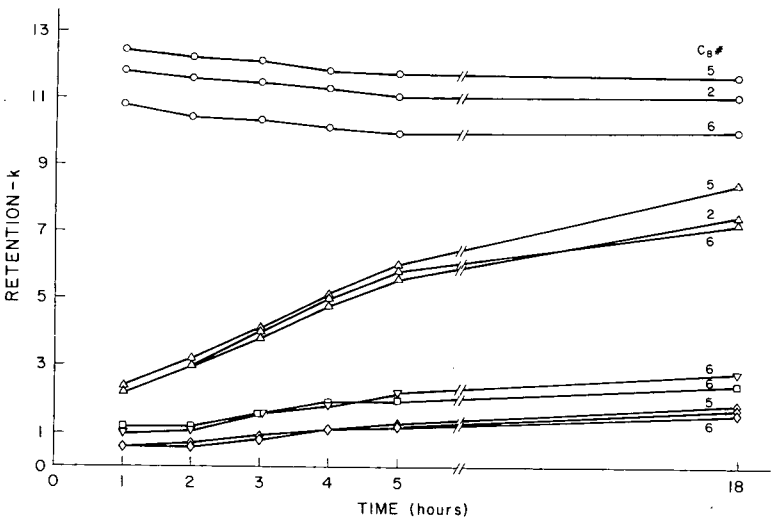

Fig. 5. Time course for equilibration of C_8-Octyl columns. Mobile phase: standard conditions, pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient. ◇, NE; □, DOPA; ▽, EPI; △, DA; ○, DOPAC.

indicates that the retention times of all catechols except DOPAC increase with time. This suggests that the rate-limiting step in the equilibration is the binding of the pairing-ion OSA to the reversed-phase packing. This is consistent with the findings of Crombeen et al. [18] who observed changing retention values of these and other substances as a function of column loading with a pairing-ion reagent. Their study suggested that a preliminary column loading with the pairing-ion reagent followed by replenishing with a minimum steady-state level furnished by the mobile phase might be an alternative approach to take to achieve equilibrium. Based on these results, it appears that the pairing-ion species binds to the reversed-phase medium and creates a dynamic cation-exchange system [18].

The results of these studies provide a sound basis for selecting the mobile phase conditions required to achieve quantitative separation of mixtures of basic, zwitterionic, and acidic catechol substances. For instance, the difficulty of separating NE from other minimally-retained solutes can be overcome by increasing the OSA concentration. The attending increase in the retention time of DA can be largely offset by increasing the methanol volume admixed which diminishes the retention time of DA and DOPAC proportionally more than that of NE, DOPA, or EPI. The DOPA also can be shifted to positions before, between, or after NE and EPI by making minor pH changes to accommodate different situations.

The general method developed from these studies has been applied to the determination of the catecholamines and their related catechols in several neuronal tissues and in plasma. Fig. 6 shows chromatograms of extracts of cat spinal cord tissues which were spiked with DOPA as an internal standard. The amount of gray matter used in the assay was 17 mg while 53 mg of white matter was analyzed. The only endogenous catecholamine detected in sig-

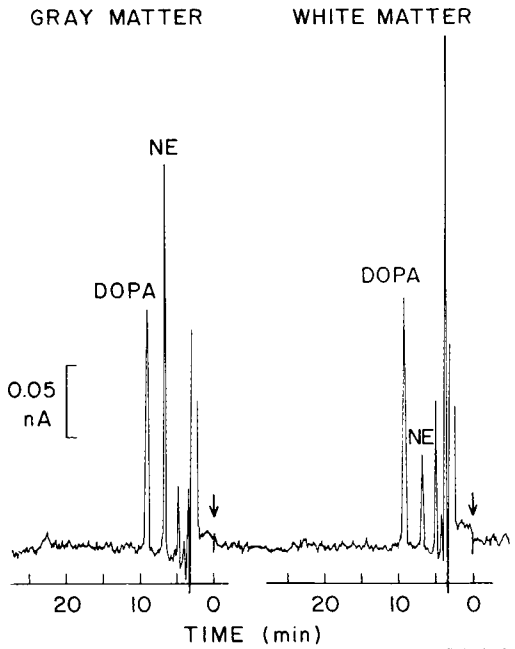

Fig. 6. Cat spinal cord chromatograms. Mobile phase: standard conditions, pH 3.0. Tissue specimens: 17 mg of gray matter; 53 mg of white matter. DOPA added as internal standard. NE content: gray matter, 330 ng/g; white matter, 24 ng/g.

nificant amounts was NE (330 ng/g in gray matter and 24 ng/g in white matter).

The chromatogram of human plasma is shown in Fig. 7. One milliliter of plasma was used for the analysis and NE, DOPA and DOPAC were easily detected in the 20-μl sample injected. Recent studies using a larger sample loop (100 μl) have facilitated the quantitation of EPI (not shown). The challenge in this assay is the resolution of NE from the solvent front components. In this case, this was achieved by increasing the OSA concentration to 0.5 m*M*. We have found that the decline in column performance is signalled by the loss of such resolution even with the use of elevated OSA concentrations. The C_{18}-I.P. columns exhibit greater NE retention and permit its resolution at 0.2 m*M* OSA [19]. The plasma NE level in this sample corresponds to a concentration of 4.4 nmole/l which is within the range observed by Hallman et al. [1] for normal human plasma samples.

The steady-state levels of NE, DA, and DOPAC for various regions of rat brain are shown in Table I. To date, the most extensive use of this method in our laboratory has been for the determination of these substances and of DOPA in discrete regions of rat brain under a variety of drug-altered conditions [15]. Fig. 8 shows one such experiment in which DOPA accumulation was studied in animals treated with a DOPA-decarboxylase inhibitor (Ro 4-4602). The chromatograms are of prefrontal cortex samples. This study measured the DOPA accumulation and catecholamine concentrations in rat corpus striatum, olfactory tubercle, as well as in prefrontal cortex [15]. The C_8-Octyl columns are admirably suited to such investigations.

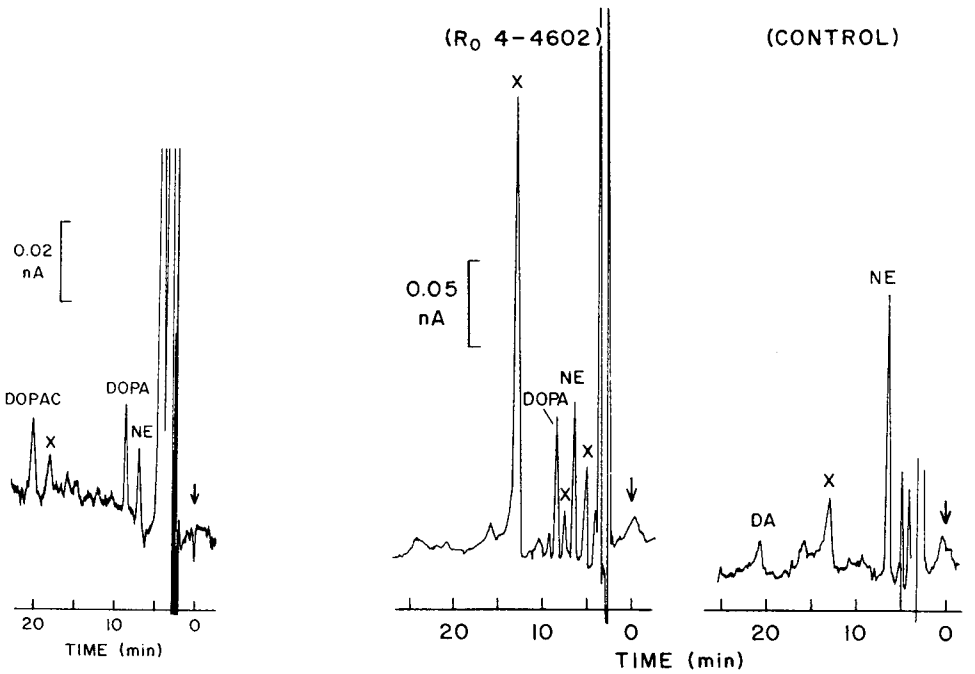

Fig. 7. Human plasma chromatogram. Mobile phase: 0.1 m*M* NaH_2PO_4, 0.1 m*M* EDTA, 0.5 m*M* OSA, and 4% methanol, pH 3.0. Flow-rate, 1.0 ml/min. Temperature: ambient.

Fig. 8. Effect of DOPA-decarboxylase inhibition on chromatogram of rat prefrontal cortex. Mobile phase: standard conditions, pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient.

TABLE I

REGIONAL VALUES FOR RAT BRAIN NE, DA AND DOPAC

Rat brain regions differing greatly in catecholamine content have been studied using this method. Values indicate the mean ± the standard error of the mean, expressed as ng per g wet weight tissue. Brain regions were dissected bilaterally and pooled before determinations. Two DA-rich areas, the corpus striatum and olfactory tubercle are shown. NE, DA, and DOPAC values for a small section of prefrontal cortex receiving a DA projection [15] are also included. The cerebellum and hippocampus receive a noradrenergic (but not dopaminergic) innervation, and the DA and DOPAC levels in these tissues are consistent with the role of DA as a precursor to NE in noradrenergic nerve terminals.

Brain area	Weight (mg)	N	NE (ng/g)	DA (ng/g)	DOPAC (ng/g)
Corpus striatum	70 ± 4	7	94 ± 6	7129 ± 166	988 ± 38
Olfactory tubercle	21 ± 1	7	287 ± 10	6820 ± 123	1093 ± 46
Prefrontal cortex	21 ± 1	7	310 ± 17	89 ± 11	47 ± 4
Hippocampus	120 ± 4	5	239 ± 12	12 ± 1	4 ± 1
Cerebellum	242 ± 5	5	171 ± 9	6 ± 1	2 ± 1

ACKNOWLEDGEMENT

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SIMULTANEOUS QUANTIFICATION OF DOPAMINE, 5-HYDROXYTRYPTAMINE AND FOUR METABOLICALLY RELATED COMPOUNDS BY MEANS OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for simultaneously quantifying dopamine, 5-hydroxytryptamine (5-HT) and four metabolically related compounds has been developed, permitting more efficient neurochemical examination of these often interrelated biogenic amine systems. The method uses high-performance liquid chromatographic separation of these compounds on a C_{18} reversed-phase column with a buffered mobile phase containing methanol as an organic modifier and heptanesulfonate as an ion-pair reagent. Using 5-hydroxy-N-methyltryptamine as an internal standard and electrochemical detection, chromatography time is less than 12 min. Sample preparation simply involves the addition of internal standard, homogenization in the mobile phase, centrifugation and injection of the supernatant into the chromatograph. The method is sensitive to a tissue content of these compounds of less than 1 ng. The utility of this method for neuropharmacological—neurochemical studies is illustrated with studies using inhibitors of monoamine oxidase (pargyline) and aromatic amino acid decarboxylase (RO 4-4602).

INTRODUCTION

The involvement of dopamine- and 5-hydroxytryptamine (5-HT)-containing neuronal systems in the function of the central nervous system (CNS) is well documented. Evidence obtained from diverse lines of investigation support the existence of functional interactions between neuronal elements containing these two putative neurotransmitters [1—3]. It is therefore of interest to be able to assess simultaneously changes in the functional state of both of these systems.

Biochemical determinations of the rate of neurotransmitter turnover have

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proven to be useful estimates of the activity of neuronal systems. For dopamine systems, the available nonradiometric techniques include: (1) measurement of the rate of accumulation of 3,4-dihydroxyphenylalanine (DOPA) following decarboxylase inhibition [4]; (2) the rate of decrease in dopamine concentration following synthesis inhibition with α-methyltyrosine [5]; and (3) the concentration of 3,4-dihydroxyphenylacetic acid (DOPAC) or 3-methoxy-4-hydroxyphenylacetic acid (HVA) at a steady state [6,7] or following inhibition of their formation or transport [8,9]. Similarly, the biochemical estimation of changes in 5-HT turnover have been employed as an index of changes in the functional state of 5-HT neurons in the CNS. These techniques include: (1) measurement of the rate of accumulation of 5-hydroxytryptophan (5-HTP) following decarboxylase inhibition [4,10]; (2) the rate of accumulation of 5-HT following inhibition of its metabolism [11,12]; and (3) the concentration of 5-hydroxyindoleacetic acid (5-HIAA) at a steady state [13] or following inhibition of its formation [11] or transport [14].

The heterogeneous distribution, function, and regulation of dopamine and 5-HT systems in the CNS necessitates the use of analytical techniques with sensitivity requisite for determinations in small brain regions. A variety of separation and detection methods have been used to quantify the precursors and metabolites of dopamine and 5-HT. Of these techniques, the greatest degrees of flexibility, sensitivity and specificity have been obtained using either gas chromatography with electron-capture or mass fragmentographic detection [15—21], or high-performance liquid chromatography (HPLC) using fluorometric or electrochemical detection [22—37]. However, each of these techniques has potential shortcomings in at least one of the following criteria: speed; sensitivity; specificity; flexibility; sample purification and/or concentration; use of internal standards to monitor recovery during sample preparation; or complexity of instrumentation.

The present study reports a method for the simultaneous determination of dopamine, DOPAC, HVA, 5-HTP, 5-HT and 5-HIAA in samples from small brain regions by means of ion-pair, reversed-phase HPLC with electrochemical detection. N-Methyl-5-hydroxytryptamine is used as an internal standard. This assay offers considerable advantages in terms of ease of sample preparation, internal standardization and versatility, and offers sufficient sensitivity to perform accurate determinations in tissue samples as small as 1 mg.

EXPERIMENTAL

Reagents and drugs

Chemicals were obtained from the following sources: dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 5-hydroxytryptamine creatinine sulfate and 5-hydroxyindoleacetic acid were obtained from Sigma (St. Louis, MO, U.S.A.); 5-hydroxytryptophan from Calbiochem (Los Angeles, CA, U.S.A.); 5-hydroxy-N-methyltryptamine oxalate (N-methyl-5-HT) from Aldrich (Milwaukee, WI, U.S.A.); sodium 1-heptanesulfonate from Eastman-Kodak (Rochester, NY, U.S.A.); pargyline hydrochloride from Abbott Laboratories (North Chicago, IL, U.S.A.); and RO 4-4602 [N-(DL-seryl-N)-(2,3,4-trihydroxybenzyl)hydrazine] from Hoff-

mann-LaRoche (Nutley, NJ, U.S.A.). Reagent grade methanol (Fisher Scientific, Fairlawn, NJ, U.S.A.) was used as supplied.

Apparatus

A Laboratory Data Control (Riviera Beach, FL, U.S.A.) ConstaMetric IIG solvent delivery pump was used in conjunction with a TL-5 glassy carbon electrode and an LC-4 controller, both from Bioanalytical Systems (West Lafayette, IN, U.S.A.). The detector potential was maintained at 0.75 V vs. a Ag/AgCl reference electrode. A six port rotary valve (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 500-μl sample loop was used for sample injection. Chromatographic separations were performed using a 25 cm × 4.6 mm I.D. stainless-steel column packed with octadecylsilane (C_{18}) on microparticulate (5 μm) silica gel (Spherisorb ODS, Applied Science Labs., State College, PA, U.S.A.). A precolumn filter with a 2-μm frit (Rheodyne) effectively minimized the accumulation of particulate matter on the analytical column.

Chromatography

The mobile phase was a mixture of 0.1 *M* citrate, 0.075 *M* Na_2HPO_4, 0.75 m*M* sodium heptanesulfonate and 14% methanol (v/v). After adjusting to pH* 3.9, the mobile phase was filtered through a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI, U.S.A.) and degassed under vacuum with ultrasonic agitation. All separations were performed isocratically at a flow-rate of 1.5 ml/min.

Standards

Stock solutions of the reference and internal standards were prepared in the mobile phase at a concentration of 1 μg/μl (calculated as the free compound) and stored at 4°C. Working solutions (1 ng/μl) were prepared at weekly intervals by a 1:1000 dilution of the stock solutions using the mobile phase as the diluent.

Calculations

Standard curves were prepared by analyzing a series of standard solutions containing a fixed amount (10 or 25 ng) of the internal standard and varying amounts of dopamine, DOPAC, HVA, 5-HTP, 5-HT and 5-HIAA. These standards, in 400-μl aliquots of the mobile phase, were subjected to the identical preparative procedures used for tissue samples. The ratio of the detector response for each compound versus that of the internal standard was determined for each sample and standard. The concentrations of dopamine, DOPAC, HVA, 5-HTP, 5-HT and 5-HIAA in tissue samples were calculated from the ratio of their detector response relative to that of the internal standard using the slope and intercept of the standard curve.

Sample preparation

Male Sprague-Dawley rats (200—275 g; Charles River Laboratories, Wilmington, MA, U.S.A.) were sacrificed by decapitation and the brain rapidly re-

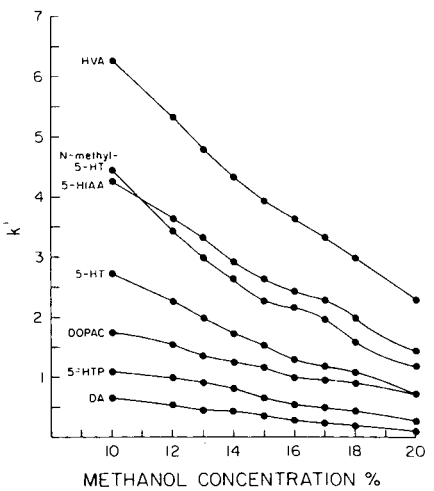
*All references indicate the measured pH ("apparent pH") of the aqueous methanol mixture referred to an aqueous standard buffer solution.

moved. The olfactory tubercles, nucleus accumbens and striata were dissected and placed in 500-μl nalgene tubes (No. 1801, Denville Scientific, Denville, NJ, U.S.A.) containing 400 μl of mobile phase and the internal standard (10 or 25 ng). Samples were homogenized with an ultrasonic cell disrupter (Model 185, Branson, Danbury, CT, U.S.A.) and centrifuged for 15 min at 4°C in a microcentrifuge (Model 5412, Brinkmann, Westbury, NY, U.S.A.). A 100—200-μl aliquot of either the standards or tissue sample supernatants was injected into the chromatograph. The homogenate pellet was assayed for protein content with the Folin reagent [38] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Effects of methanol, heptanesulfonate, and pH on compound retention

Functional group selectivity was optimized by alterations in the nature and relative concentrations of the mobile phase components. The relative retention (capacity factor, k') of dopamine, DOPAC, HVA, 5-HT, N-methyl-5-HT, 5-HTP and 5-HIAA was found to be a function of the methanol and heptanesulfonate concentrations, as well as of the pH of the mobile phase. Decreasing the aqueous characteristics of the mobile phase by increasing the methanol concentration produced a concentration-dependent decrease in k' for all compounds (Fig. 1).



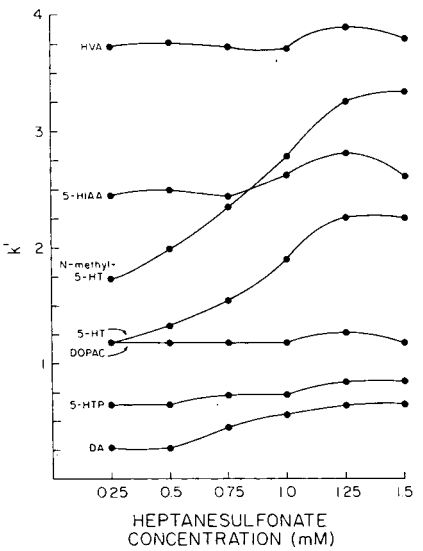


Fig. 1. Effect of methanol concentration of the mobile phase on the retention of DA, 5-HTP, DOPAC, 5-HT, 5-HIAA, N-methyl-5-HT and HVA. Mobile phase: citrate—Na_2HPO_4, 0.75 m*M* heptanesulfonate, pH 3.9. The capacity factor, k', was calculated by $k' = (t_R - t_{vv})/t_{vv}$ where t_R is the retention time of the compound of interest and t_{vv} the retention time of an unretarded component (representing the time to displace one column void volume).

Fig. 2. Effect of heptanesulfonate concentration of the mobile phase on the retention of DA, 5-HTP, DOPAC, 5-HT, N-methyl-5-HT, 5-HIAA and HVA. Mobile phase: citrate—Na_2HPO_4, 14% methanol, pH 3.9. See legend to Fig. 1 for derivation of k'.

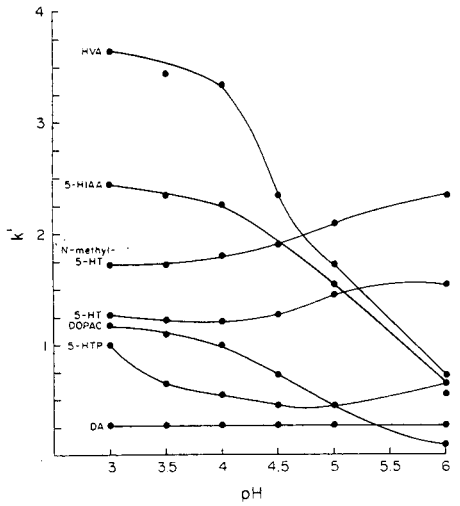


Fig. 3. Effect of pH of the mobile phase on the retention of DA, 5-HTP, DOPAC, 5-HT, N-methyl-5-HT, 5-HIAA and HVA. Mobile phase: citrate—Na_2HPO_4, 0.75 m*M* heptanesulfonate, 14% methanol. Orthophosphoric acid (85%) or 1 *N* sodium hydroxide were used to titrate the mobile phase to the desired pH. See legend to Fig. 1 for derivation of k'.

Ion-pair chromatography has been demonstrated to be effective in minimizing the peak tailing attributed to secondary interactions between polar ionogenic compounds and unreacted silanol groups on the silica surface and, more importantly, in controlling the relative retention of such compounds [39—42]. The addition of heptanesulfonate to the mobile phase produced a concentration-dependent increase in the retention of the protonated amines, i.e. dopamine, 5-HT and N-methyl-5-HT (Fig. 2). Pentanesulfonate, a less hydrophobic ion-pair reagent, did not facilitate the chromatographic separation of dopamine, DOPAC, HVA, 5-HTP, 5-HT, N-methyl-5-HT and 5-HIAA as completely as was possible using heptanesulfonate.

The observed influence of mobile phase pH on the relative retention of dopamine, DOPAC, HVA, 5-HTP, 5-HT, N-methyl-5-HT and 5-HIAA is in good agreement with predictions based on the degree of ionization at a given pH (Fig. 3). As the mobile phase pH increases relative to the pK_a values of DOPAC, HVA and 5-HIAA, they are deprotonated to a greater extent, resulting in a decrease in their capacity factors (Fig. 3). Conversely, the calculated capacity factors for the amines (dopamine, 5-HT and N-methyl-5-HT) are relatively unaffected by the pH range (3—6) examined in the present study since their pK_a values exceed the highest mobile phase pH tested. The capacity factors for the amino acid 5-HTP exhibit a concave downward relationship with increasing mobile phase pH. This is probably due to initial deprotonation of the carboxyl group to form the more polar zwitterion followed by deprotonation of the amino group to yield the amino carboxylate anion.

Internal standardization

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration. The addition of N-methyl-5-

HT to standards and tissue samples effectively controlled for variable volumes injected and within-run variations in column or detector electrode performance. The ratio of the detector response to varied amounts (0.1—50 ng) of dopamine, DOPAC, HVA, 5-HTP, 5-HT and 5-HIAA relative to a constant amount of N-methyl-5-HT (10 ng) was a linear function of concentration. Correlation coefficients for each compound were generally greater than 0.99. 5-Hydroxy-2-indolecarboxylic acid (Aldrich) elutes after HVA and can be used in place of, or in addition to, N-methyl-5-HT as an internal standard. Although this compound simplifies establishing optimal mobile phase parameters for a given column, it increases the total chromatography time, thereby decreasing sample throughput.

Tissue protein precipitation

The relative effectiveness of the mobile phase in precipitating proteins from tissue homogenates was also determined. The whole brain from a rat was minced and duplicate groups of 10, 50 or 100 mg samples were collected. One group was homogenized in 400 μl of the mobile phase and the other homogenized in 400 μl of 0.1 *M* perchloric acid. Following centrifugation and removal of the supernatants, the protein content of the pellet was determined (Table I). It is apparent from these data that the mobile phase is at least as effective as 0.1 *M* perchloric acid in precipitating tissue protein.

TABLE I

PROTEIN CONTENT (mg) OF BRAIN MINCES HOMOGENIZED IN MOBILE PHASE* OR 0.1 *M* PERCHLORIC ACID

Values represent mean ± S.E.M. for 3 separate determinations.

Approximate tissue weight (mg)	Solvent	
	Mobile phase	0.1 *M* perchloric acid
10	0.87 ± 0.09	1.05 ± 0.15
50	5.4 ± 0.4	4.4 ± 0.2
100	11.5 ± 0.6	9.8 ± 0.4

*See text for mobile phase composition.

Chromatographic interference

The utility of any analytical method is limited by the extent to which endogenous or exogenous substances interfere with the separation and detection of the compounds of interest. In this assay, interference will occur only when such substances coelute with one or more of the compounds of interest and are significantly oxidized at the detector electrode potential used. A series of catechol and indole derivatives were examined to determine if either or both of these criteria were applicable. Epinephrine (EPI), norepinephrine (NE) and DOPA had significant electrochemical activity within the range of detector potentials examined (0.55—0.75 V). However, these polar compounds elute in the solvent front and thus would not interfere. Conversely, tyramine and tryptophan coelute with 5-HTP and N-methyl-5-HT, respectively, but produce little

or no detector response at detector potentials less than 0.9 V (tryptophan) or 0.8 V (tyramine). The 3-O-methylated metabolites of NE and EPI, normetanephrine and metanephrine, respectively, eluted immediately prior to and following dopamine and were electrochemically inactive at detector potentials less than 0.6 V. The deaminated metabolites of NE, 3-methoxy-4-hydroxymandelic acid (VMA) and 3-methoxy-4-hydroxyphenylglycol (MHPG), elute in the solvent front and prior to 5-HTP, respectively. The 3-O-methylated metabolite of dopamine, 3-methoxytyramine (3-MT), eluted between and was incompletely resolved from DOPAC and 5-HT. In agreement with Shoup and Kissinger [43] no detector response was obtained for 3-MT at detector potentials less than 0.6 V.

Of these potentially interfering compounds, only 3-MT is a particular source of concern when assaying dopaminergic systems. Although it interfered slightly with the quantification of DOPAC and 5-HT, the 3-MT content of the rat brain is relatively small, and does not compromise the overall accuracy of the assay. Moreover, interference attributed to 3-MT may be eliminated by sacrificing the rats by head focused microwave irradiation, or by decreasing the detector electrode potential to 0.6 V. Either course effectively eliminates this problem, although the latter method results in a concomitant decrease in the electrochemical activity of HVA.

Sensitivity and within-run reproducibility

Detection limits (in terms of compound injected from an aqueous solution) ranged from 20—60 pg for dopamine, DOPAC, 5-HTP, 5-HT, N-methyl-5-HT and 5-HIAA and 50—150 pg for HVA, depending on column condition. The within-run precision of the assay was determined by processing and assaying ten 50-μl aliquots of a sample obtained by homogenizing a single rat striatum in 500 μl of mobile phase containing 250 ng of N-methyl-5-HT. The estimated coefficients of variation ranged from 6.3% (5-HIAA) to 3.1% (dopamine).

Effects of volume of sample injected

It is generally accepted that as the volume injected into the HPLC system increases, peak broadening may occur, resulting in a deterioration of the separation of adjacent chromatographic peaks. This assumption has limited the number of liquid—liquid or liquid—solid sample purification techniques which can be used in conjunction with HPLC, as their use compromises the overall sensitivity by generating dilute solutions from which a finite volume can be injected. The effect of injection volume on peak symmetry and resolution was examined by injecting solutions containing 50 ng of dopamine, DOPAC, HVA, 5-HTP, 5-HT, N-methyl-5-HT and 5-HIAA in varying volumes (20—200 μl). The peak to peak resolution (R_s) was calculated according to Brown and Krstulovic [44]:

$$R_s = \frac{V_2 - V_1}{(W_1 + W_2)/2}$$

where V and W are the retention volume and base peak width, respectively, for compounds 1 and 2. No significant differences in the resolution of adjacent

TABLE II

EFFECTS OF PARGYLINE OR RO 4-4602 ON THE CONCENTRATIONS OF DA, DOPAC, HVA, 5-HTP, 5-HT AND 5-HIAA IN THE STRIATUM, OLFACTORY TUBERCLE AND NUCLEUS ACCUMBENS

Animals were sacrificed 45 min after saline (1 ml/kg), pargyline (75 mg/kg) or RO 4-4602 (750 mg/kg). Brain regions were dissected and the samples assayed as described in Methods. Values represent means ± S.E.M. for 3 or 4 determinations.

Treatment	Protein (ng/mg)					
	DA	DOPAC	HVA	5-HTP	5-HT	5-HIAA
	Striatum					
Saline	90.3 ± 6.7	17.2 ± 1.2	9.3 ± 0.9	–	4.6 ± 0.5	6.4 ± 0.6
Pargyline	112.0 ± 6.5*	0.58 ± 0.12*	1.4 ± 0.08*	–	8.3 ± 0.8*	4.1 ± 0.4*
RO 4-4602	57.4 ± 5.3*	16.1 ± 4.9	8.2 ± 0.4	6.0 ± 0.5	2.8 ± 0.4*	6.3 ± 0.7
	Olfactory tubercle					
Saline	41.3 ± 4.0	7.8 ± 1.0	3.1 ± 0.8	–	10.1 ± 1.1	5.3 ± 0.8
Pargyline	55.9 ± 6.8*	0.39 ± 0.01*	0.26 ± 0.04*	–	15.7 ± 0.5*	1.4 ± 0.2*
RO 4-4602	29.5 ± 3.9*	5.8 ± 0.8	2.2 ± 0.1	9.9 ± 1.0	6.3 ± 0.9*	4.2 ± 0.3
	Nucleus accumbens					
Saline	59.3 ± 5.0	15.4 ± 1.0	5.7 ± 0.6	–	5.9 ± 0.9	5.5 ± 0.6
Pargyline	96.4 ± 2.5*	1.1 ± 0.2*	1.5 ± 0.09*	–	9.0 ± 0.9*	2.4 ± 0.2*
RO 4-4602	64.6 ± 7.2	13.8 ± 1.2	5.0 ± 0.8	10.0 ± 1.6	5.9 ± 0.6	4.9 ± 1.2

*Indicates values different from saline-injected controls at $p < 0.05$.

peaks ($R_s \geqslant 1.35$ in all cases) were noted with increasing injection volume.

Quantitative determinations

The present method permits the simultaneous determination of dopamine, 5-HT, their deaminated metabolites, as well as 5-HTP (the immediate precursor of 5-HT), from samples of small brain regions. Representative chromatograms of standards and of samples of rat striatum or olfactory tubercle are shown in Fig. 4. The effects of pargyline and RO 4-4602 on the concentrations of these compounds in the striatum, olfactory tubercle and nucleus accumbens were determined to demonstrate the utility of the present method (Table II). The tissue concentrations of dopamine, 5-HT and DOPAC are in agreement with those previously reported [45—47]. The inhibition of monoamine oxidase activity by pargyline increased the concentrations of dopamine and 5-HT, and decreased the concentrations of DOPAC, HVA and 5-HIAA, in all brain regions examined. The inhibition of aromatic amino acid decarboxylase activity by RO 4-4602 increased the 5-HTP concentrations in all three brain regions to detectable concentrations. The concentrations of dopamine and 5-HT in the striatum and olfactory tubercle, but not the nucleus accumbens, were significantly decreased following RO 4-4602 treatment.

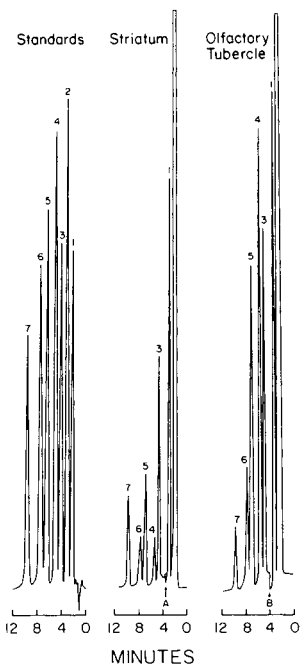


Fig. 4. Chromatograms of 50 μl of a mixture containing 1 ng/μl of the reference and internal standards and of supernatants from rat striatum or olfactory tubercles. Peaks: 1 = DA; 2 = 5-HTP; 3 = DOPAC; 4 = 5-HT; 5 = N-methyl-5-HT; 6 = 5-HIAA; 7 = HVA. Detector sensitivities (using a 1-V recorder) were: 50 nA/V for standards; A = change of sensitivity from 200 to 50 nA/V; B = change of sensitivity from 50 to 20 nA/V. See text for chromatography conditions.

Considerations during daily use

It should be noted that differences in the chromatographic performance of commercially available C_{18} reversed-phase columns supplied by different manufacturers, or changes in performance that occur with continuous use of a given column, necessitate the periodic reoptimization of mobile phase parameters (heptanesulfonate and methanol concentrations, and pH). However, due to the weak surface energies of the bonded hydrocarbonaceous phase, a rapid re-equilibration with the column packing is possible when altering mobile phases [48]. Therefore, optimization of column performance, using the data in Figs. 1—3, can generally be accomplished in a few hours. In our experience, for columns supplied by different manufacturers, the necessary mobile phase alterations usually represent a change of less than 10% in any given parameter compared to values established with prior columns.

In summary, there are several salient features of the present method. The absolute and relative recovery of the compounds of interest was essentially complete. Furthermore, the ease of sample preparation permits the assay of 35—40 tissue samples plus a standard curve in one working day. The versatility of this method is demonstrated by the simultaneous determination of dopamine, DOPAC, HVA, 5-HTP, 5-HT, 5-HIAA and N-methyl-5-HT in a single sample. Obviously, use of the assay in whole or part can be tailored to the analytical demands of a particular experimental design.

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MICROASSAY OF INORGANIC SULFATE IN BIOLOGICAL FLUIDS BY CONTROLLED FLOW ANION CHROMATOGRAPHY*

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SUMMARY

The application of controlled flow anion chromatography to the assay of inorganic sulfate in biological fluids is described. The sulfate anion is separated from other anions by ion-exchange chromatography and quantitated conductimetrically. Coefficient of variance is 3.4%, about half that for the barium precipitation assay. Interference from heparin in plasma samples and unknown sources in tissue extract analysis is avoided. Sulfate levels in plasma are not different from those measured in serum after protein precipitation. Normal levels for sulfate concentration in human plasma, cerebrospinal fluid and hepatic tissue extract are reported.

INTRODUCTION

Inorganic sulfate is the substrate for a wide variety of sulfoconjugation reactions, including sulfation of glycosaminoglycans, steroids and drugs [1]. The degree to which homeostasis of inorganic sulfate is achieved and influences the in vivo rate of sulfoconjugation has not been determined. Following drug administration, large demands for inorganic sulfate may occur in liver, lung and intestine, and may exceed the capacity of local stores and local production of sulfate by sulfur amino acid oxidation [2—4]. Conversely, reduced ambient levels of inorganic sulfate may be rate-limiting with regard to sulfation of glycosaminoglycans and drugs [5, 6].

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The presence of specific, energy-requiring absorptive and reabsorptive transport mechanisms in gut and kidney respectively [7—9] provides further evidence that maintenance of adequate inorganic sulfate pools is necessary for homeostasis.

Measurements of free sulfate anion in biological fluids are essential to studies of sulfate metabolism. The anion has been measured by precipitation with barium or organic agents such as benzidine. Although we were able to adapt a radiolabelled barium assay to small volumes of serum [10] formidable difficulties arose when we attempted to apply these methods to tissue extracts, a problem noted by several previous investigators [11—13]. This stimulated us to look at an alternative method.

The technique of controlled flow anion chromatography, by which sulfate is separated from attendant anions and quantitated directly by electrical conductance, represents a new method for sulfate estimation in biological samples [14, 15]. It is inherently more accurate than previous methods and it requires only small sample volumes. When modified for use with biological fluids, this method is an effective analytic tool for determination of free sulfate anion in serum, urine, or cerebrospinal fluid (CSF).

The lack of interference from sulfated glycosaminoglycans and other compounds permits assay of 15—30 μl of heparinized plasma samples and allows accurate assay of tissue extracts. We report values for serum and plasma for humans, levels of sulfate in CSF from infants and children, and hepatic tissue concentrations in mouse and humans.

MATERIALS AND METHODS

Apparatus

The D-10 Ion Analyzer (Dionex, Sunnyvale, CA, U.S.A.) was outfitted with 3 × 50 mm concentrator precolumns containing Dionex anion separator resin®, a 3 × 500 mm anion separator column, also containing Dionex anion separator resin®, and a 6 × 250 mm anion suppressor column, in series. A standard eluent, consisting of 2.4 m*M* Na_2CO_3 and 3 m*M* $NaHCO_3$, was utilized and the flow-rate was adjusted to approximately 140 ml/h.

Sensitivity range of the conductance cell was set to 0—1 μS, which is sufficiently low to assure linear correlation between ion concentration and electrical conductance. Electrical output was fed to a linear strip chart recorder and peak height was estimated from the baseline drawn by free hand using vernier calipers.

Reagents

Reagent grade chemicals were purchased from Fisher Scientific (Montreal, Canada) and prepared in deionized water (resistance $\geqslant$ 10 MΩ). Disposable polyethylene tubes, syringes, and pipette tips were used, to minimize contamination by exogenous sulfate.

Biological samples

Blood samples, obtained at random by venipuncture from healthy ambulant adults and hospitalized children were centrifuged and the serums frozen at

−20°C until utilized in further studies. Heparinized plasma (100 μl) was obtained from premature infants between 28 and 36 weeks gestation (kindly provided by Dr. Stanley Zlotkin, Dept. of Nutrition, University of Toronto). Matching serum and CSF samples obtained from patients who required lumbar puncture were kindly provided by Dr. Claire Dupont, Department of Clinical Chemistry, Montreal Children's Hospital.

Serum samples from C57B1/6J adult mice were obtained at decapitation; blood was collected from the neck in serum separator tubes (Microtainer, Becton-Dickinson, Toronto, Canada) centrifuged and stored at 20°C until time of assay.

Preparation of sample for injection

Solutions of Na_2SO_4 diluted in 1.5 ml of deionized water were assayed at the beginning of each run to generate a standard curve. Biological samples were diluted with 1 m*M* NaOH to a final volume of 1—1.5 ml and a final sulfate concentration of 5—25 μ*M*.

Effect of other anions on sulfate peak

To evaluate possible interference from other anions, we determined the peak locations relative to sulfate (R_{SO_4}), and maximum conductances, at physiological serum concentrations, of seven other anions: bromide, urate, citrate, oxalate, ascorbate, malate and succinate. The anions, as sodium salts, were diluted with 1 m*M* NaOH, then chromatographed under standard conditions. The elution profiles of 1 m*M* dehydroepiandrosterone sulfate (DHEA-SO_4) and chondroitin sulfate (1%, w/v) were also determined.

Determination of sulfate by barium precipitation

Inorganic sulfate was measured in 22 serum samples by our modification of the radiolabelled barium method [10] and compared with results obtained on the same samples by controlled flow anion chromatography.

Assay of inorganic sulfate in tissue extracts

We studied liver because sulfate metabolism is prominent in this tissue. Livers were removed from C57B1/6J mice immediately after sacrifice, rinsed briefly with saline, blotted on filter paper, minced, and placed in a chilled Potter-Elvehjem homogenizer. Exactly four volumes of chilled 1 m*M* NaOH were added and the tissue homogenized with at least three strokes. The homogenate was then spun for 10 min at 2000 *g* and the supernatant transferred to tubes and centrifuged at 105,000 *g* for 60 min. Aliquots of the supernatant were diluted ten-fold with 1 m*M* NaOH.

Human liver samples were obtained from frozen autopsy material (courtesy of Dr. T.A. Seemayer and Dr. P. Hechtman). After thawing, they were processed as above with the exception that the final extract was diluted only five-fold prior to chromatography.

Determination of hepatic water spaces

Whole mouse livers were weighed after removal (as described above), then placed in a drying oven at 110°C for 24 h and reweighed to obtain tissue dry

weight and the value for total tissue water. The extracellular space was measured with [^{14}C]methoxyinulin (New England Nuclear, Boston, MA, U.S.A.). Animals were given inulin [0.1 ml/kg body weight as 10% solution (w/v) in saline] containing 10 μCi/ml tracer, at zero time by intraperitoneal injection; followed by subcutaneous injections of 5% inulin (containing 5 μCi/ml tracer) at 20 and 35 min. At about 1 h, when plasma inulin was shown to reach steady state, the animal was sacrificed, serum collected and the liver removed. Samples were counted after tissue solubilization with Protosol (New England Nuclear). Results were corrected for quenching. Intracellular water was calculated by subtracting the extracellular volume from total tissue water.

RESULTS

Sulfate standard calibration

Linear correlation of concentration and measured conductance was obtained in the range 0—25 μM with the sulfate standard. The correlation coefficient was greater than 0.99 in all experiments. The method assays absolute quantities of sulfate as low as 50 ng (ca. 0.5 nmol). Occasionally the regression may not pass through the origin, indicating a small amount of contaminating sulfate (always less than 5% of sample values); the latter may originate from surface adsorption of air-borne sulfate particulates.

The relative elution time during controlled flow elution anion chromato-

TABLE I

RELATIVE RETENTION TIMES AND CONDUCTANCES OF SOME PHYSIOLOGICAL ANIONS ASSAYED BY CONTROLLED FLOW ANION CHROMATOGRAPHY

Anion	R_{SO_4}*	Maximum conductance ($\mu S \times 10^3$)**	Maximum physiological concentration*** (μmol/l)
Urate	—	0	481
Citrate	—	0	135
Succinate	0.52	18	5
Malate	0.54	2§	1
Phosphate	0.56	5960	3000
Bromide	0.65	104	166
Sulfate	1.00	1783	1000§§
Oxalate	1.15§§§	8	31
Ascorbate	1.21§§§	3	80

*Retention time relative to sulfate value of 1.00.

**Conductance for samples of standards at maximum physiological concentrations diluted as for human serum.

***Values for human serum, taken from ref. 16.

§With hemolysis, malate concentration could rise to 5 mmol/l; the corresponding conductance is given.

§§Taken from ref.17.

§§§At high, non-physiological concentrations, these substances emerge in the tail of the sulfate peak (e.g. in urine after ingestion of very large doses of ascorbic acid, unpublished observation).

graphy of seven anions encountered in physiological solution is shown in Table I. Fluoride (a calibration standard) co-elutes with organic anions (results not shown); iodide, another physiological anion, is present only in nanomolar amounts [16] and is therefore not detected by the system. Values for chloride are not shown, since the very large chloride peak is easily identified on all chromatographic runs (Fig. 1).

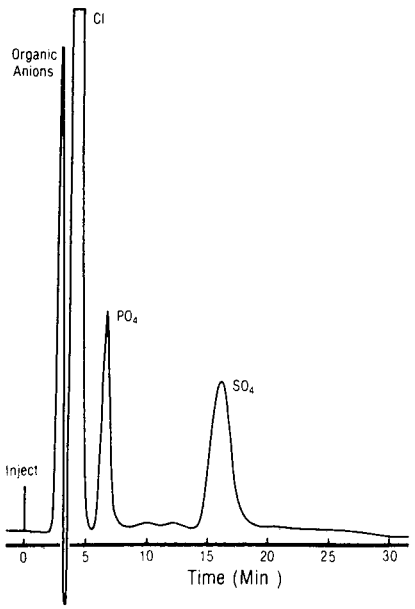

Fig. 1. Elution profile of human infant plasma. A 30-μl aliquot was diluted in 1.5 ml of 1 m*M* NaOH and the whole volume placed in the sample loop. A fraction (100 μl) is injected automatically from the sample loop to the column.

Anions eluting near sulfate do not interfere with its measurement. However, precipitating agents commonly used to remove protein from biological fluids, such as trichloroacetic acid, perchloric acid and sulfosalicylic acid, interfere with the elution profile. In fact, contamination of sulfosalicylic acid with free inorganic sulfate in various lots could be accurately monitored on the ion chromatograph. We omitted protein precipitation and utilized precolumns to prevent pollution of the main separator column and degradation of its resolving power. Precolumns were changed and cleaned after about ten runs (200 samples).

Sulfoesters comprise about 10% of total sulfate in serum, and are presumed to be stable at pH 11 (1 m*M* NaOH) and 24°C. We examined the sulfoesters, DHEA-SO_4 and chondroitin sulfate, and found that both yielded distinct but insignificant peaks co-chromatographing with inorganic sulfate (data not shown). No contamination of the inorganic sulfate elution region was detected at physiological concentrations of sulfoesters (less than 1% of standards chromatographed). We did not detect free sulfate or interfering material in the deionized water effluent from heparinized capillary tubes (Fisherbrand Red Tips) containing porcine intestinal ammonium heparin (2 USP per tube).

Elution profile for human plasma

A typical profile generated by application of dilute human plasma to the D-10 column is shown in Fig. 1. Separate phosphate and sulfate peaks are easily distinguished by their characteristic elution times. Sulfate concentration in heparinized blood plasma is no different from that measured in serum obtained from the same blood sample (data not shown).

Sulfate profiles for CSF, urine and tissue extract were essentially similar (data not shown) although tailing, due to extraneous unidentified compounds, was seen with some samples. Tailing does not effect sulfate quantitation by peak height. We demonstrated this by removing sulfate with excess barium and observing that the extraneous material did not co-eluate at the sulfate peak maximum, but eluated as a small amorphous peak in the tail of the sulfate peak.

Recovery of added sulfate

The average recovery of sodium sulfate added to serum, CSF and tissue extracts was complete (101 ± 3%, mean ± S.E.M., $n = 9$) within the range found in the physiological samples (Fig. 2). Cumulative retention of sulfate on the column during the daily analytical program was not observed.

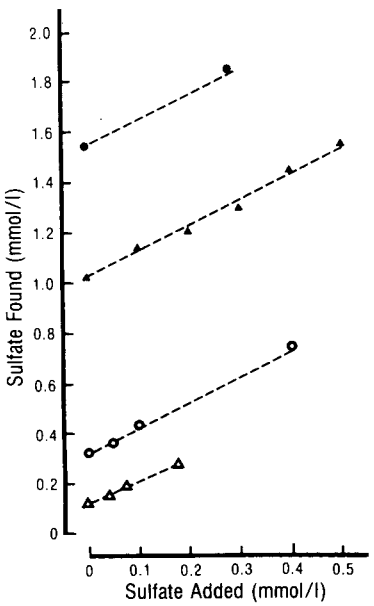

Fig. 2. Recovery of sulfate added to biological fluids. •, Mouse hepatic tissue extract; ▲, mouse serum; ○, human serum; △, human CSF. The dashed lines indicate expected values.

Comparison with barium precipitation method

Measurement of serum sulfate by anion chromatography correlated well with our barium-133 method (Fig. 3) [10] ($r = 0.87$, $p < 0.001$ and not different from unity, $y = 0.987X + 0.003$).

We measured 20 paired samples on different days by the two methods; coefficient of variance was 3.6%, by anion chromatography, and 7.4% for barium precipitation.

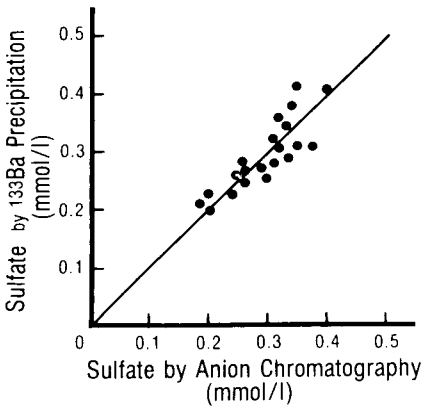

Fig. 3. Comparison of sulfate assayed by the barium-133 precipitation method [10] with assay by controlled flow anion chromatography in 22 serum samples. The line of identity is indicated.

Normal levels of sulfate in CSF and serum plasma

Sulfate concentrations in serum and CSF in man are indicated in Fig. 2, and described for serum and plasma in Table II. Sulfate levels in premature infants were significantly higher than in children and adults ($p < 0.05$, one-way analysis of variance). The data obtained by anion chromatography corroborate those reported earlier for the barium precipitation method [17]. Sulfate levels in CSF were about half those found in the matched serum sample. The difference between the concentrations in the two fluids is highly significant ($p < 0.0001$, paired t-test). Serum sulfate is higher in mouse than in man (Fig. 2) as reported earlier [17].

TABLE II

INORGANIC SULFATE IN HUMAN SERUM, PLASMA AND CEREBROSPINAL FLUID

Sample	n	Sulfate (mmol/l) (mean ± S.D.)
Plasma		
Premature newborns	7	0.41 ± 0.14
Serum		
Children	16	0.33 ± 0.12
Adults	16	0.30 ± 0.05
Cerebrospinal fluid		
Infants and children	9	0.16 ± 0.09
CSF:Serum ratio (matched samples)	9	0.46 ± 0.27

Sulfate concentration in liver

Dry weight of mouse liver was 33 ± 3% wet weight (mean ± S.E.M., $n = 11$); inulin space was 21 ± 6% and cytosol volume, 46 ± 7%. These are expected values. The mean serum sulfate concentration in mice fed normal diet was 1.19 ± 0.03 mmol/l (mean ± S.D.). The corresponding hepatic values ($n = 6$) were:

undiluted tissue extracts, 0.17 ± 0.01 mmol/l; cytosol concentration was 1.23 ± 0.06 mmol/l, or 104 ± 6% of serum. Human liver sulfate (average cytosol concentration) is 0.6 mmol/l (n = 2), or about two-fold greater than the corresponding serum concentration.

DISCUSSION

Controlled flow anion chromatography is a useful assay of free sulfate anion in biological fluids and tissues [15]. Recovery of sulfate at physiological concentrations is satisfactory and the coefficient of variance is half that of the radiolabelled barium method. Absence of interference by other anions and related compounds avoids the difficulties inherent in methods based on barium or benzidine precipitation. The possibility of incomplete precipitation at low sulfate concentrations [10] is avoided. Freedom from the heparin artefact with the chromatographic method permits assay of plasma. The ability to assay small volumes permits study of infants, various body fluids and tissue biopsy material.

We present previously unobtainable data for the premature human infant. Plasma sulfate corresponds to that predicted from our earlier studies [17]. We have also obtained preliminary data on inorganic sulfate in CSF in relation to serum of children. An earlier investigation, in adults only, used a modified benzidine technique [18]; serum levels in that study were unaccountably high.

Liver cytosol sulfate is equivalent to or exceeds extracellular sulfate. Mulder and Keulemans [2] reported that sulfate concentration, determined in rat liver cytosol by an isotope dilution method, was about 94% of the serum concentration, in close agreement with our findings in mouse. Human autopsy liver sulfate exceeds serum levels but we do not yet know whether this is an artefact of the postmortem preparation. Hepatocyte cytosol is normally −60 mV relative to the extracellular space and the Gibbs—Donnan equation predicts an equilibrium intracellular concentration about two orders of magnitude less than that of the extracellular fluid. The relatively high intracellular concentration of sulfate that we and others have found may be the result of active accumulation, compartmentation, binding, or an artefact of methodology. The first alternative is attractive although detailed studies carried out on Ehrlich ascites cells show that equilibrium exchange diffusion serves sulfate uptake and efflux in somatic cells [19, 20]. That tissue sulfate may be over-estimated because of methodological problems seems unlikely. There remains the intriguing possibility that sulfate is accumulated selectively in one intracellular compartment or another. Winters et al. [21] reported that mitochondria rapidly accumulate sulfate, and Crompton et al. [22] characterized the kinetics of transport into those organelles. Whether sufficient quantities could be sequestered by intracellular organelles or bound intracellularly at unspecified sites merits further investigation.

In summary, controlled flow anion chromatography allows accurate assay of inorganic sulfate anion. Analysis of tissue sulfate is also feasible. The method represents a distinct advance over all previous methods. It will allow investigation of sulfate homeostasis in various biological systems in health and disease.

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CHROMBIO. 947

ISOTACHOPHORESIS OF URINARY PURINES AND PYRIMIDINES

THE USE OF SPACERS AND ENZYMES FOR IDENTIFICATION

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SUMMARY

An isotachophoretic system is described for the separation and identification of urinary purine and pyrimidine bases and nucleosides. For a better discrimination and interpretation of the UV profiles, well-defined non-UV-absorbing substances were introduced as spacers. Treatment of urine samples with purified enzymes before analysis resulted in specific shifts in the metabolite profiles, providing a sensitive and specific means of identifying a number of metabolites.

With an injected volume of 3 μl (untreated urine diluted 1 : 5) the present method allows reproducible separations within 20 min of at least twenty different nucleosides and bases.

INTRODUCTION

Considerable progress has been made in the understanding of pathophysiological mechanisms, using analytical techniques that enable the simultaneous identification of metabolites participating in the same metabolic pathway. High-performance liquid chromatography (HPLC) especially has created new possibilities for, for example, the study of inborn errors of purine and pyrimidine metabolism and the pharmacokinetic analysis of purine and pyrimidine drug metabolism (for example, in cancer chemotherapy) [1]. Isotachophoresis

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is another analytical technique that is suitable for monitoring metabolic intermediates [2]. It has been used for screening of purines and pyrimidines in urine and serum [3, 4].

During isotachophoretic separation, the ions (for example, metabolites) are separated in a buffered system according to differences in net mobility. The separated ions form zones, which move consecutively as a train at equal speed, with sharp boundaries between them [2]. In the case of purines and pyrimidines the zones are generally monitored with UV light at 254 nm and/or 280 nm [2–4].

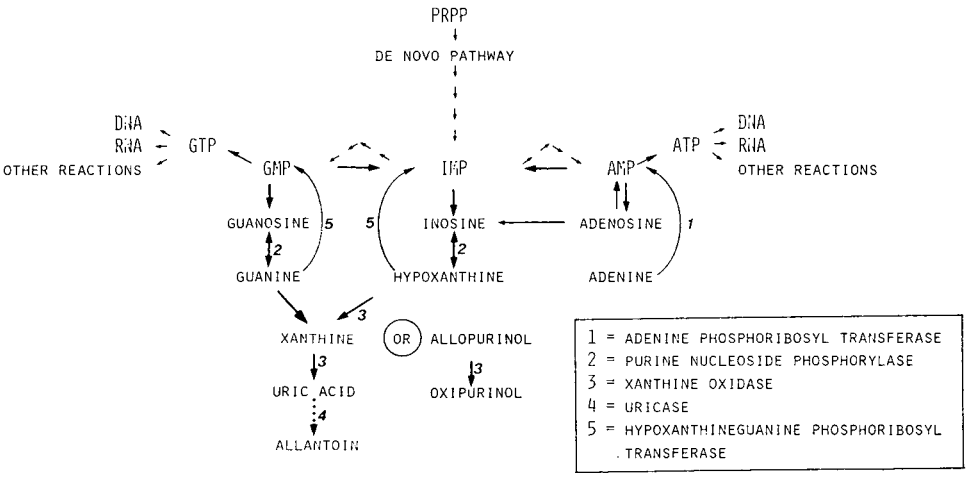

Fig. 1. Simplified scheme of purine metabolism in man.

Problems with the identification and quantification of metabolites may sometimes arise when a given compound forms an extremely small zone or when adjacent zones exhibit similar UV-absorption characteristics. The latter problem can be solved by using conductimetric detection (see Fig. 5 in ref. 5). Another solution might be provided by the use of non-UV-absorbing ionic spacers, which form discriminating zones between UV-absorbing compounds. Preincubation of a sample with a purified enzyme that acts specifically on a compound, might provide another solution. Disappearance of a certain metabolite will be accompanied by the formation of a new metabolite (product of the enzymatic reaction), which in some cases can be identified in the same isotachopherogram.

The purpose of this paper is to communicate the analysis of urinary purine and pyrimidine bases and nucleosides using a system of spacers and enzymes. A number of metabolites and enzymatic reactions relevant to the present study are depicted in Fig. 1.

MATERIALS AND METHODS

Equipment

The analyses were performed with an LKB 2127 Tachophor equipped with a 43-cm PTFE capillary tube (I.D. 0.5 mm) and thermostated at 20°C. The separation was monitored with a UV detector at 254 nm.

Electrolyte system

The operation system, as used in this study, is given in Table I.

TABLE I

OPERATIONAL SYSTEM FOR THE SEPARATION OF PURINE AND PYRIMIDINE METABOLITES BY ISOTACHOPHORESIS

	Leading electrolyte*	Terminating electrolyte*
Anion	Chloride	β-Alanine/OH^-
Concentration	0.005 *M*	0.02 *M*
Counter-ion	Ammediol$^+$	H^+/Ba^+
pH	8.55 ± 0.02	10.4—10.5
Additive	0.3% hydroxyethylcellulose	None

*β-Alanine, ammediol (2-amino-2-methyl-1,3-propanediol), HCl and $Ba(OH)_2$ were all purchased from Merck and were of analytical grade. The first two chemicals were further purified by recrystallization with methanol. Hydroxyethylcellulose was purchased from Polysciences Inc. (Warrington, PA, U.S.A.) and was purified by ion exchange using mixed-bed exchanger No. V (Merck).

Spacers

For better discrimination and interpretation a study was made to test non-UV-absorbing compounds as spacers. Table II shows, on the left, a list of purines and pyrimidines, according to their net mobility, which can be separated by the system described in Table I. On the right-hand side Table II presents a list of non-UV-absorbing compounds. Those that have net mobilities equal to certain purines and pyrimidines are indicated on the same lines. Those compounds that have intermediate net mobilities are listed between the relevant purines and pyrimidines.

Enzymatic identification

The following enzymes were used: PNP (purine-nucleoside phosphorylase EC 2.4.2.1, from calf-spleen) was purchased from Boehringer (Mannheim, G.F. R.); XO (xanthine oxidase EC 1.2.3.2, from buttermilk) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); uricase (EC 1.7.3.3) was purchased from Lφvens Kemische Fabrik (Malmö, Sweden); A-PRT (adenine phosphoribosyltransferase EC 2.4.2.7) was purified according to the method of Hershey and Taylor [6].

As co-substrates for the PNP and A-PRT reactions ribose-1-phosphate (Boehringer) and phosphoribosylpyrophosphate (Sigma), respectively, were used.

For enzymatic identification 100 μl of diluted (1 : 5) urine were incubated for various periods of time at 37°C. For the PNP incubation 3 μl of enzyme-solution were added in the presence of 0.5 m*M* ribose-1-phosphate; incubation time was 2.75 h. For the A-PRT reaction 3 μl of purified enzyme fraction were added in the presence of 1 m*M* phosphoribosylpyrophosphate; incubation time was 2 h. For the XO reaction 3 μl of enzyme solution were used; incubation time was 3 h. For the uricase reaction 6 μl of enzyme solution were added; incubation time was 22 h.

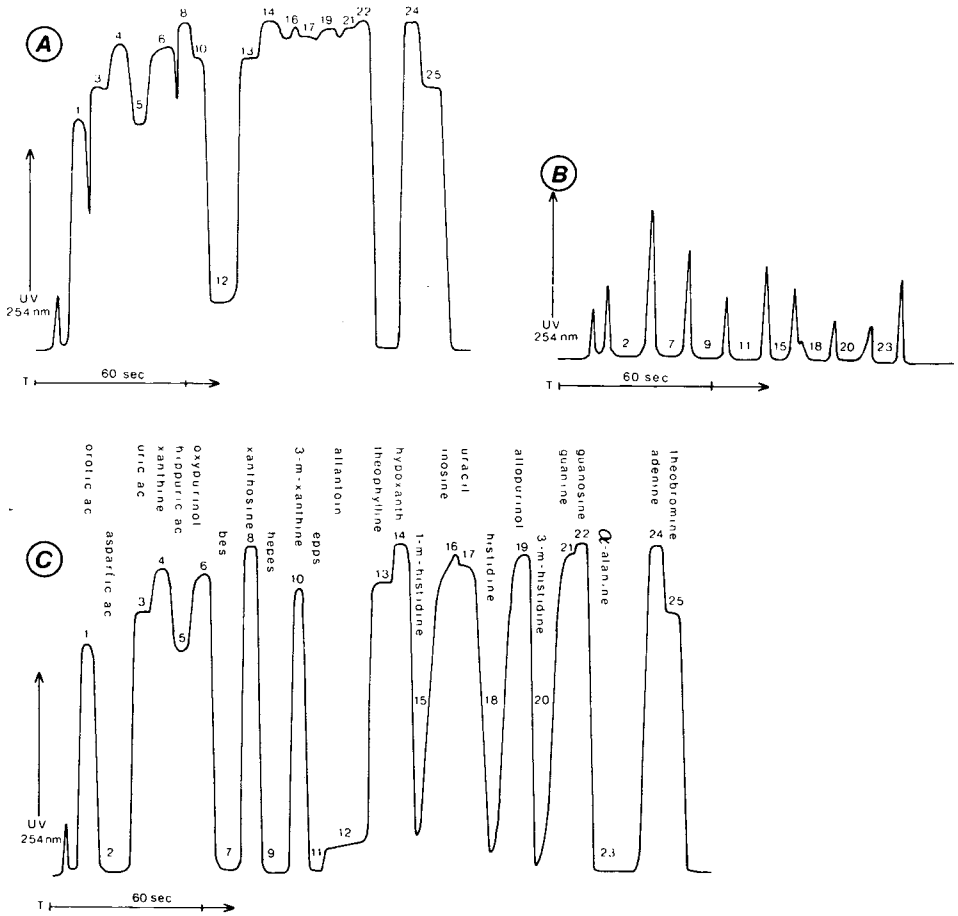

Fig. 2. The isotachophoretic analysis of 17 UV-absorbing compounds (A), 8 non-UV-absorbing spacers (B) and a mixture of the two types of compounds (C). 1 = orotic acid; 2 = aspartic acid; 3 = uric acid; 4 = xanthine; 5 = hippuric acid; 6 = oxypurinol; 7 = BES; 8 = xanthosine; 9 = HEPES; 10 = 3-methylxanthine; 11 = EPPS; 12 = allantoin; 13 = theophylline; 14 = hypoxanthine; 15 = 1-methylhistidine; 16 = inosine; 17 = uracil; 18 = histidine; 19 = allopurinol; 20 = 3-methylhistidine; 21 = guanine; 22 = guanosine; 23 = α-alanine; 24 = adenine; 25 = theobromine. The zone-lengths represent approx. 1.5 nmol of each substance. For abbreviations see Table II.

RESULTS

An operational electrolyte system that gives reproducible separations of urinary nucleosides and bases is given in Table I, the leading ion being Cl^-, the counter-ion ammediol and the terminating ion β-alanine. Analysis time is approximately 20 min. In order to speed up the analysis, during the initial separation a driving current of 180 μA was applied; during the last 15 min the current was 40 μA. The injected volume of the diluted* (1 : 5) urine sample was

*Dilution guarantees that certain substances, for example urate and oxalate, which might be present as precipitates will redissolve.

TABLE II

PURINES AND PYRIMIDINES AND A NUMBER OF NON-UV-ABSORBING COMPOUNDS, LISTED ACCORDING TO THEIR RELATIVE NET MOBILITIES

Purines and pyrimidines	Compounds* useful as spacers (non-UV-absorbing)	Non-UV-absorbing compounds**
Orotic acid (1)**		Capronic acid/isobutyric acid
	Aspartic acid (1)	
Uric acid (1)		Caproic acid/glutamic acid/ACES
Xanthine (1)		
	MES (2)	
Hippuric acid (1)		
Oxypurinol (3)		MOPS
Orotidine (1)		
	BES (1)	
Xanthosine (1)		Cystine/TES
	HEPES (1)	
3-Methylxanthine (1)		TRICINE
	EPPS (1)	TAPS
Allantoin (1)		
Theophylline (1)		
Hypoxanthine (2)		Asparagine
	1-Methylhistidine (1)	
Inosine (2)		Serine
d-Inosine (1)		
Uracil (1)		
	Histidine (2)	Methionine/glutamine/cysteine
Pseudouridine (1)		
Allopurinol (3)		
	3-Methylhistidine (1)	
Guanine (2)		
Uridine (1)		CHES
Guanosine (1)		
d-Guanosine (1)		
	α-Alanine (2)	Glycine/leucine/valine
Adenine (2)		
Theobromine (1)		
		β-Alanine/OH^- (terminator)

*Chemicals were purchased from Sigma (1), Merck (2) and Wellcome (London, Great Britain) (3).

**ACES = 2-(2-amino-2-oxyethylamino)ethanesulfonic acid; BES = N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid; CHES = 2-(N-cyclohexylamino)ethanesulfonic acid; EPPS = N-2-hydroxyethylpiperazinepropanesulfonic acid; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES = 2-morpholinoethanesulfonic acid; MOPS = morpholinopropanesulfonic acid; TAPS = tris-(hydroxymethyl)-methylaminopropanesulfonic acid; TES = N-[tris-(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; TRICINE = N-[tris-(hydroxymethyl)-methyl]-glycine. All chemicals were of analytical grade.

3 μl. No further sample pretreatment was carried out. The non-UV-absorbing compounds that were found to be useful as spacers, are listed in italics in Table II.

Two different analyses of the same standard mixture are shown in Fig. 2. In Fig. 2A a separation without spacers is shown; Fig. 2C shows that with the

spacers the various UV-absorbing zones are more clearly visibly separated from each other. The UV trace of the blank run, i.e. the analysis of the spacer mixture, is shown in Fig. 2B. The standard mixture contained 17 UV-absorbing compounds and 8 spacers (Fig. 2C). Deoxyinosine, uridine and deoxyguanosine can also be separated; in the electrolyte system used (Table I) these compounds form adjacent zones behind inosine, guanine and guanosine, respectively. No appropriate spacers have been found until now. Discrimination of deoxyinosine and deoxyguanosine is possible with conductimetric detection. Unidentified

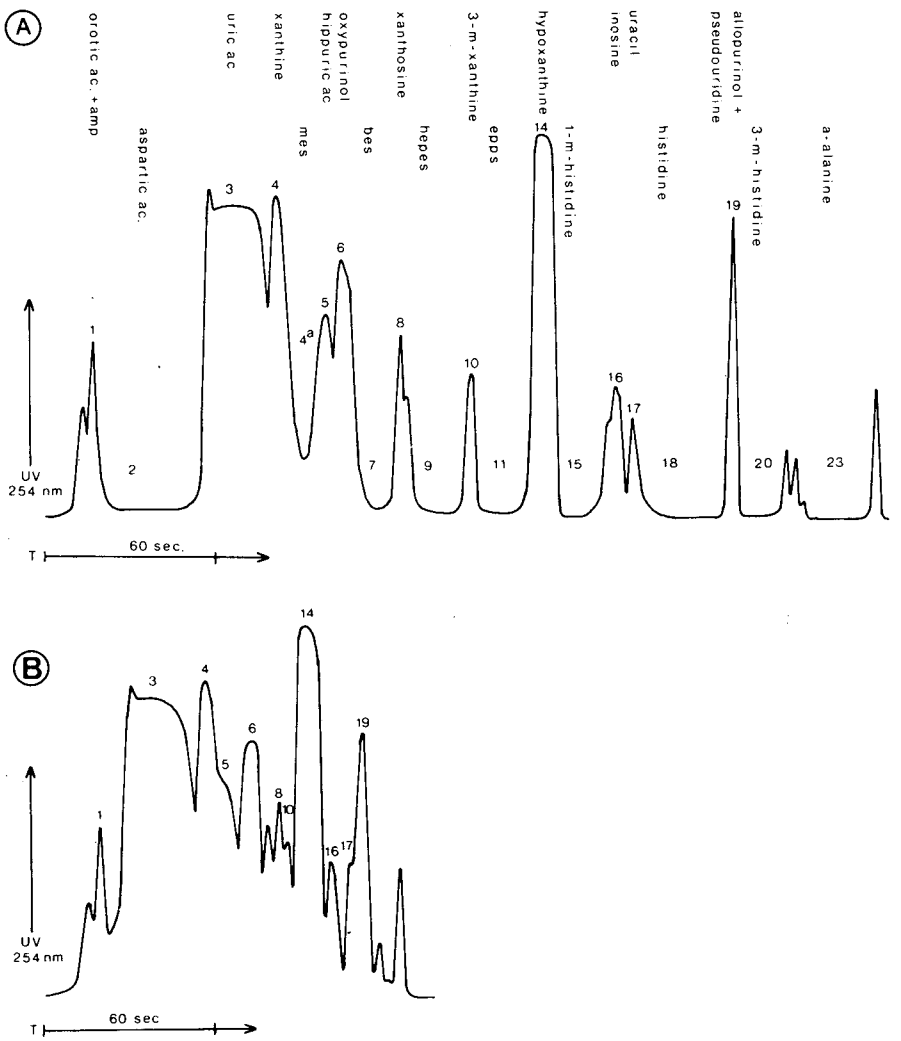

Fig. 3. UV traces of isotachophoretic analyses of urine from a Lesch—Nyhan patient under allopurinol treatment: (A) with spacers; (B) without spacers. Injected were 3 μl of urine (diluted 1:5) and 1 μl of spacer-mixture [a solution of aspartic acid (4 m*M*), MES (1 m*M*), BES (1.5 m*M*), HEPES (2 m*M*), EPPS (2 m*M*), 1-methylhistidine (2 m*M*), histidine (3 m*M*), 3-methylhistidine (2 m*M*) and α-alanine (2 m*M*)]. The numbered peaks are identified in Fig. 2.

substances normally occurring in urine can function as spacers [3]. In the case of uridine, the extinction ratio or the transmission ratio (for example, 280 nm and 254 nm) can also be used for identification. Pseudouridine forms a "steady-state" mixed zone with allopurinol in urine (Fig. 3). Adenosine and deoxyadenosine do not migrate in the electrolyte system (Table I) in the isotachophorese stack (leading electrolyte—terminating electrolyte configuration) and consequently will not be detected. Severe deficiency of hypoxanthine—guanine phosphoribosyltransferase (HG-PRT, EC 2.4.2.8; see also Fig. 1) is mostly associated with a neurologic disease known as the Lesch—Nyhan syndrome [7]. One of the metabolic disturbances in this disease is increased purine

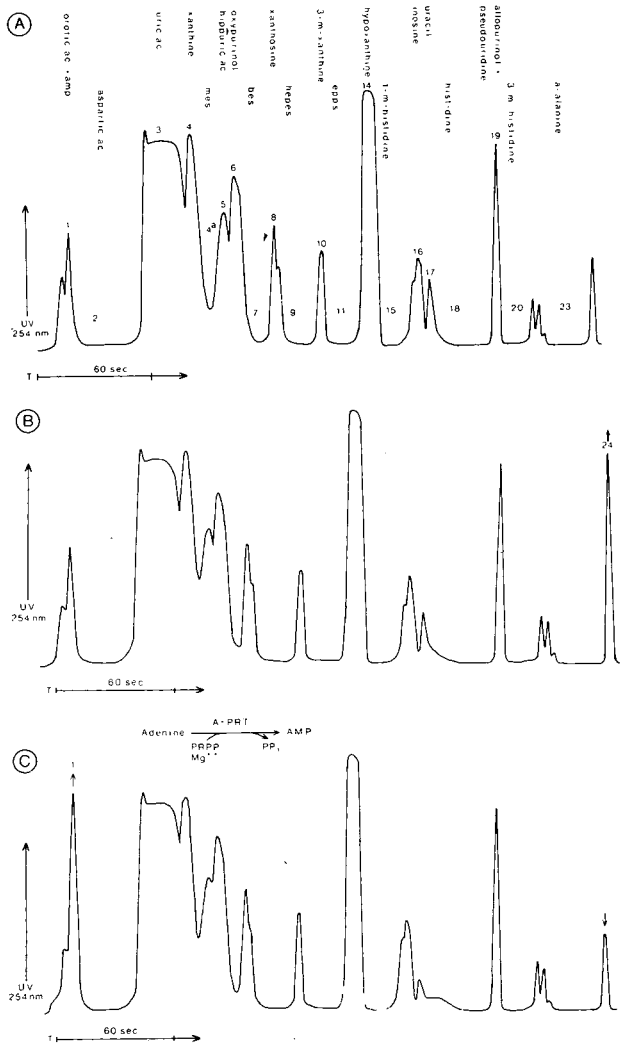

Fig. 4. Analysis of urine of a Lesch—Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after adding 50 μM adenine to the urine; (C) after preincubation of the same urine as in (B) with A-PRT. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

biosynthesis, resulting in hyperuricemia [8]. Consequently, urine from a Lesch—Nyhan patient contains a higher amount of uric acid as compared to urine from a normal control [3]. Fig. 3 shows UV scans of urine from a Lesch—Nyhan patient under allopurinol treatment. This drug reduces the amount of uric acid formed by inhibiting the xanthine oxidase reaction (see Fig. 1). This leads to the accumulation of the more soluble purine bases xanthine and hypoxanthine [3]. Allopurinol itself is converted to oxipurinol by xanthine oxidase, whereas small amounts of unchanged allopurinol are also excreted [3] (Fig. 3A and B). Addition of the various spacers allows a better discrimination between purines and pyrimidines (Fig. 3A). In Fig. 3B a run without any extra spacers is shown. In all further analyses the urine of the same allopurinol-treated Lesch—Nyhan patient was used.

Preincubation of a Lesch—Nyhan urine sample with purified A-PRT did not result in the disappearance of the zone at the place where adenine would be expected: a UV trace identical to that in Fig. 3A was obtained (Fig. 4A). Extra addition of adenine (50 μM) resulted in a clearly increased zone-length (Fig. 4B). After preincubation of this sample with A-PRT the adenine added was converted to AMP (Fig. 4C; see also Fig. 1). The original zone (Fig. 4A) was still present, indicating that this zone is definitely not adenine. PNP converts

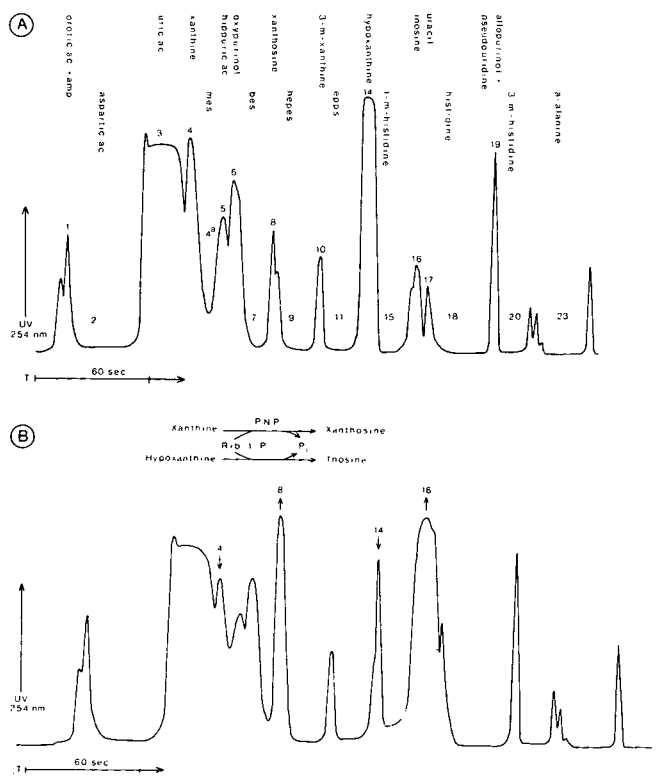

Fig. 5. Analysis of urine of a Lesch—Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after preincubation of the urine with PNP. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

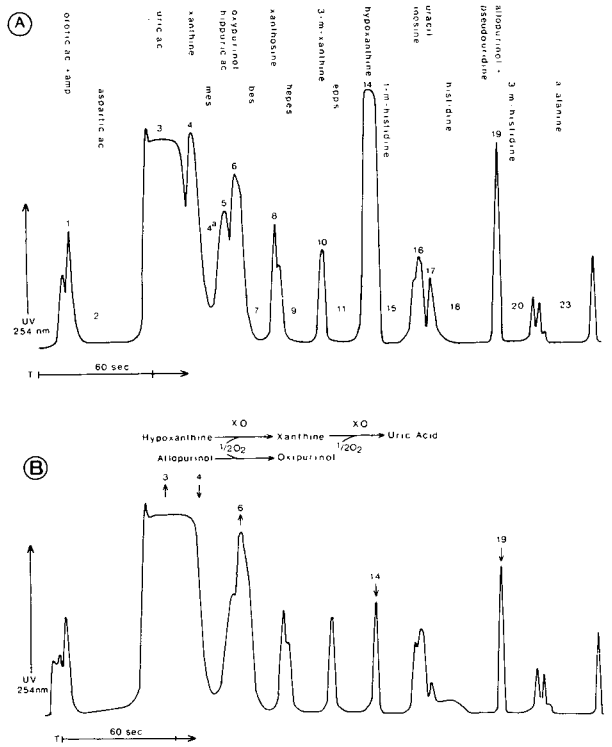

Fig. 6. Analysis of urine of a Lesch—Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after preincubation of the urine with XO. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

hypoxanthine, xanthine and guanine in the presence of ribose-1-phosphate to inosine, xanthosine and guanosine, respectively (Fig. 1). The hypoxanthine zone decreased after preincubation with PNP, whereas the inosine zone increased*; the xanthine present was converted to xanthosine (Fig. 5A and B). No guanine was detected (Fig. 5A) and consequently no formation of guanosine was observed (Fig. 5B).

Xanthine oxidase acts on hypoxanthine and xanthine to form uric acid (Fig. 1). Both purine bases are present in the Lesch—Nyhan urine sample tested (Fig. 6A) and both disappeared after preincubation with xanthine oxidase (Fig. 6B). The uric acid zone increased (Fig. 6A and B). The free allopurinol which is present in the urine of the Lesch—Nyhan patient (Fig. 6A) is converted to oxipurinol by the action of xanthine oxidase (Fig. 6B).

The uricase reaction, which does not normally occur in human cells, removes the uric acid zone from the UV trace, giving rise to the formation of allantoin (Fig. 7B; see also Fig. 1), which runs ahead of hypoxanthine. It can be seen that after 22 h of preincubation uric acid is completely converted to allantoin (Fig. 7B).

*This zone is suspected of containing another UV-absorbing compound, which has not yet been identified.

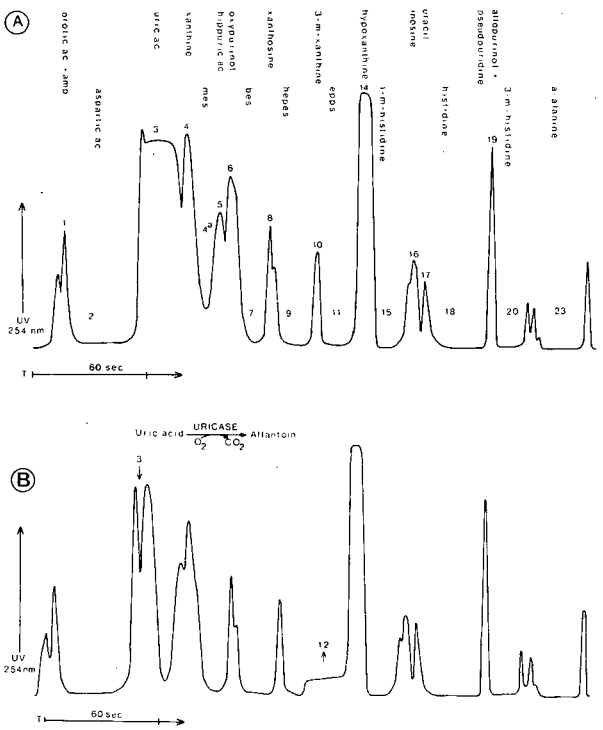

Fig. 7. Analysis of urine of a Lesch—Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after preincubation of the urine with uricase. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

DISCUSSION

The isotachophoretic separation system presented in this paper offers a simple and rapid means of determining urinary purines and pyrimidines. The reproducibility is sufficiently high, variation coefficients being below 2%. In the present study, samples (ca. 3 μl) containing 17 nucleosides and bases of purines and pyrimidines could be separated conveniently within 20 min.

To obtain optimal results with the system given in Table I, several points should be considered: (1) the terminating electrolyte should be prepared freshly every day, filtered through a 0.22-μm Millipore filter and stored until use in closed electrolyte reservoirs (syringes) at room temperature; (2) after each run the terminator compartment must be emptied completely and refilled with fresh terminator; (3) the pH of the leading electrolyte should be checked every two runs and eventually be adjusted to pH 8.55 with ammediol. At a slightly deviating pH a poor separation of xanthine, hippurate and oxipurinol was obtained; (4) the counter-electrode compartment contains 5 m*M* HCl—ammediol (pH 8.55) (without hydroxyethylcellulose), and should be replenished every 4—5 runs; (5) the sample should be injected carefully into the leading electrolyte, due to the high pH of the terminating electrolyte.

This study concentrates on the analysis and identification of a number of purines and pyrimidines by means of spacers and enzymatic shifts. No attempts

were made to quantify the amounts of the various compounds. However, this can be done conveniently by measuring the integrated UV-absorbance peak area or zone length of a given compound [9].

The UV tracing of the electrolyte system, with spacers (Fig. 2B), showed several UV-absorbing and non-UV-absorbing zones, as could be anticipated. These compounds will also feature in the metabolite profiles and should accurately be discriminated from possible coincident sample zones. As evidenced by the findings shown in Fig. 3A and B, the use of non-UV-absorbing compounds as spacers (Table II) facilitates the interpretation of the metabolite profiles.

Possibilities of identifying a UV-absorbing compound include measurement of extinction ratio $\epsilon_{280}/\epsilon_{254}$ [3], and addition of the presumed compound ("spiking") and measuring the step height from the conductivity signal [2, 9, 10]. From Figs. 4—7 it follows that a specific and sensitive alternative is the enzymatic conversion of metabolites by purified enzymes.

The present isotachophoretic technique allows routine analyses of urinary purines and pyrimidines with a high degree of simplicity and reproducibility, for both diagnostic and experimental purposes.

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CHROMBIO. 949

QUANTITATIVE DETERMINATION OF THE CHOLINESTERASE INHIBITOR PHYSOSTIGMINE IN BRAIN TISSUE SAMPLES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method, based on a dynamic cation-exchange system was used for the determination of physostigmine in brain tissue extracts. The precision and detection limit of the method as well as the extraction efficiency were established. The distribution of physostigmine over several parts of the brain after intravertebral application is reported.

INTRODUCTION

Previous pharmacological experiments have demonstrated that physostigmine changes haemodynamic parameters in the anaesthetized cat. The cardiovascular effects evoked could be attributed to an action of the drug upon the pontomedullary region [1]. In order to localize the site of action and to study the kinetic behaviour of the drug within the central nervous system, a sensitive, precise and accurate analytical method is necessary for the measurement of drug concentrations. Moreover, such a method would make studies concerning concentration—activity relationships possible. Until now quantitative methods are only available to determine physostigmine in pharmaceutical preparations [2, 3]. The fluorimetric determination of physostigmine in tissues as described by Laverty et al. [4] and Taylor [5] was not sensitive enough for the detection of the low drug amounts present in brain tissue after the administration of pharmacologically relevant doses.

In the present study a suitable quantitative method for the determination of physostigmine in brain tissue of the cat using high-performance liquid chromatography (HPLC) is described. Moreover, the distribution of the drug within

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the brain following intravenous administration and infusion via the left vertebral artery is discussed.

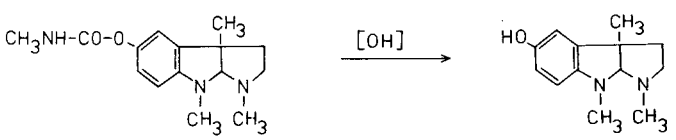
EXPERIMENTAL

Apparatus

The liquid chromatograph comprised a reciprocating pump (LC3-XP, Pye-Unicam), a high-pressure injection valve (Rheodyne 7105) equipped with a 175-μl sampling loop, and a variable-wavelength UV detector (Pye-Unicam LC3). The wavelength was set at 245 nm. All chromatograms were recorded on a linear potentiometric recorder (Servogor RE 542). In all experiments stainless-steel 316 columns, with the dimensions 125 × 3 mm, were used.

Materials

In all experiments double-distilled water was used. All solvents and chemicals were of analytical grade and used without further pretreatment. Sodium dodecyl sulphate (SDS) was obtained from Merck (Darmstadt, G.F.R.). The column was packed with octyl-modified silica (LiChrosorb RP-8, Merck) with a mean particle size of 5 μm. Physostigmine sulphate was obtained from BDH (Poole, Great Britain). The structure of the hydrolysis product of physostigmine is represented in Fig. 1.



physostigmine eseroline

Fig. 1. Physostigmine and its degradation product.

Chromatography

The HPLC columns were packed by a pressurized balanced slurry method [6]. The slurry liquid consisted of a mixture of chloroform and tetrabromomethane of specific gravity 1.82 for the alkyl-modified silica (RP-8). After packing, the columns were washed with 100 ml of methanol and subsequently equilibrated with the eluent until constant retention of physostigmine was obtained. A standard solution of physostigmine sulphate was freshly prepared from a stock solution, containing 4.7 mg per 100 ml of methanol, and was stored at 4°C. The mobile phase used for the analysis of physostigmine in extracts of brain tissue consisted of 0.005 *M* NaH_2PO_4, 0.3% (w/v) SDS and 0.1 *M* $NaClO_4$ in 500 volumes of methanol and 500 volumes of water (pH 3.00). The flow-rate was 0.60 ml/min.

Administration of drugs

Mongrel cats of either sex (weight 2—4 kg) were anaesthetized with 60 mg of α-glucochloralose per kg, given intraperitoneally. After inserting a tracheal canula the animals were subjected to artificial respiration. The intravenous application of physostigmine was carried out by the infusion of the drug into a femoral vein in a volume of 140 μl during 1 min. For central application

physostigmine was infused into the left vertebral artery in a total volume of 140 μl during 1 min. For a detailed description of the vertebral artery model the reader is referred to Van Zwieten [7]. After administration via the left vertebral artery the drug will accumulate mainly within the right part of the brain stem [8, 9]. In order to obtain a more uniform distribution pattern within the pontomedullary region the opposite (right) vertebral artery was ligated [10]. After left-sided thoracotomy the aorta was ligated. At different intervals after dosing the circulation was arrested by occluding the aorta. Immediately afterwards, various brain regions were isolated on ice and weighed.

Sample preparation

Both the medulla oblongata and pons were divided into a left and a right part. Subsequently, the hypothalamus and a part of the cortex (gyrus marginalis) were isolated. After weighing, an homogenate 20% (w/v) was prepared in appropriate glass tubes with Teflon pads at 0°C, using the mobile phase as the homogenising fluid. The mixture was centrifuged at 68,000 *g* for 15 min at ca. 2°C. The clear supernatant was stored for 24 h at 4°C, filtered through a porous metal frit (2 μm) and 100 μl of the clear filtrate were injected.

RESULTS AND DISCUSSION

Chromatography

A cation-exchange system, dynamically generated by addition of SDS to the mobile phase, was chosen as the separation mode for the analysis of physostigmine in brain tissue extracts. Dynamic cation-exchange systems have been found to be very attractive for the separation of amino acids [11] and basic compounds such as tricyclic antidepressants [12], catecholamines [13], butyrophenones and diphenylalkylpiperidines [14]. Compared with a normal reversed-phase system the dynamic cation-exchange system shows a greater flexibility with respect to selectivity. In complex samples such as extracts of brain tissue this system has definite advantages for the analysis of small

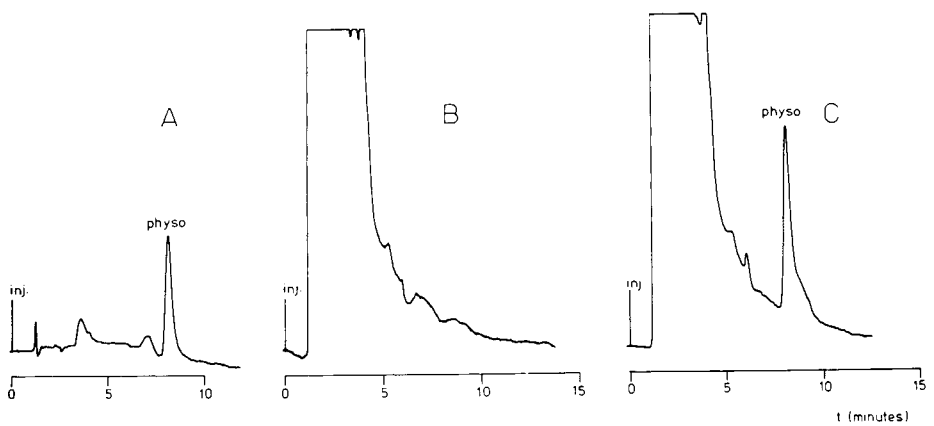


Fig. 2. Chromatograms of (A) a standard amount of physostigmine, (B) a blank brain tissue sample, and (C) a tissue sample obtained after a pharmacological experiment, containing physystigmine (10.7 ng/mg).

amounts of basic drugs. Moreover, columns with a greater theoretical plate height are obtained [12, 13]. Furthermore, the pH of the mobile phase can be adjusted to obtain minimal hydrolysis of drugs containing labile structures such as physostigmine (Fig. 1) without any loss of column efficiency. In general, minimal hydrolysis of ester-type drugs occurs often between pH 3 and 5 [15].

In order to determine the optimal chromatographic conditions for the separation of the solutes the capacity ratio of solutes as a function of a number of parameters, i.e. organic modifier, SDS, Na^+ and H_3O^+ concentrations in the mobile phase, were investigated. As was expected physostigmine behaves like a cationic solute in the chosen system. In Fig. 2 some typical examples of chromatograms are represented.

Quantitative aspects

The precision and linearity of the method were determined by injecting 50-μl or 100-μl solutions of physostigmine in different concentrations. The linear regression of peak height versus injected amount (10—500 ng) of physostigmine yielded a correlation coefficient of 0.9994, which indicates a high degree of linearity.

The precision of the method was estimated by repeated injections (n = 5) of 50 ng of solute dissolved in mobile phase. The standard deviation was found to be 1.8 ng (3.6%).

The peak-to-peak value of the baseline noise was determined to be $5 \cdot 10^{-5}$ a.u. This leads to a calculated limit of detection of 2 ng of physostigmine for a signal-to-noise ratio of 3. This corresponds to a theoretical, calculated limit of detection of 100 ng per gram of brain tissue for an injection volume of 100 μl.

Efficiency of the extraction

The recovery and reproducibility of the extraction have been determined. Thus, different amounts of physostigmine were added to blank brain tissue and subsequently extracted as described above. The efficiency of extraction was 89.9 ± 7.2%.

Distribution of physostigmine in different brain regions after intravenous administration and infusion into the left vertebral artery

The method described above for the quantitative analysis of physostigmine was applied to brain tissue samples of cats that had received physostigmine intravenously and via the left vertebral artery. The latter animal model is often used for the study of drug actions upon the pontomedullary region [7].

Table I represents drug concentrations in different brain regions following intravenous administration of $1 \cdot 10^{-6}$ mol kg^{-1} physostigmine (ca. 270 μg of physostigmine base per kg). Concentrations were established in the medulla oblongata, pons and in the higher brain structures like the hypothalamus and cortex, 5 min after dosing. At that time the pharmacological effect, i.e. the hypotensive effect, was maximal. The results indicate that the drug is equally distributed in the brain; similar amounts of drug per gram of tissue are found in the left and right parts of the pontomedullary region, hypothalamus and cortex. This observation is in agreement with distribution studies in cats after systemic application of clonidine [16], isoarecaidine propyl ester [17] and R28935 [18].

TABLE I

DISTRIBUTION OF PHYSOSTIGMINE IN DIFFERENT BRAIN REGIONS AFTER INTRAVENOUS ADMINISTRATION

Physostigmine, $1 \cdot 10^{-6}$ mol kg^{-1}, was administered intravenously. Results are expressed as the mean ± S.E.M., $n = 4$.

Brain part*	ng per brain part	ng per gram wet tissue	Percentage of the administered dose
R pons	168 ± 20	559 ± 51	0.022 ± 0.003
L pons	138 ± 9	510 ± 46	0.017 ± 0.001
R medulla oblongata	350 ± 31	542 ± 41	0.044 ± 0.003
L medulla oblongata	348 ± 29	546 ± 50	0.044 ± 0.003
Hypothalamus	148 ± 8	492 ± 35	0.018 ± 0.001
Cortex	—	543 ± 52	—
Brain stem (total)			0.127 ± 0.003

*R = right; L = left.

TABLE II

AMOUNTS OF PHYSOSTIGMINE IN VARIOUS BRAIN REGIONS AT DIFFERENT TIME INTERVALS FOLLOWING INFUSION INTO THE VERTEBRAL ARTERY

Amount of physostigmine infused was $3 \cdot 10^{-8}$ mol kg^{-1}. Values are expressed as the mean ± S.E.M., $n = 5$.

Brain part*	Time after infusion					
	2 min		5 min		30 min	
	ng per brain part	ng per gram wet tissue	ng per brain part	ng per gram wet tissue	ng per brain part	ng per gram wet tissue
R pons	193 ± 64	512 ± 188	166 ± 40	582 ± 185	n.d.	n.d.
L pons	255 ± 83	750 ± 292	176 ± 50	648 ± 214	n.d.	n.d.
R medulla oblongata	535 ± 115	791 ± 207	660 ± 129	1048 ± 238	n.d.	n.d.
L medulla oblongata	1932 ± 266	2638 ± 463	2241 ± 321	3349 ± 497	n.d.	n.d.
Hypothalamus	n.d.†	n.d.**	n.d.	n.d.***	n.d.	n.d.
Cortex	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*R = right; L = left.
**120 ng per gram of tissue measured in one cat; in the other cats no detectable amounts of physostigmine.
***In two cats 192 ng and 137 ng of physostigmine per gram of tissue; no detectable amounts in the other cats.
†n.d. = not detectable (< 120 ng per gram of tissue).

In Table II the distribution of the carbamate is shown in the various brain parts after injection of the drug ($3 \cdot 10^{-8}$ mol kg^{-1}, ≡ 8.3 μg of base per kg) into the vertebral artery. The drug concentrations were determined 2, 5 and 30 min following application of physostigmine. Most of the drug accumulates in

the medulla oblongata and pons whereas the higher brain parts like the hypothalamus and cortex contain no detectable amounts or insignificant amounts of the drug. Similar observations have been described for other drugs [16, 19].

According to the results of El Sherbini-Schepers and Van Zwieten [10], we found a similar discrepancy in drug concentration between the left and the right part of the medulla oblongata (a ratio of between 3 and 4) after administration of the drug via the left vertebral artery. These findings illustrate the validity of the developed HPLC method for physostigmine. The drug could not be detected 30 min after application of physostigmine. This suggests a rapid elimination of the drug (possibly due to redistribution and hydrolysis) from the lower brain stem. In a separate paper (to be published) the brain concentrations are discussed in relation to the depression of acetylcholinesterase and the haemodynamic effects induced by physostigmine.

Finally, it is concluded that this newly developed HPLC method is sensitive, relatively simple and is suitable for the determination of physostigmine in brain tissue with respect to pharmacological experiments.

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REVERSED-PHASE CHROMATOGRAPHY OF URINARY METABOLITES OF PARACETAMOL USING ION SUPPRESSION AND ION PAIRING

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SUMMARY

High-performance liquid chromatography (HPLC) has proven particularly useful for the study of paracetamol metabolism. Two alternative methods were developed using reversed-phase C_{18} columns. A rapid ion suppression technique was used for the analysis of free paracetamol, paracetamol mercapturic acid and cysteine conjugate in urine samples obtained from isolated perfused rat kidney preparations, which has conveniently demonstrated the oxidative metabolic capacity of the kidney towards paracetamol. A somewhat longer, but higher resolution, ion-pair HPLC procedure was developed for the analysis of paracetamol metabolites in urine samples from experimental animals. The ion-pairing solvent was composed of tetrabutylammonium hydroxide, Tris and EDTA buffered to pH 7.2 with phosphoric acid. Gradient programming was further used to enhance resolution. Using this system two new metabolites, the sulphate and glucuronide conjugates of 3-thiomethylparacetamol were detected and routinely determined along with other known paracetamol metabolites, viz. free paracetamol, paracetamol sulphate, glucuronide, mercapturic acid, and cysteine conjugates, 3-methoxyparacetamol glucuronide and sulphate, *p*-aminophenol and its O-glucuronide and O-sulphate conjugates. Phenolic O-substituted glucuronide and sulphate conjugates of N-hydroxyparacetamol were also separated.

INTRODUCTION

The toxicity of many drugs is a result of their biotransformation to toxic metabolites and this is well illustrated by the metabolism of paracetamol [1]. To understand the mechanisms involved it is useful to correlate the pattern of excreted metabolites with the toxicity in experimental animals which are chosen so as to exhibit variations in both the site and extent of toxicity. Having chosen specific animal models it is then essential to develop rapid and reliable methods by which the drug and its metabolites may be separated and quantitated.

When one considers the array of known paracetamol metabolites [2—7] (Fig. 1) it is obvious that many different compound types are present: highly

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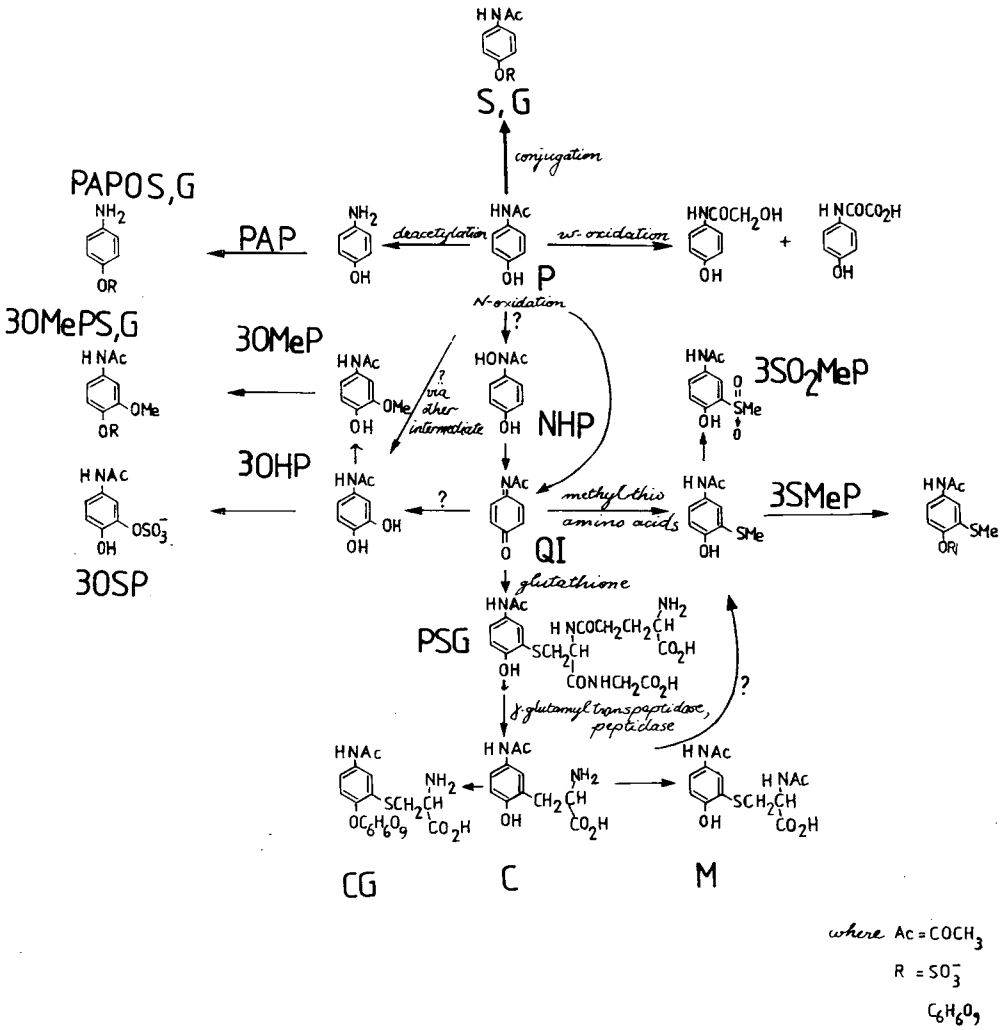


Fig. 1. Paracetamol metabolites. Major excretory products are paracetamol sulphate potassium salt (S), paracetamol-β-D-glucuronic acid (G), paracetamol (P), paracetamol mercapturic acid (M), and paracetamol cysteine conjugate (C) [2—5]. 3-Hydroxylated metabolites, 30MePS, 30SP and 30MePG have been reported by Andrews et al. [4]. Glycolyl and oxanilic acid derivatives formed by ω-oxidation have also been observed [6]. 3-Thiomethyl-substituted paracetamol (3SMeP) has been observed both as the sulphone ($3SO_2MeP$) [3] and conjugated with glucuronic acid and/or sulphate [7]. The double conjugate CG was also postulated [5]. Conjugates of *p*-aminophenol (PAPOS, PAPOG) would be expected if deacetylation occurs.

water soluble conjugates of paracetamol and its oxidized metabolites, the relatively less polar nonconjugated products including N-hydroxyparacetamol [8] which has been considered until recently to be the precursor of the reactive intermediate, N-acetyl-*p*-benzoquinone imine [9], and finally amino acid conjugates derived from glutathione condensation with the reactive intermediate [10]. In order to separate all of these compound types in one separation,

reversed-phase high-performance liquid chromatography (HPLC) offered the largest scope over conventional methods of thin-layer chromatography (TLC) and ion-exchange chromatography. Bonded reversed-phase packings were most suitable since columns packed with these materials are eluted with aqueous eluents. This allows direct application of biological fluids without preliminary extraction. However under typical conditions of reversed-phase chromatography using neutral eluents, e.g. water—methanol mixtures, only those compounds which are non-ionic are retained [11].

This problem is avoided by modifying the eluents used so as to render the ionic conjugates non-polar. This can be achieved in two ways. Firstly by pairing the anionic conjugate with a bulky non-polar cation such as tetrabutylammonium ion (TBA). Ion pairs so formed are well retained by the column and excellent selectivity can be obtained by the choice and concentration of pairing ion [12]. Alternatively the pH of the eluent may simply be reduced so that the non-ionic free acid forms of the conjugates are retained by the column [11]. The latter technique is known as ion suppression.

Buckpitt et al. [13] have illustrated all three reversed-phase HPLC separations for the specific separation of 3-substituted paracetamol metabolites: paracetamol mercapturic acid (M), paracetamol cysteine conjugate (C) and the glutathione conjugate (PSG). These separations while useful for the specific study described, were limited for the full range of urinary metabolites.

Howie et al. [14] and Knox and Jurand [15] used an ion suppression solvent of dilute acetic acid solution containing 15% methanol to investigate urinary excretion of paracetamol metabolites over a 24-h period. While they concluded that excellent separation was achieved the early section of their chromatograms allowed little definition of glucuronide and sulphate conjugates from endogenous urine components. Thus quantitative estimation of these particular conjugates was made difficult. In their study, Knox and Jurand [15] confirmed the presence of 3-methoxyparacetamol sulphate as a human paracetamol metabolite [4]. However, separation of the sulphate conjugates of paracetamol and its 3-methoxy analogue was insufficient, so the authors turned their attention to ion-pairing reagents [16]. Dioctylamine and tetrabutylammonium were both evaluated as pairing ions. Separations with these additives were a major improvement over those using ion suppression because increased retention of paracetamol sulphate enabled the resolution of other possible sulphate metabolites. However, the separation of paracetamol-β-D-glucuronic acid (G) and C was not entirely satisfactory at the front of the chromatograms particularly with the TBA columns where endogenous urine constituents appear to be difficult to resolve from the metabolites.

In the present study the techniques of ion suppression and ion-pairing reversed-phase HPLC for urinary paracetamol metabolites have been further investigated and conditions optimized to obtain maximum separation of the major metabolites.

EXPERIMENTAL

Apparatus

A Spectra-Physics 3500B dual-pump liquid chromatograph fitted with a

solvent programmer, a Waters Assoc. U6K loop injector and an Aerograph Variscan variable-wavelength ultraviolet—visible detector were used for all HPLC analyses.

Commercially packed columns (30 cm × 3.9 mm I.D.; particle size, 10 μm) containing microporous bonded phase support, μBondapak C_{18} were purchased from Waters Assoc.

Solvents

Water, doubly distilled and deionized, was obtained from Marine Chemistry Laboratory (University of Melbourne, Australia). Methanol (AR) was used without further treatment.

Mobile phases

Flow-rates of 2 ml/min were used throughout.

Ion suppression HPLC. Two alternative systems were used. (a) Solvent was prepared according to Howie et al. [14], i.e. 1% acetic acid—methanol—ethyl acetate (900:150:1.0). (b) Methanol was added at 15 volumes to 85 volumes of 0.05 *M* phosphoric acid. The pH of the solvent mixture was adjusted to the required value using 10% potassium hydroxide (AR). After studying a range of pH values a final pH of 2.8—2.85 was chosen for routine analysis.

Ion-pair HPLC. An aqueous solution of tetrabutylammonium (TBA) hydroxide (0.4 *M*, Eastman-Kodak, Rochester, NY, U.S.A.) was diluted to 0.005 *M* and neutralized to pH 7.2 with phosphoric acid (AR). Addition of 0.01 *M* Tris (tris-hydroxymethylaminomethane) (Sigma, St. Louis, MO, U.S.A.) and 0.005 *M* disodium EDTA (ethylenediaminotetraacetic acid disodium salt) was also made to control the selectivity of the separation. Various concentrations of methanol ranging from 0—50% were added to the above solution to control retention times.

Gradient elution ion-pair HPLC

In order to obtain all paracetamol metabolites on a single chromatogram with satisfactory resolution, it was necessary to use gradient elution. Two solvents were prepared: Solvent A = 0% methanol in water containing 0.005 *M* TBA, 0.010 *M* Tris, 0.005 *M* EDTA, pH 7.2 (H_3PO_4), and Solvent B = as for A, but containing 50% methanol.

With solvent A passing through the column at a flow-rate of 2 ml/min the sample was injected. After a delay time of 4 min, solvent B was introduced at a linear rate of 5.6%/min over 18 min. The column was held at 100% solvent B for a minimum of 10 min or until the next sample was ready for analysis. The column was then returned to 0% methanol at a rate of 20%/min over 5 min. After an equilibration period of 18—25 min, the next sample was injected. Approximately 1 h was required in total for sample analysis and column re-equilibration.

Column regeneration

For periods when the HPLC system was not in use, buffers were washed from the pumps and the column with water followed by dilute methanol solution. After the injection of more than 30—50 urine samples on reversed-phase

columns, non-polar impurities were removed by eluting the column with a neutral water—methanol solution in which the methanol concentration was gradually increased to 100%.

Standard compounds

Table I lists authentic drug conjugates which were synthesised and used to establish HPLC conditions. The glucuronides were prepared by the reaction of methyl (tri-O-acetyl-α-D-glucupyranosyl bromide) uronate [17] and the appropriate phenol in the presence of silver carbonate and subsequent transformation of the substituents and protecting groups was achieved by standard methods [18, 19]. The sulphates were prepared as potassium salts by standard methods from the corresponding phenols [20].

TABLE I

SYNTHETIC DRUG CONJUGATES

Conjugate	m.p. (°C)	Symbol
β-Phenyl-D-glucuronic acid	159—161	PhG
β-Quinolyl-D-glucuronic acid	107—109	QG
Paracetamol-β-D-glucuronic acid	260 d	G
p-Aminophenyl-β-D-glucuronic acid	220 d	PAPOG
Potassium phenyl sulphate		PhS
Paracetamol sulphate potassium salt		S
p-Aminophenyl-N-sulphate		PAPNS
p-Aminophenyl-O-sulphate		PAPOS

N-Hydroxyparacetamol (NHP) was chromatographically pure [10]. Paracetamol was obtained from Aldrich (Milwaukee, WI, U.S.A.). Paracetamol mercapturic acid (M) and paracetamol cysteine conjugate (C) were synthesized by reaction of N-acetylcysteine and cysteine respectively dissolved in phosphate buffer, pH 7.2, with N-hydroxyparacetamol at 22°C [10]. The reaction was allowed to proceed for several hours and was monitored by the disappearance of the peak for N-hydroxyparacetamol and the appearance of the paracetamol conjugates by ion-pair HPLC. The filtered reaction mixtures were used directly as standards. *p*-Nitrosophenol was observed as a by-product of the reactions. *p*-Aminophenol hydrochloride (AR) (PAP) was recrystallized.

Radioactively labelled paracetamol

Ring-labelled [^{14}C]paracetamol of specific activity 9.263 μCi/mmol and ring-labelled [^{3}H]paracetamol of specific activity 12 μCi/mg were administered to rats for the collection of radioactively labelled paracetamol metabolites.

Sample preparation

Standard solutions. Standard compounds were dissolved in double distilled deionized water and filtered through 0.5-μm cellulose Millipore membrane filters using a stainless-steel Swinney holder attached to a Luer-lok hypodermic syringe.

Urine samples. These were obtained from Sprague-Dawley albino and Gunn

rats following administration of paracetamol or N-hydroxyparacetamol. Urines were filtered through 0.5-μm cellulose filters held in washable plastic Swinney holders which were also fitted with borosilicate microfibre glass prefilters (Type AP25; Millipore, Bedford, MA, U.S.A.). Samples greater than 2 ml were undiluted whereas smaller volumes were first mixed with water to a known volume before filtration. Filtered urine samples were kept frozen at −20°C in acid-washed screw-capped vials (3 ml capacity) until ready for analysis. Injection volumes varied according to the concentration of metabolites expected in the urine. Typically 1—40 μl of urine were injected for gradient ion-pair HPLC. There was no necessity to add ion-pair reagent to the sample before injection onto the column as ion-pair equilibrium was quickly obtained.

Perfusion urines. These were injected without any treatment [21]. Injections (100 μl) were made in order to determine mercapturic acid. This was necessary because of the limit of sensitivity of the detector (0.1 a.u.f.s.). A 5-μl injection of the same sample was made to determine the larger paracetamol peak.

HPLC analyses of radioactive metabolites

A 0—6 h collection of urine from a female Sprague-Dawley rat given 1 mmol/kg [^{14}C]paracetamol, specific activity 9.263 μCi/mmol, intravenously (i.v.) was used to obtain a radioactive chromatogram of simple paracetamol metabolites. Further metabolites were discerned in the 6—24 h urine from a heterozygous Gunn rat given 2 mmol/kg i.v. [^{3}H]paracetamol of specific activity 1800 μCi/mmol.

Urine samples were separated by HPLC and fractions collected for scintillation counting to positively identify paracetamol metabolites. The column eluent following sample injection was collected in 0.4-ml fractions using an LKB Ultro-Rac 7000 fraction collector filled with plastic scintillation vials. Scintillation fluid (5 ml) (2,5-diphenyloxazole (4 g, Beckman, Fullerton, CA, U.S.A.) dissolved in a small quantity of toluene plus 333 ml Triton X-100 diluted to 1 l with toluene) was added to each vial using an Oxford pipettor. Fraction numbers greater than 100 collected from gradient elution ion-pair separations contained excess methanol which caused turbidity in the scintillation fluid and a consequent decrease in counting efficiency. This was overcome by the addition of 100 μl of water to these fractions. Radioactivity was determined in a Beckman LS-133 liquid scintillation counter. Counts were corrected for quench using calibration graphs. A radioactive chromatogram was thus constructed by plotting number of counts per minute (dpm) versus fraction number, i.e. elution volume.

Thin-layer chromatography

The two-dimensional TLC method of Andrews et al. [4] was used to separate metabolites in urine from rats given both paracetamol and N-hydroxyparacetamol. Spots assigned to paracetamol metabolites were removed from the plates, extracted with phosphate buffer, filtered and separated by ion-pair HPLC to confirm retention times of metabolites in the latter system. Similarly, the cysteine—N-hydroxyparacetamol reaction mixture was run on TLC plates and the fraction ascribed to cysteine conjugate rechromatographed by HPLC.

Rechromatography of separated fractions

Metabolites collected from previous HPLC or TLC separations were reinjected into the chromatograph via the U6K injector. Up to 2 ml of dilute solution could be reinjected, thus solvent evaporation proved to be unnecessary. Samples from TLC plates were scraped off, extracted into a suitable solvent and filtered through membrane filters before injection onto the column. Fractions derived from HPLC columns did not require filtration.

RESULTS AND DISCUSSION

Ion suppression chromatography

Initially an unbuffered ion suppression solvent [1% acetic acid—methanol—ethyl acetate (90:15:0.1)] was used. Synthetic paracetamol conjugates and paracetamol metabolites in a standard urine sample were well retained by the column and a good separation resulted (Fig. 2), although it was different to that reported with the same solvent system [14]. In that case C moved much later than all other metabolites and there was little resolution between S and G. Endogenous urine constituents interfered in this section of the chromatogram (Fig. 2). M was well resolved from all other components. Continued use

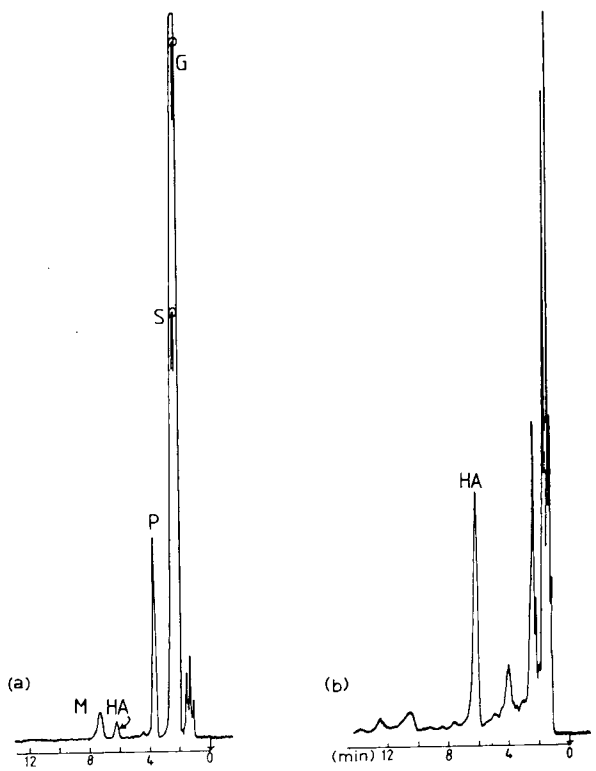


Fig. 2. Ion suppression HPLC of (a) paracetamol metabolites in rat urine and (b) control rat urine. Eluent, 1% aqueous acetic acid—methanol—ethyl acetate (90:15:0.1). Detector, UV, 254 nm, 1.0 a.u.f.s. Peak HA is endogenous urine constituent hippuric acid.

of this solvent failed to give reproducible retentions. These changes were dependent upon the presence of residual ion-pairing salts and changes in pH (Fig. 3b—d). Thus a buffered solvent was prepared according to Molnár and Horváth [22] at pH 2.1. Retentions became longer in this system (Fig. 3e). Optimization of selectivity and retention was achieved by observing variations of retention time with pH (Fig. 4). This was a useful experiment since it clearly differentiated between ionic species such as S, G and M and neutral compounds such as paracetamol. This aided in the identification of a previous-

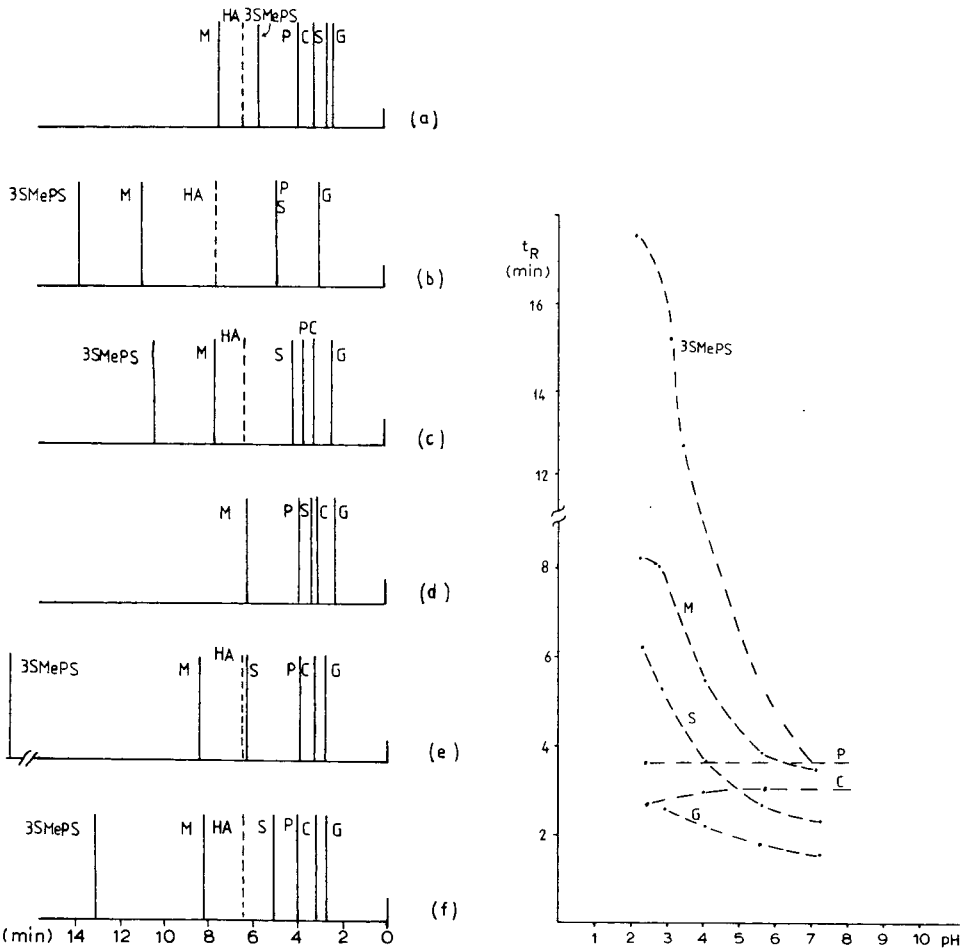


Fig. 3. Change in retention of paracetamol metabolites in ion suppression HPLC with different eluent: (a) as in Fig. 2; (b) as in (a) following the use of TBA phosphate; (c) freshly prepared solvent as in (a) and long column washing to eliminate residual pairing and buffer ions; (d) as in (a) plus 0.1 *M* KH_2PO_4; (e) 15% methanol in 0.05 *M* potassium phosphate buffer (pH 2.1), eluent pH 2.4; (f) as in (e), eluent pH 2.85.

Fig. 4. Change in retention time in ion suppression chromatography versus pH. Eluent, 15% methanol in potassium phosphate buffer (0.05 *M* re phosphate).

ly unidentified metabolite 3-thiomethylparacetamol sulphate (3SMePS) [23]. Fifteen per cent methanol in 0.05 *M* phosphate buffer (pH eluent 2.85) (Fig. 3f) was eventually chosen as a standard eluent for ion suppression chromatography and was used quite successfully to analyse a number of whole animal urine samples and perfusion urines.

There are various advantages and disadvantages in routinely using an ion suppression method. With whole urine samples, G, S, C and P moved very close together making it difficult to estimate each of these metabolites individually especially since endogenous urine constituents eluted close to them also. In whole urine samples G and S represent a large proportion of the drug and large peaks in this section of the chromatogram easily became overloaded, masking other constituents.

By contrast urines from isolated perfused kidneys [21] contained only small amounts of G and S and thus were well suited to ion suppression chromatography. An interesting aspect of the perfusion study was the detection of M and C formed by the kidney. In the absence of large quantities of G and S, the glutathione-derived conjugates were easily monitored as was the proportion of free drug recovered (Fig. 5). In the context of kidney metabolism, ion suppression chromatography was extremely valuable, the separations being completed in 10—15 min without solvent programming and column regeneration, thus enabling the analysis of many samples in a single day. The technique was further refined with the use of a dual-wavelength detector set at two different sensitivities for 254 nm, to simultaneously monitor the small levels of

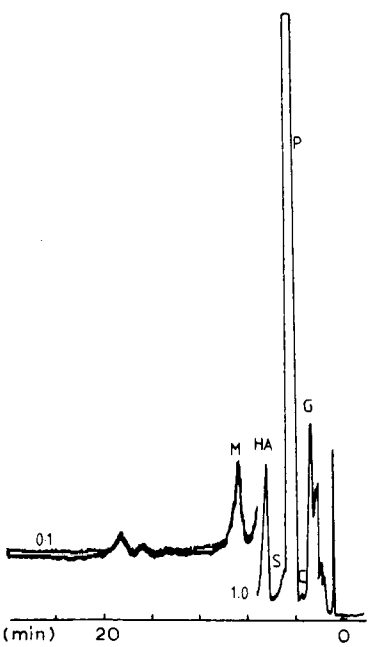


Fig. 5. Ion suppression chromatography of perfusion urine. Eluent, 15% methanol in potassium phosphate buffer (0.05 *M*), pH 2.85. Detector, UV, 254 nm; sensitivity as indicated.

M and C, and the relatively high concentrations of P [24].

In the case of whole animal urine samples such a rapid separation, while desirable, was not so important. Rather, slightly longer separation times could be tolerated, provided the resolution was superior to the ion suppression method. The logical choice for these samples was thus ion-pair partition chromatography on reversed-phase columns.

Optimization of conditions for ion-pair chromatography

TBA was used as the pairing ion since it covered a large range of log E_{QX} values [25] suitable for those metabolites likely to be encountered. Various synthetic paracetamol and *p*-aminophenol conjugates (Table I) were chosen to optimize concentrations of the various solvent components. The complete range of ion-paired conjugates could not be eluted at a single methanol concentration. For example, under conditions whereby P and G were only just resolved at around $k' = 2$—3, S had a k' value of more than 20. Therefore retention times for conjugates were also determined at different methanol concentrations.

Fig. 6 shows the retention data (relative to benzyl alcohol) obtained using the simplest composition of 0.005 *M* TBA only in the mobile phase. The pH was adjusted to 7.2 with phosphoric acid; however, little buffering capacity was evident and pH control was limited. There was quite acceptable resolution

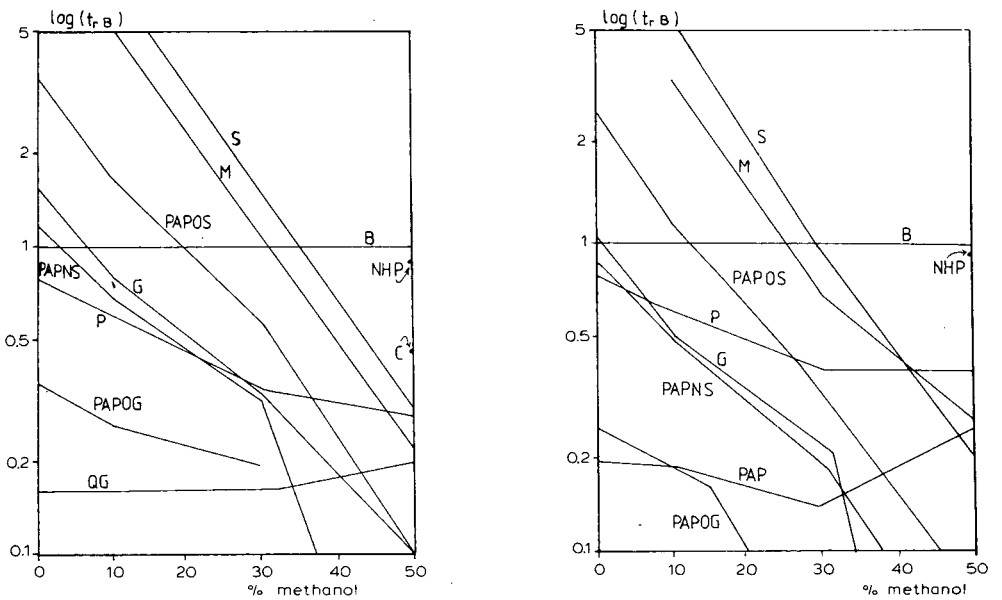
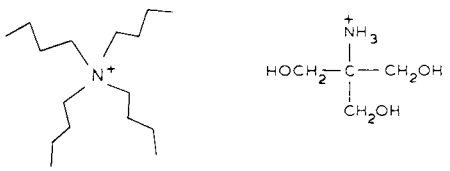


Fig. 6. Relative retention of synthetic paracetamol derivatives in ion-pair HPLC. Solvent, 0—50% methanol in water containing 0.005 *M* TBA adjusted to pH 7.2 with phosphoric acid. k' for benzyl alcohol (B) at 0, 10, 30 and 50% methanol are 7.0, 4.8, 2.0 and 0.8 respectively.

Fig. 7. Relative retentions of synthetic paracetamol derivatives in ion pair HPLC. Solvent as in Fig. 6, but also containing 0.01 *M* Tris. k' for benzyl alcohol (B) at 0, 10, 30 and 50% methanol are 6.2, 4.6, 2.0 and 0.7 respectively.

of all of the conjugates although retentions for M and S were very long. An endogenous urine constituent, hippuric acid, was unresolved from M under these conditions. A further disadvantage was that C and NHP could only be eluted at methanol concentrations of at least 50%, that is, after all the other conjugates. Their peak shapes were also quite poor and inadequate for analytical purposes.

In order to increase buffering capacity Tris was used. This was found to give selectivity superior to simple sodium or potassium phosphate buffers. In Fig. 7, the effect of added Tris is shown. Two concentrations were used, 0.005 *M* and 0.01 *M*. As there was little difference in retention and selectivity between the two concentrations, the higher level of Tris was chosen since it provided greater buffering capacity. Addition of Tris altered the elution pattern quite significantly. By inspection of the structures of TBA and protonated Tris ion,



it would be expected that ion pairs formed with Tris would be more water soluble and hence less strongly retained on the column. Under these conditions endogenous urine constituents in a control urine sample did not interfere with paracetamol conjugates, in particular M. Unfortunately the decrease in retention for paired conjugates meant that resolution between P, G and PAPNS had diminished. NHP and C still did not chromatograph well.

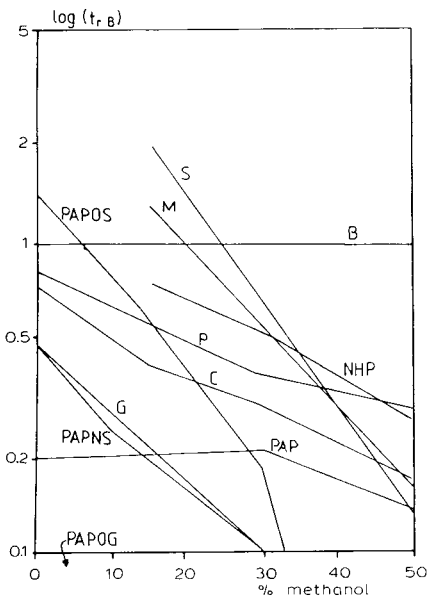


Fig. 8. Relative retentions of synthetic paracetamol derivatives in ion pair HPLC. Solvent as in Fig. 7, but also containing 0.005 *M* EDTA. *k'* for benzyl alcohol (B) at 0, 15, 30 and 50% methanol are 5.9, 3.3, 1.8 and 0.7 respectively.

The effect of introducing 0.005 *M* EDTA into the mobile phase is shown in Fig. 8. EDTA was also incorporated since it prevented excessive tailing of N-hydroxyparacetamol (unpublished work in this laboratory). Retentions for the conjugates once again decreased suggesting involvement of EDTA in ion pairing. The effect was beneficial in many respects. Much less methanol was required to elute S and M, G now moved sufficiently ahead of P to improve the resolution of these two compounds, although now G coincided exactly with *p*-aminophenol-N-sulphate. Loss of resolution for this pair of conjugates however was considered to be less important since improved resolution of the paracetamol metabolites had been achieved and *p*-aminophenol has not been observed as a quantitatively significant metabolite of paracetamol [26]. Perhaps the most beneficial result was that NHP and C eluted at all concentrations of methanol. It appeared that this solvent composition offered the best separation and was used for all future urine analysis.

Gradient programming

Urine samples were tested with the standardized ion-pair reagent at 10% methanol. S appeared as a broad peak taking approximately 20 min to elute, while the early part of the chromatogram was moderately well resolved (Fig. 9a). When the methanol concentration was increased to 20% (Fig. 9b) the sulphate conjugate appeared as a sharp peak at a shorter retention, and 3SMePS which had not appeared at 10% methanol was now detected. These benefits

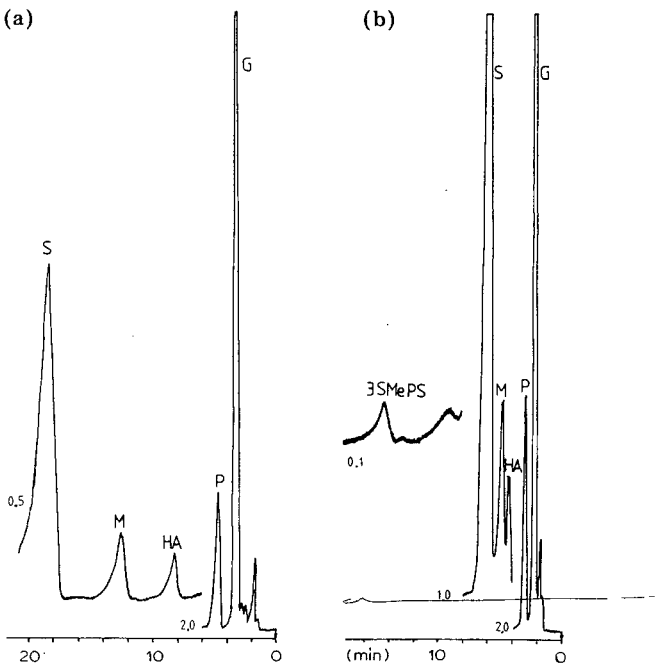


Fig. 9. Isocratic ion-pair HPLC of urinary paracetamol metabolites. Eluent (a) 10% methanol and (b) 20% methanol in standard ion-pair reagent, i.e., 0.005 *M* TBA—0.01 *M* Tris—0.005 *M* EDTA buffered to pH 7.2 with phosphoric acid. Detector, UV, 254 nm, sensitivity as indicated.

were overshadowed however by crowding at the beginning of the chromatogram which was poorly resolved.

Thus it was clear that solvent programming with increasing concentration of methanol was necessary. This was quite possible since no stationary phase was used on the column [27—31]. By reference to Fig. 8 it was noted that adequate resolution was maintained at 0% methanol and all conjugates were eluted with 50% methanol. Therefore these solvent concentrations were chosen as initial and final values respectively. After some trial and error a gradient programme as outlined in Experimental was adopted with samples run every hour.

Identification and quantitation of paracetamol metabolites

Fig. 10 illustrates a relatively simple metabolic profile following the administration of a low dose of paracetamol. P, S, G, M and *p*-aminophenol-O-sulphate were observed and identified by comparison with standard compounds. Further proof of identity was obtained when the urine from a rat given radioactively labelled paracetamol was analysed (Fig. 11a) and compared

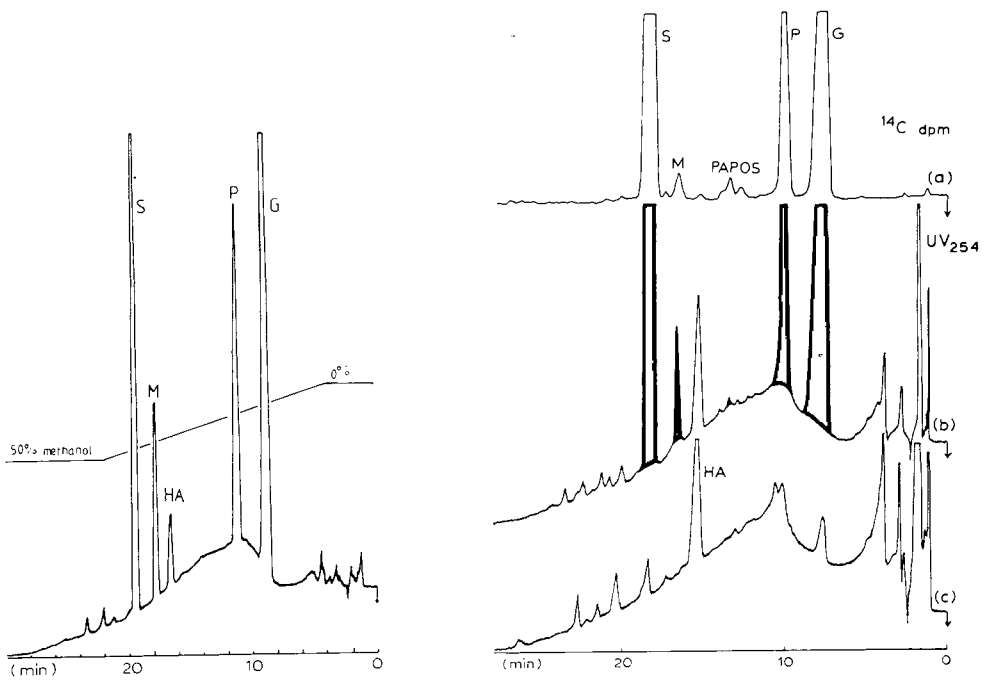


Fig. 10. Solvent programmed ion-pair HPLC of urinary paracetamol metabolites. Eluent, standard ion-pair reagent linearly programmed from 0—50% methanol over 18 min after an initial hold time of 4 min. Detector, UV, 254 nm, 1.0 a.u.f.s. Sample, 6-h urine collection from a female Sprague-Dawley rat following the intravenous administration of 3 mmol/kg body weight of paracetamol.

Fig. 11. Identification of paracetamol derived metabolites using radioactive detection. HPLC conditions as in Fig. 10. Detection, (a) ^{14}C-counts per 0.4-ml fraction; (b) UV detection at 254 nm, 1.0 a.u.f.s. Urine samples (a) and (b) from a heterozygous Gunn rat given 2 mmol/kg body weight [^{14}C]paracetamol, (c) control rat urine, i.e. no paracetamol administered.

with the conventional 254-nm chromatogram (Fig. 11b). The chromatogram of control urine from a rat which was not given paracetamol is shown for comparison (Fig. 11c). It can be seen that there is little interference from endogenous constituents.

The radioactive chromatogram served a second purpose since it enabled the calculation of response factors for each metabolite. A formula was derived for the response factor by comparison of the 254-nm peak area with the number of counts per peak as calculated from the radioactive chromatogram.

$$\text{Response factor} = \frac{\text{number of counts per peak}}{\text{peak area}_{254/1.0}} \times \frac{\text{mmol paracetamol administered}}{\text{total counts administered}} \times 10^6$$

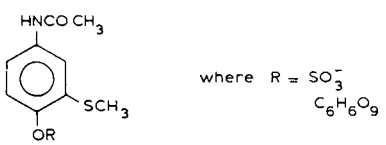
Units for the response factor nm per cm^2 peak area at 254 nm, sensitivity 1.0 a.u.f.s. Once the response factor for each metabolite was determined, this value was used to calculate the mass of the same metabolite, and hence percentage of the dose excreted in any subsequent chromatogram. Table II lists response factors for all the metabolites recovered in this study.

TABLE II

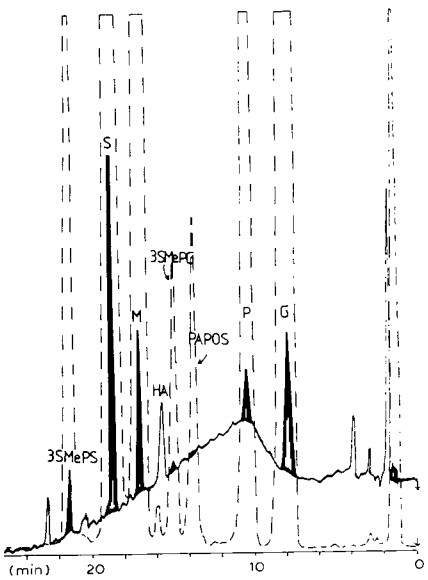
RESPONSE FACTORS FOR PARACETAMOL METABOLITES

Metabolite	Response factor nmol cm^{-2} (254/1.0)
Paracetamol	23.5
Paracetamol glucuronide	21.5
Paracetamol sulphate	19.5
Paracetamol mercapturic acid	20.0
3-Thiomethylparacetamol sulphate	18.5
3-Thiomethylparacetamol glucuronide	21.5
Cysteine conjugate	20.0
p-Aminophenol-O-sulphate	150.0

Inspection of the radioactive chromatogram of the urine from a different species of rat given ^{3}H-radiolabelled paracetamol (Fig. 12) revealed the presence of another new metabolite, 3-thiomethylparacetamol glucuronide (3SMePG) as well as 3SMePS. The isolation and identification of these new metabolites is described elsewhere [23]. They were found routinely in animals receiving high



doses of paracetamol [26]. Previously 3-thiomethylparacetamol has only been isolated from urine after treatment with glucuronidase—aryl sulphatase enzymes [7].



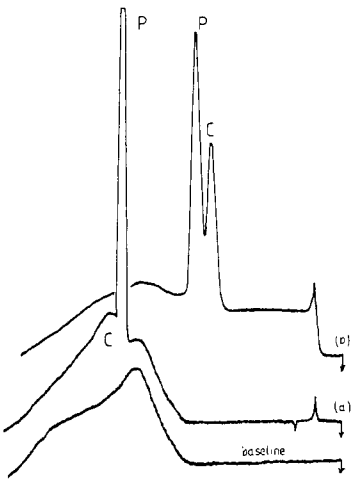


Fig. 12. Chromatograms of urinary [^{3}H]paracetamol metabolites from a heterozygous Gunn rat given 2 mmol/kg body weight paracetamol. HPLC conditions as in Fig. 10. Detection, solid line, UV, 254 nm, 1.0 a.u.f.s.; broken line, ^{3}H-counts per 0.4-ml fraction.

Fig. 13. Effect of column age on recovery of paracetamol cysteine conjugate, C. HPLC conditions as in Fig. 10; (a) new column, (b) old column.

Cysteine conjugate proved to be an elusive metabolite and was originally expected to be present in rat urine as a metabolite of paracetamol. It was not found in rats given paracetamol, but was found however in rats given relatively large quantities of N-hydroxyparacetamol. A spot assigned to C in two-dimensional TLC was rechromatographed in the ion-pair system. It eluted near paracetamol; however, unless present in sufficiently large quantities, it was difficult to discern, especially since at this point in the chromatogram the baseline changed under the solvent programming conditions. Recovery of cysteine conjugate was improved with older EDTA-saturated columns (Fig. 13) and this is consistent with the ability of amino acids to coordinate with metal ions or silica hydroxyls of the column packing. Ion suppression HPLC or two-dimensional TLC [4] were used as alternatives to the ion-pair method for detection of C. These systems demonstrated that over very wide ranges of paracetamol administered to rats, cysteine conjugate was not present as a metabolite. On the other hand in the urines from mice or humans it was observed in significant quantities [9]. It was noted that cysteine conjugate is relatively non-polar and in fact elutes close to paracetamol in neutral reversed-phase chromatography (Fig. 14a). By contrast the N-acetylated cysteine conjugate, that is paracetamol mercapturic acid, eluted with other water soluble conjugates, which included the two 3-thiomethylparacetamol conjugates, under these conditions. Rechromatography of fractions collected under neutral conditions by ion pair chromatography confirmed their identity (Fig. 14b).

Sulphate and glucuronide metabolites of 3-methoxyparacetamol were not

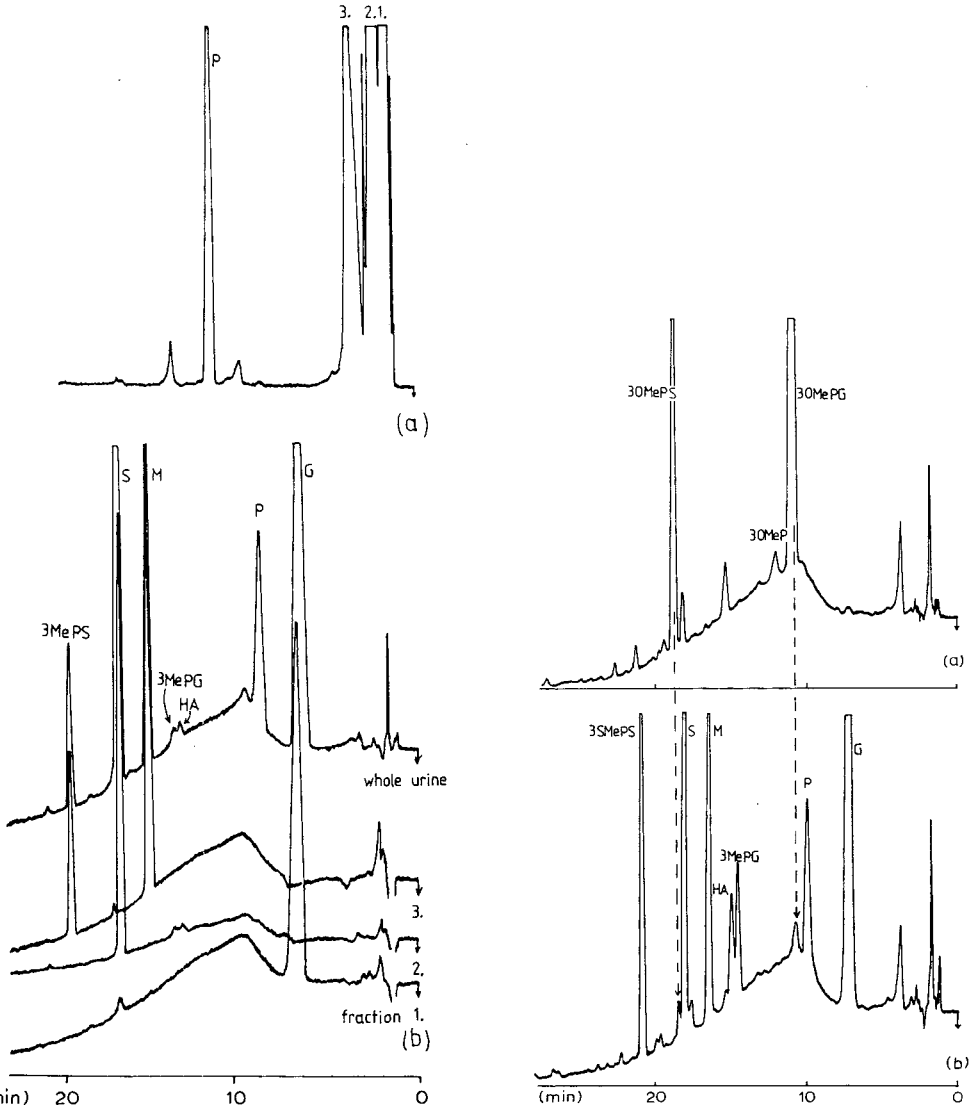


Fig. 14. (a) Neutral reversed-phase chromatograms of urinary paracetamol metabolites. Eluent, water programmed from 0—50% methanol linearly over 18 min after 4 min delay. Three water-soluble fractions 1, 2 and 3 were collected and rechromatographed (b) by standard ion-pair gradient programming as in Fig. 10.

Fig. 15. Chromatograms of urinary metabolites of (a) 3-methoxyparacetamol (30MeP) and (b) paracetamol. HPLC conditions as in Fig. 10.

ordinarily found in rat urines, although their occurrence has been mentioned by other workers, in particular from human urines, [4, 15, 16] (Fig. 1). As standards of 30MePS and 30MePG were unavailable, the free compound was administered to rats at a dose of 1.0 mmol/kg. Chromatography of the urine confirmed these compounds as the principal metabolites (Fig. 15). 30MePG ran just after paracetamol while 30MePS ran very close to S.

N-Hydroxyparacetamol metabolites

For some time N-hydroxylation [32—34] has been considered to be the initial metabolic step in the toxicity of paracetamol, although evidence is now accumulating to suggest that direct oxidation of paracetamol to the reactive N-acetyl-*p*-benzoquinone imine occurs [9]. In this context it was interesting to observe phenolic O-substituted conjugates of N-hydroxyparacetamol, i.e. N-hydroxyparacetamol-O-sulphate and -O-glucuronide in the urine of animals given N-hydroxyparacetamol. They were identified by their close retention to the analogous paracetamol conjugates and by their chromatographic behav-

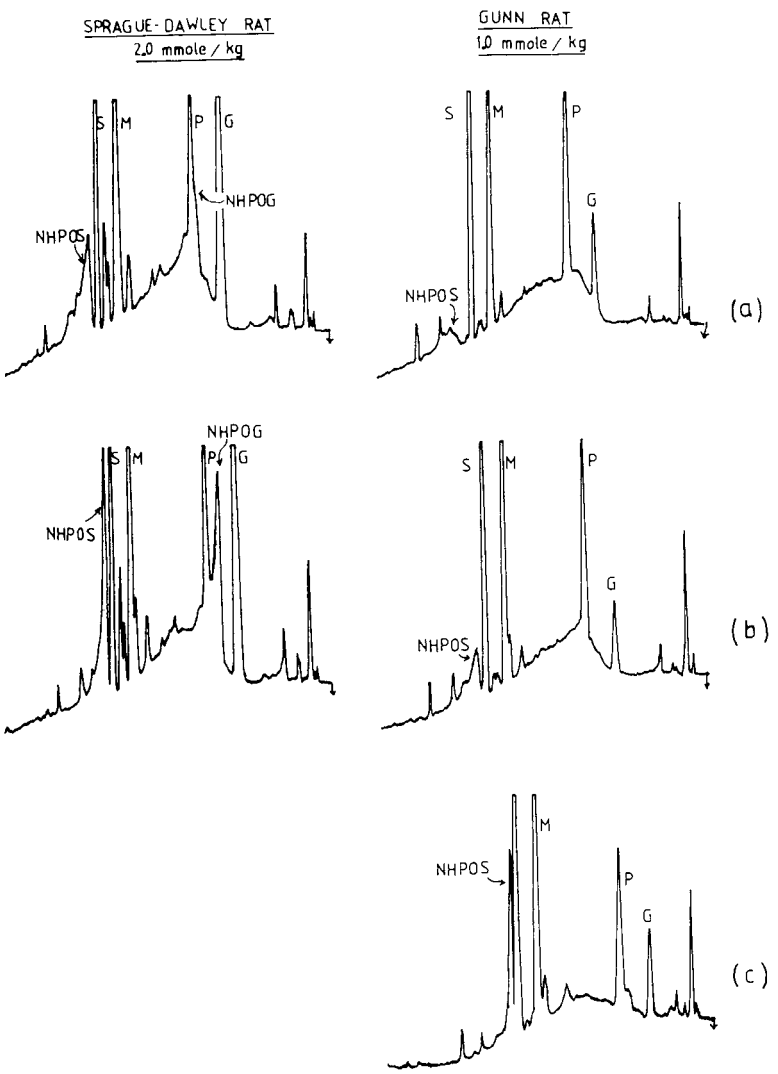
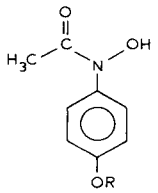
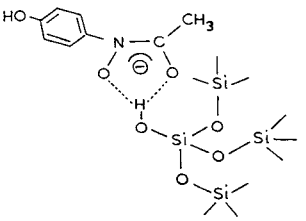


Fig. 16. Chromatograms of urinary N-hydroxyparacetamol metabolites from two different species of rat. HPLC conditions as in Fig. 10. Column conditions, (a) new column; (b) column partly deactivated with EDTA and (c) fully saturated with EDTA.



where R = $C_6H_6O_9$
= SO_3^-

iour, which was very similar to N-hydroxyparacetamol itself. That is, with a new column these peaks moved well after the analogous paracetamol conjugates and tailed badly (Fig. 16a) due to coordination with metal ions or silica hydroxyls of the column packing. As the column aged and became fur-



ther satured with EDTA the peaks eluted progressively earlier with increased sensitivity and decreased tailing (Fig. 16b, c). N-Hydroxyparacetamol glucuronide showed identical chromatographic properties to an authentic sample synthesized in this laboratory [9]. Interestingly with Gunn rats the peak ascribed to N-hydroxyparacetamol glucuronide virtually disappeared while the corresponding sulphate conjugate increased. Limited glucuronidation of paracetamol with Gunn rats is well known [35]. This observation further confirmed the occurrence of phenolic N-hydroxyparacetamol-O-conjugates. To date these compounds have not been detected in the urine from animals given paracetamol alone [9, 26].

CONCLUSION

The methods developed for the separation of paracetamol metabolites have opened a wide field of metabolic profiling universally applicable to a number of different species, and to biological fluids other than urine. The methods have been especially useful for the study of paracetamol metabolism in isolated organ and cell fraction preparations.

ACKNOWLEDGEMENTS

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SIMULTANEOUS DETERMINATION OF MISONIDAZOLE AND DESMETHYLMISONIDAZOLE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of misonidazole and desmethylmisonidazole in plasma is described. After plasma is deproteinized with methanol and the diluted supernatant is chromatographed on a C_{18} reversed-phase column, both compounds are quantitated by means of an internal standard. The coefficients of variation of within-day and day-to-day precision are below 5.0% for misonidazole in the concentration range of 25—250 mg/l and below 6.1% for desmethylmisonidazole in the concentration range of 2.5—25.0 mg/l. Calibration curves are linear and an analytical recovery varying from 97.6 to 99.8% is obtained. The detection limits for misonidazole and desmethylmisonidazole in plasma are 1.4 mg/l and 0.7 mg/l, respectively.

INTRODUCTION

Misonidazole, 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (Fig. 1, I), has been shown to increase the sensitivity of hypoxic cells to the effects of ionizing radiation in vitro [1]. This so-called radiosensitizing effect of misonidazole could also be demonstrated in vivo in various experimental tumours in animals [2, 3] and in human tumours [4, 5].

The radiation enhancement ratio of hypoxic cells increases with the concentration of misonidazole in the cells [1]. Consequently, to obtain the greatest benefit of the treatment of patients with tumours by a combination of radiotherapy and administration of misonidazole it is desirable to attain levels of misonidazole as high as possible during irradiation. However, the administration of misonidazole to man is limited by its neurotoxicity. Side-effects and neurotoxic symptoms [6—12] have been reported in several studies of the administration of misonidazole to humans. The incidence of neurotoxic symptoms

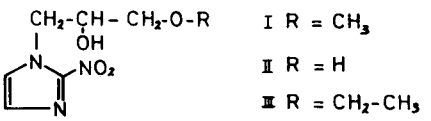

Fig. 1. Chemical structures of misonidazole (I), desmethylmisonidazole (II) and the internal standard (III).

seemed to be related to the total dose of misonidazole administered in a certain period [5—8, 11, 13, 14]. In order to perform toxicological and pharmacokinetic studies of misonidazole in man it was necessary to develop a rapid, sensitive and specific method for the determination of misonidazole and its O-demethylated metabolite, desmethylmisonidazole (Fig. 1, II), in biological material.

Several methods for the determination of misonidazole have been published. Previously reported UV spectrophotometric procedures [10, 15, 16] are not specific for misonidazole, because these methods do not differentiate between the parent compound and desmethylmisonidazole, which has an almost identical UV-absorption spectrum. Polarographic procedures [16, 17] also lack specificity, because all drug-related species with the intact nitroimidazole nucleus are determined simultaneously.

Although paper (PC) [18], thin-layer (TLC) [19] and gas—liquid chromatography (GLC) [16, 19] do separate misonidazole and desmethylmisonidazole, these separation techniques are not optimal. PC and TLC procedures are inadequate for pharmacokinetic studies, because they lack sensitivity and accuracy. GLC methodology requires a rather tedious extraction and derivatization procedure and is unsuitable for the analysis of large numbers of samples usually encountered in pharmacokinetic studies. A high-performance liquid chromatographic (HPLC) method [20] involving protein precipitation by methanol, centrifugation and injection of the methanolic supernatant shows serious problems. Column contamination probably due to accumulation of plasma proteins led to a continuously increasing column pressure. Moreover, even small injection volumes of 10 μl produced peak distortion, which became more of a problem if larger volumes were injected. Another HPLC method [21] lacks the use of an internal standard and also the supernatant is injected directly. Therefore, a rapid, sensitive and specific procedure for the simultaneous determination of misonidazole and desmethylmisonidazole in plasma has been developed. The procedure involves a simple protein precipitation followed by aqueous dilution of the supernatant obtained after centrifugation. Then misonidazole and desmethylmisonidazole are separated on a microparticulate reversed-phase column and detected by monitoring the UV absorbance of the column effluent.

EXPERIMENTAL

Chemicals and reagents

Misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (Ro 07-0582)], desmethylmisonidazole [1-(2-nitroimidazol-1-yl)-2,3-propandiol (Ro 05-9963)] and the internal standard [1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913)] (Fig. 1, III) were kindly provided by Hoffmann-La Roche

& Co. (Mijdrecht, The Netherlands). Analytical reagent grade methanol (Merck, Darmstadt, G.F.R.) and double-distilled water were used. Both solvents were filtered through a 0.45-μm filter before use.

Apparatus

The high-performance liquid chromatograph consisted of a Model 995 isochromatographic pump coupled to a Model 970A variable-wavelength UV—Vis absorbance detector with auto scan option (both from Tracor Instruments, Austin, TX, U.S.A.). Injections were achieved with a Model 7120 syringe loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). This six-port rotary valve was fitted with a 100-μl sample loop. Loading of the sample loop was accomplished with a low-pressure syringe (Scientific Glass Engineering, Ringwood, Australia).

Separations were performed on a reversed-phase column (25 cm × 4.6 mm I.D.) constructed of stainless steel and prepacked with a C_{18} hydrocarbon stationary phase, chemically bonded to silica gel with a mean particle size of 10 μm (LiChrosorb 10 RP-18, from Chrompack, Middelburg, The Netherlands). Detector output was recorded at 1 mV on a flat-bed recorder (Technicorder F Type 3052, Yokogawa Electric Works, Tokyo, Japan).

A Fi-streem 4-litre bidistillation unit (Fisons Scientific Apparatus, Loughborough, Great Britain) was used to prepare double-distilled water. An all-glass filter apparatus with appropriate 0.45-μm filters (Solvent Clarification Kit, Waters Associates, Milford, MA, U.S.A.) was always used to filter solvents before use. An ultrasonic bath was employed to degass the mobile phase before use. Other equipment included a vortex-type mixer, reciprocating shaker, high-speed centrifuge and 100 × 16.25 mm disposable glass tubes with polypropylene caps.

Chromatographic conditions

Chromatography was performed at ambient temperature. The mobile phase, consisting of a methanol—water mixture (20:80, v/v), was delivered at a constant flow-rate of 2.0 ml/min. The resulting operating pressure was approximately 9.65 MPa (1400 p.s.i.). Detection is made by monitoring the UV absorbance of the column effluent at 323 nm. The sensitivity of detection was 0.010 absorbance unit full-scale deflection (AUFS). The detector output was recorded at 1 mV and the chart speed of the recorder was 20 cm/h.

Standards

Aqueous stock solutions of desmethylmisonidazole (250 mg/l) and of misonidazole (2500 mg/l) and a methanolic stock solution of the internal standard (220 mg/l) were prepared. Working standard solutions were made by appropriate dilution of the stock solutions with water or methanol.

Plasma standards containing both misonidazole and desmethylmisonidazole were prepared by adding 9 ml of drug-free plasma to 1 ml of aqueous working standard solutions containing both drugs at various concentrations. Concentrations in the plasma standards ranged from 2.5 to 25.0 mg/l for desmethylmisonidazole and from 25 to 250 mg/l for misonidazole. The standard solutions were stored in the dark at 4°C and were stable for at least two months.

Procedure

Add 9 ml of methanolic internal standard solution (22.0 mg/l) to 1 ml of plasma or working standard solution in a glass tube. Extract the mixture on a reciprocating shaker for 5 min and centrifuge at 2000 *g* for 10 min. After centrifugation transfer 1 ml of the supernatant to a second glass tube and dilute with 4 ml of bidistilled water. Vortex for 30 sec and inject an aliquot into the high-performance liquid chromatograph.

Quantitation

Plasma standards were always included to calculate unknown plasma concentrations of misonidazole and desmethylmisonidazole. Peak height ratios of the drug or its metabolite to the internal standard were calculated. The respective calibration curves were constructed and the best fitting line was found using a least-squares linear regression method. All chromatographic results reported were the mean of duplicate or triplicate injections of every sample or solution, unless otherwise stated.

RESULTS AND DISCUSSION

Chromatographic system

A chromatographic system of a reversed-phase column and a polar mobile phase was preferred, because in such a system polar compounds including drug metabolites are eluted quickly.

Methanol—water mixtures were tested as mobile phases. Volume ratio and

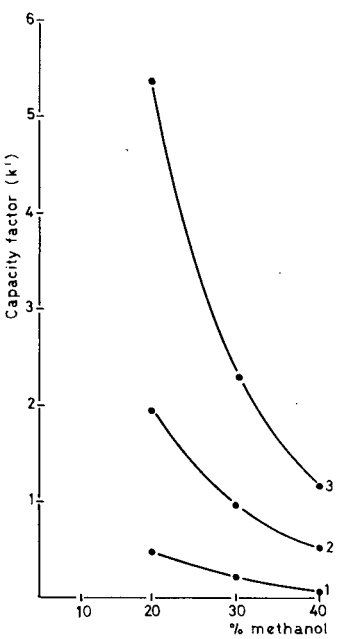

Fig. 2. Plot of capacity factors (k') of some nitroimidazoles against methanol content of the mobile phase. Unretained compound: methanol. Flow-rate: 2.0 ml/min. Chromatographic conditions as described. 1 = Desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

flow-rate of the methanol—water mixture were varied to achieve optimum chromatographic conditions. The influence of the percentage of methanol in the mobile phase on the capacity factors of the respective nitroimidazoles is shown in Fig. 2. A methanol—water mixture (20:80, v/v) at a flow-rate of 2.0 ml/min was found to be optimum, because separation and resolution of the three nitroimidazoles was achieved adequately. The UV absorption spectra of the three nitroimidazoles in the proposed mobile phase all show absorption maxima at 223 nm and 323 nm. We preferred 323 nm as wavelength of detection, because at this wavelength the highest absolute peak heights were obtained. Under the conditions of this chromatographic system the three nitroimidazoles were eluted as sharp and symmetrical peaks, allowing use of peak heights to quantitate detector response. Retention times for desmethylmisonidazole, misonidazole and internal standard were 2.0, 4.0 and 8.6 min, respectively. The chromatograms of a plasma blank (A), a standard solution in eluent (B) and a plasma standard (C) are shown in Fig. 3. A chromatogram of a plasma sample of a patient with carcinoma of the cervix 5.5 h after oral intake of 0.8 g/m^2 misonidazole is shown in Fig. 4.

Procedure

In order to obtain complete protein precipitation 9 ml of methanol were added to 1 ml of plasma. When the undiluted methanolic supernatant of a plasma standard containing 100 mg/l misonidazole and 10 mg/l desmethylmisoni-

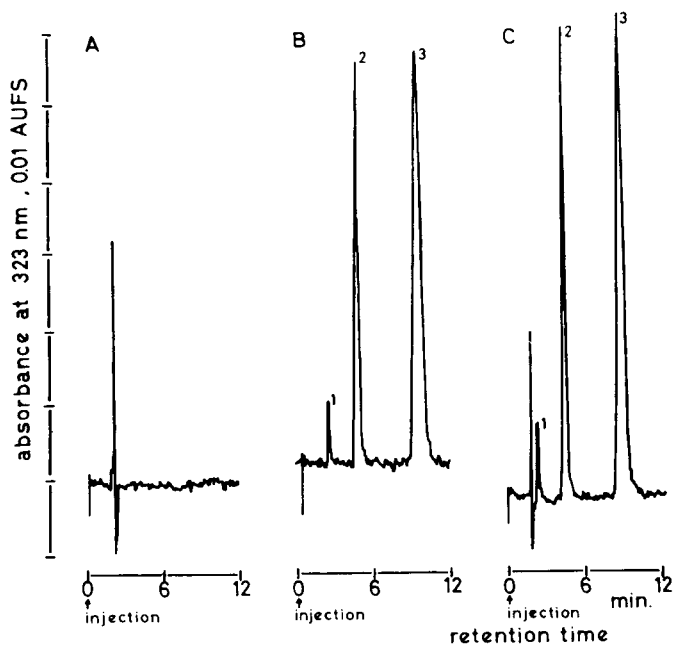

Fig. 3. (A) Chromatogram of blank plasma. (B) Chromatogram of a chromatographic standard solution containing 0.2 mg/l desmethylmisonidazole, 2 mg/l misonidazole and 4 mg/l internal standard. (C) Chromatogram of a plasma standard containing 10 mg/l desmethylmisonidazole and 100 mg/l misonidazole. Chromatographic conditions as described. Injection volume: 75 µl. Peaks: 1 = desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

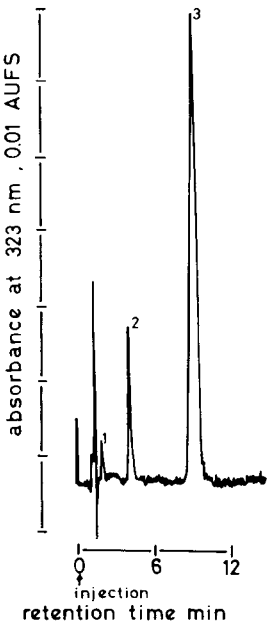

Fig. 4. Chromatogram of a plasma sample of a patient with carcinoma of the cervix 5.5 h after oral intake of 0.8 g/m² misonidazole. Chromatographic conditions as described. Injection volume: 75 µl. Peaks: 1 = desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

dazole was injected, it became evident that even at a small injection volume of 10 µl the peak shape was not ideal and peak heights were low, although peak height ratios were correct. However, on further increase of the injection volume the peaks became increasingly distorted and at injection volumes greater than 50 µl even peak height ratios became low. After aqueous dilution of the methanolic supernatant of the plasma standard, distortion of the peaks became less as the volume ratio of methanol and water in the injected dilution approximated more closely to that in the mobile phase. Only supernatants, that were diluted with a three- to ninefold volume of water, showed correct peak shape, peak heights and peak height ratios. Therefore, we may conclude that it is not recommended to inject the methanolic supernatant of a plasma sample directly as described previously [20].

Precision

Precision was always evaluated with four pools of plasma containing 25, 50, 100 and 150 mg/l misonidazole and 2.5, 5.0, 10.0 and 15.0 mg/l desmethylmisonidazole. After processing 1-ml aliquots of the plasma pools according to the described procedure and injecting the resulting solutions, precision was calculated as the coefficient of variation of the peak height ratio of misonidazole or desmethylmisonidazole to the internal standard.

The precision of the chromatographic process from injection to detection was determined by making eight identical, successive injections of the resulting solution from one sample of each pool of plasma. The precision of the whole

method was determined by establishing the within-day and day-to-day precision. Within-day precision was determined by processing eight aliquots of 1 ml from each plasma pool and injecting the resulting solutions the same day. Day-to-day precision was established by analyzing one 1-ml aliquot of each plasma pool on each working day during a two-week period. Data on precision of the chromatographic process, within-day and day-to-day precision are presented in Table I.

TABLE I

PRECISION DATA OF THE PROPOSED METHOD FOR THE DETERMINATION OF NITROIMIDAZOLES IN PLASMA

	Plasma conc. (mg/l)	Coefficient of variation (%)		
		Chromatographic process (n = 8)	Within-day (n = 8)	Day-to-day (n = 10)
Desmethylmisonidazole	2.5	5.9	4.5	6.1
	5.0	3.6	4.1	5.4
	10.0	4.3	3.5	4.7
	15.0	2.6	1.5	2.3
Misonidazole	25	0.9	1.9	5.0
	50	1.1	0.6	3.2
	100	1.3	0.7	2.5
	150	0.7	1.2	3.3

Recovery

The results of the recovery study summarized in Table II demonstrate complete recovery of the nitroimidazoles from plasma at the indicated plasma concentrations. The recovery data represent the average value of four extractions at each concentration.

Linearity

The calibration curves for misonidazole and desmethylmisonidazole showed good linearity over the respective concentration ranges 25—250 mg/l and 2.5—25.0 mg/l both in water and in plasma. Plasma levels of misonidazole and desmethylmisonidazole in patients after oral intake of therapeutic doses of misonidazole are reported to be in the concentration ranges mentioned above [6, 22, 23]. The data for the calibration curves of both drugs are summarized in Table III.

Sensitivity and detection limit

Misonidazole and desmethylmisonidazole may be determined at the highest detector sensitivity because the described procedure yields an almost clean extract. The lower limits of detection measured at a detector sensitivity of 0.005 AUFS, with a detector noise of 1×10^{-4} absorbance unit and allowing a signal-to-noise ratio of 2, are 2.0 ng and 1.0 ng for misonidazole and desmethylmisonidazole, respectively.

TABLE II

ANALYTICAL RECOVERY OF NITROIMIDAZOLES FROM PLASMA

	Plasma conc. (mg/l)	Recovery* (%)
Desmethylmisonidazole	2.5	97.6
	5.0	98.1
	10.0	99.4
	15.0	98.7
Misonidazole	25	98.4
	50	99.8
	100	99.0
	150	99.3
Internal standard	50	99.2
	100	98.8
	200	99.5
	300	99.8

*Means of four determinations at each concentration.

TABLE III

DATA FOR THE CALIBRATION CURVES OF DESMETHYLMISONIDAZOLE AND MISONIDAZOLE IN PLASMA

	Concentration (mg/l)	Peak height ratio*		Linear regression parameters**		
		Mean	C.V. (%)	Slope	y-intercept	Correlation coefficient
Desmethyl-misonidazole	2.5	0.039	5.4	0.016	−0.004	0.9998
	5.0	0.075	4.2			
	10.0	0.157	1.1			
	15.0	0.239	1.3			
	20.0	0.326	2.3			
	25.0	0.402	0.2			
Misonidazole	25	0.248	2.2	0.010	−0.010	0.9999
	50	0.488	1.3			
	100	0.987	0.9			
	150	1.483	0.9			
	200	1.986	0.3			
	250	2.500	0.9			

*Data represent mean and coefficient of variation (C.V.) of three replicate injections at each concentration.

**Peak height ratio of drug to internal standard plotted on the y-axis versus drug concentration in plasma (in mg/l) on the x-axis.

Analyzing a 1-ml plasma sample according to the procedure of this method and injecting 75 μl of the resulting solution the lower limits of quantification are 1.4 mg/l and 0.7 mg/l for misonidazole and desmethylmisonidazole, respectively.

Clinical application

The method described has been applied to the determination of plasma levels of misonidazole and desmethylmisonidazole in patients with various tumours receiving therapeutic doses of misonidazole in combination with radiotherapy. The results of these clinical studies will be reported soon.

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MICRO-DETERMINATION OF TOBRAMYCIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

A procedure for the high-performance liquid chromatographic determination of tobramycin in serum is described using pre-column derivatisation with 1-fluoro-2,4-dinitrobenzene and subsequent chromatographic analysis on a reversed-phase column with ultraviolet detection. Gentamicin is used as the internal standard. The sensitivity is 0.5 mg/l with 50-µl samples. Precision, expressed as the coefficient of variation, is 3% or better in the concentration range 0.5—16 mg/l. The absolute recovery of tobramycin is 41%.

The analyses of serum samples obtained in an in vivo experiment correlated well with the results from a microbiological assay. The influence of variation of derivatisation conditions and the implications for the reliability of the internal standardisation were studied. The 2,4-dinitrophenyl tobramycin derivative was synthesized and its structure was proved to be the fully derivatized tobramycin. Side-products of the derivatisation reaction were isolated.

INTRODUCTION

A high-performance liquid chromatographic (HPLC) method for the determination of gentamicin and sisomicin in serum was previously reported by us [1, 2]. The assay is based on pre-column derivatisation of the aminoglycoside with 1-fluoro-2,4-dinitrobenzene (FDNB) and subsequent analysis of the derivatised products on a reversed-phase type column with ultraviolet (UV) detection. In the present paper we describe the determination of tobramycin in serum, using gentamicin as the internal standard.

For "perfect" internal standardisation, tobramycin and gentamicin should react in a completely similar way with the derivatisation reagent. The reliability of internal standardisation was tested under varying derivatisation conditions. In order to establish its structure, the dinitrophenyl tobramycin derivative was

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synthesized on a preparative scale. With the help of this purified derivative, the absolute recovery of tobramycin was measured.

FDNB reacts with primary and secondary amino groups [3]. The tris(hydroxymethyl)aminomethane (Tris) added to the reaction mixture also contains a primary amino group, so a reaction between FDNB and Tris can be expected to take place. Furthermore, FDNB hydrolyses in alkaline solutions to 1-hydroxy-2,4-dinitrobenzene [3]. To establish the presence of these products, and of possible other side-products of the derivatisation reaction, the composition of the reaction mixture was investigated.

Other chromatographic determinations of tobramycin in serum have been reported [4—11]. The method described in the present paper has the advantages of small sample volume (50 μl), simple sample preparation, and the convenience offered by HPLC with UV detection.

EXPERIMENTAL

Instrumentation

The chromatographic equipment was described previously [1]. UV absorption measurements, UV spectra and infrared (IR) spectra were obtained with a Pye-Unicam SP-500 Series 2 single-beam spectrophotometer, a Shimadzu UV-200 double-beam recording spectrophotometer, and a Jasco IRA-1 grating infrared spectrophotometer, respectively. Mass spectra were recorded on an AEI MS 902 electron-impact mass spectrometer. Element analyses were carried out by the Element Analytical Section of the Institute for Organic Chemistry TNO, Utrecht, The Netherlands, under the supervision of W.J. Buis. The microbiological assays were carried out by the National Institute of Public Health (RIV), Bilthoven, The Netherlands, using the agar-well diffusion technique previously described [2].

Materials

Demineralized water was used. Tris(hydroxymethyl)aminomethane (Tris), 1-fluoro-2,4-dinitrobenzene (FDNB), acetic acid, methanol, acetone and ethyl acetate were p.a. grade from Merck (Darmstadt, G.F.R.). Acetonitrile, "zur Synthese", silica-gel 60, 70—230 mesh, and pre-coated silica-gel 60 F_{254} plates were also from Merck. Chloroform, 4 *M* sodium hydroxide, 25% ammonia and 10% hydrochloric acid were of Dutch Pharmacopoeia quality (Ph. Ned. VI and VIII, respectively) all from Brocacef (Maarssen, The Netherlands).

The preparations for injections — Obracin® (Eli Lilly, St.-Cloud, France) and Garamycin® (Essex, Heist-op-den-Berg, Belgium) — contained the equivalents of 40 g/l tobramycin and 40 g/l gentamicin, respectively. Tobramycin, lot OEJ 90, stated potency 975 μg/mg, was obtained by courtesy of Eli Lilly Nederland (Utrecht, The Netherlands). All antibiotic concentrations were calculated relative to potency [12, 18]. When the weight of the chemical substance tobramycin is meant it is indicated by the notation: (by weight).

Pooled human serum from ambulatory patients was frozen and stored at −18°C within three days of collection.

Stoppered polypropylene centrifuge tubes of 1.5 ml capacity, and ampoules of 0.5 ml capacity were also used.

HPLC conditions

The mobile phase was prepared by mixing 340 ml of water (filtered through a 0.2-μm filter) with 660 ml of acetonitrile (filtered through a 0.2-μm filter) and 1 ml of acetic acid, and deaerated ultrasonically. The flow-rate was 3.0 ml/min. A μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) was used (Waters Assoc., Milford, MA, U.S.A.). Ultraviolet detection was made at 365 nm. Chromatography was performed at room temperature.

Procedures

Procedure A: serum sample preparation. Dispense into a centrifuge tube 50 μl of the serum sample, add 50 μl of a solution containing 20 g/l Tris in water, also containing the equivalent of 20 mg/l gentamicin (the internal standard), and vortex. Add 200 μl of acetonitrile and vortex. Centrifuge at 2500 *g* for 5 min. Transfer 200 μl of the supernatant into an ampoule, add 20 μl of FDNB in acetonitrile (170 g/l) and heat-seal the ampoule. Place in a water-bath at 80°C for 45 min. Break the seal and inject 150 μl into the chromatograph.

Procedure B: in vivo experiment and bioassay comparison study. A healthy 81-kg volunteer received 80 mg of tobramycin by intramuscular injection. Blood samples were collected at regular time intervals, and the serum was separated and stored at −18°C. Tobramycin standards were prepared in pooled human serum. Standards and serum samples were analysed in duplicate in one run according to procedure A. The same serum samples and standards were also analysed in duplicate by the microbiological assay.

Procedure C: synthesis of the 2,4-dinitrophenyl tobramycin derivative. Obracin® (2 ml) was mixed with 4 ml of acetonitrile containing 180 mg of FDNB and 225 μl of 4 *M* sodium hydroxide. The mixture was transferred to an 8-ml vessel, closed with a septum, and placed in a water-bath at 80°C. At regular time intervals the mixture was tested for alkalinity; if necessary, more sodium hydroxide solution was added by means of a syringe. During the derivatisation reaction an oily, reddish precipitate developed, especially upon the addition of sodium hydroxide. The reaction was considered to be complete when addition of more sodium hydroxide solution did not give rise to the formation of more precipitate. The reaction mixture was neutralised by the addition of hydrochloric acid and diluted with acetonitrile. After filtration, the acetonitrile solution was evaporated under reduced pressure and the yellow residue was reconstituted in the smallest possible volume of a chloroform—acetonitrile mixture (11.5:8.5). A glass column (100 cm × 3.5 cm I.D.) was filled with a slurry, prepared by mixing about 400 ml of silica gel, activated at 100°C for 12 h, and about 400 ml of a chloroform—acetonitrile mixture (11.5:8.5). After drainage of the column, the yellow solution was put on top of the column. After washing the column with 50 ml of chloroform, the column was eluted with a chloroform—acetonitrile mixture (11.5:8.5) and 20-ml fractions were collected. The fractions were screened by thin-layer chromatography on silica-gel plates (activated at 100°C) with chloroform—acetonitrile (11.5:8.5) as the mobile phase. The appropriate fractions (160—260 ml of the eluate) were combined and the solvent was evaporated under reduced pressure. The residue was recrystallized from a water—acetone mixture and dried overnight at 105°C under reduced pressure and over diphosphorus pentoxide, since the substance showed hygroscopic properties.

Procedure D: recovery measurements. Tobramycin lot OEJ90 was investigated for tobramycin content and moisture content by non-aqueous titration and Karl Fischer titration, respectively, according to the U.S. Pharmacopeia [12]. The purity of tobramycin lot OEJ90 was investigated by thin-layer chromatography as described by Pauncz and Harsányi [13], with the detection of the spots carried out according to the method of Wilson et al. [14].

The absolute recovery of tobramycin was estimated by spiking blank serum with tobramycin in lot OEJ90 to obtain serum spiked at a concentration of 4 mg (by weight)/l. Also, a solution of the purified 2,4-dinitrophenyl tobramycin derivative in the mobile phase was prepared. Serum sample preparation and chromatography were performed in quadruplicate according to procedure A (no internal standard). The solution of the purified derivative was injected in quadruplicate.

The yield of the derivatisation reaction was estimated by preparing from blank serum a quantity of supernatant in a way analogous to procedure A (no internal standard). A quantity of this supernatant was spiked with tobramycin lot OEJ90 to obtain a concentration of 0.67 mg (by weight)/l, corresponding to a serum concentration of 4 mg (by weight)/l. The spiked supernatant was derivatised and chromatographed, in quadruplicate, according to procedure A. A solution of the purified tobramycin derivative was also injected in quadruplicate.

As a check on the results of the two former experiments, the recovery of tobramycin from the deproteinisation step was estimated by spiking two identical quantities of blank serum samples with the same amount of tobramycin, one before and the other after the addition of acetonitrile. The added amount of tobramycin corresponded to a serum concentration of 4 mg/l.

RESULTS AND DISCUSSION

For the determination of gentamicin and sisomicin, with tobramycin as the internal standard, a mobile phase composed of 700 ml/l acetonitrile was used. No separation of the tobramycin derivative from an interfering peak in the blank was then obtained [2]. However, with a mobile phase composed of 660 ml/l acetonitrile, separation of the tobramycin derivative from the interfering peak was obtained. Gentamicin was added as the internal standard; the peak of the gentamicin C_{1a} derivative was used for the calculation of the peak height ratios. Representative chromatograms are shown in Fig. 1.

Precision, linearity and sensitivity

Serum samples with eight different concentrations of tobramycin, ranging from 0.25 to 16 mg/l, were analysed in one run. The results are summarised in Table I. In the range 0.5—16 mg/l a linear calibration line was obtained; throughout this range the coefficient of variation was 3% or less. At lower concentrations deviations from linearity were observed. The equation for the linear least-squares regression line in the range 0.5—16 mg/l is: PHR = 0.037 (± 0.050) + 0.398 × tobramycin concentration, r = 0.998 (39 samples), in which PHR is the peak height ratio (tobramycin:gentamicin C_{1a}), and the number between brackets is the 95% confidence interval of the intercept. The intercept has a

95% confidence interval which includes the origin, so the calibration line passes through the origin. In the therapeutic use of tobramycin, peak serum concentrations of 5—8 mg/l and trough concentrations of 1—2 mg/l are encountered [15]. So, the linear part of the calibration line includes the therapeutic range.

TABLE I

PEAK HEIGHT RATIOS (PHR) OF DERIVATISED TOBRAMYCIN TO INTERNAL STANDARD (GENTAMICIN C_{1a}) AND COEFFICIENTS OF VARIATION (C.V.) OBTAINED WITH SERUM SAMPLES (50 µl) CONTAINING 0.25—16 mg/l TOBRAMYCIN

Concentration (mg/l)	n*	PHR	PHR/ concentration	C.V. (%)
0.25	6	0.166	0.66	4
0.50	6	0.252	0.50	2
1	6	0.420	0.42	2
2	6	0.873	0.44	3
4	6	1.67	0.42	2
8	6	3.17	0.40	2
12	6	4.68	0.39	2
16	3	6.65	0.42	1

*n = number of determinations.

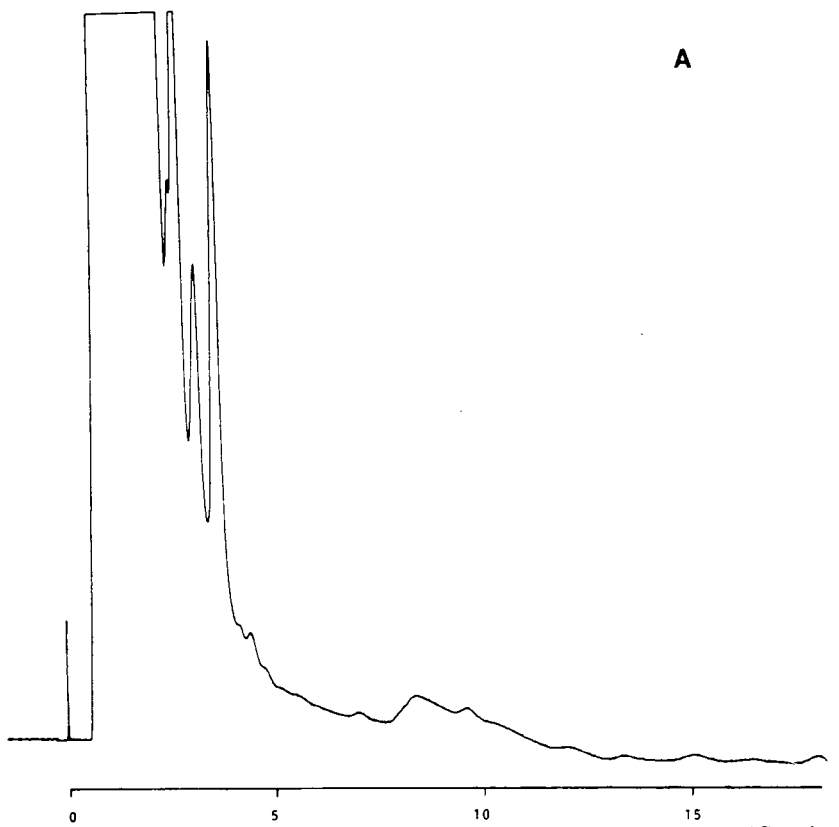

Fig. 1.

(Continued on p. 422)

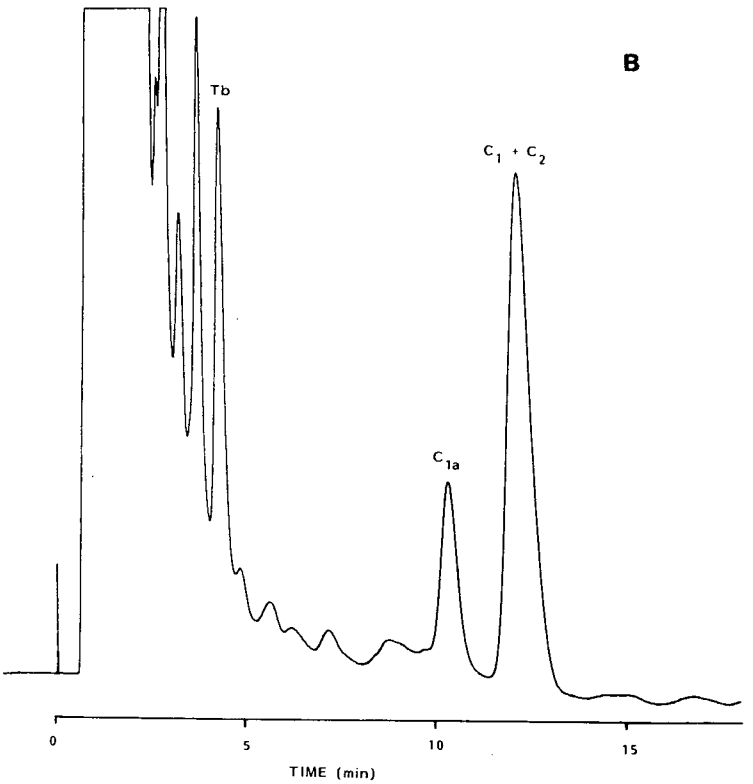

Fig. 1. HPLC of serum samples. The detector setting was 0.02 a.u.f.s. Chromatogram A was obtained from 50 μl of blank serum. No internal standard was added. Chromatogram B was obtained from 50 μl of blank serum, spiked at a concentration of 4 mg/l tobramycin, with gentamicin as the internal standard. Tb = tobramycin derivative; C_{1a} = gentamicin C_{1a} derivative; $C_1 + C_2$ = gentamicin C_1 and gentamicin C_2 derivatives (not separated).

In vivo experiment and bioassay comparison study

The results are summarized in Fig. 2. The correlation between the values found by the two methods is good. The correlation line shows no significant intercept, and the slope differs not significantly from one, so both methods can be regarded as free of systematic errors.

Characterisation of the derivative of tobramycin

(1) A solution of the recrystallised yellow substance in the mobile phase was chromatographed as described under Experimental (HPLC conditions). Only one peak was obtained with the same retention time as the tobramycin derivative after derivatisation of tobramycin-containing serum samples.

(2) The purity of the derivative was also investigated by thin-layer chromatography on silica gel, activated at 100°C with mobile phase chloroform—acetonitrile (11.5 : 8.5). Only one yellow spot with R_F = 0.48 was observed. The obtained chromatogram was sprayed with ninhydrin, according to the description of Wilson et al. [14]. The yellow spot did not change colour, indicating that all amino groups were derivatised.

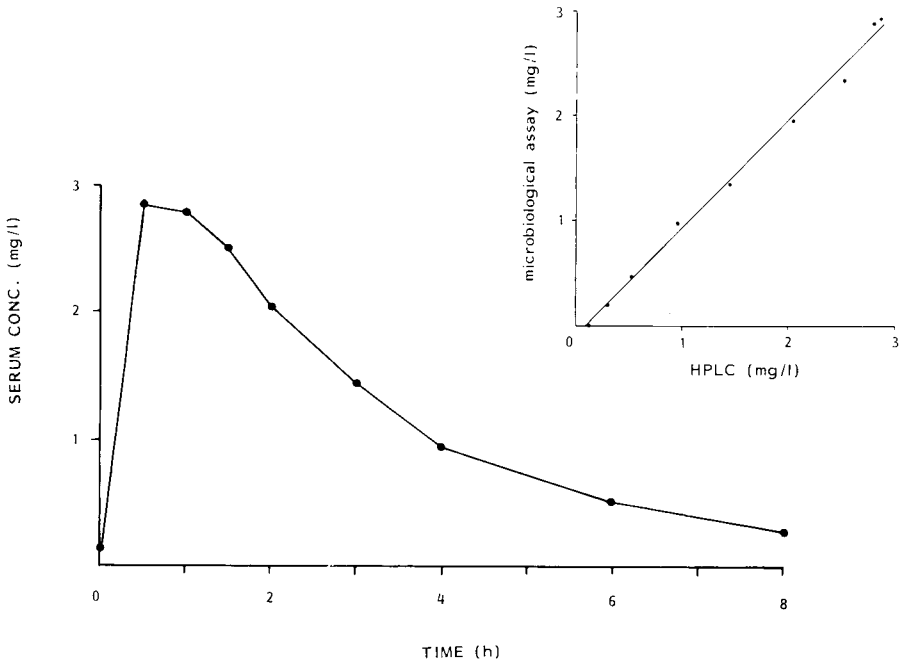

Fig. 2. Serum concentration of tobramycin as a function of time obtained in one volunteer following intramuscular injection of 1 mg/kg body weight. Each value is the mean of duplicate HPLC determinations. Inset: linear least-squares regression analysis of the tobramycin concentrations in the sera from the in vivo experiment, determined by HPLC and microbiological assay. Equation: $Y = -0.11\ (\pm\ 0.13) + 1.04\ (\pm\ 0.07)\ X$, $r = 0.997$ (9 data pairs), where Y = result of the HPLC assay (mean of duplicate determinations), and X = result of the microbiological assay (mean of duplicate determinations). Numbers in parentheses are 95% confidence intervals.

(3) The results of the element analyses were: C 43.58%, H 3.63%, N 15.62%, O 36.08%, found; C 44.42%, H 3.65%, N 16.19%, O 35.75%, calculated for $C_{48}H_{47}N_{15}O_{29}$ = tobramycin-(2,4-dinitrophenyl)$_5$.

(4) A solution of the substance in methanol showed the UV spectrum of the N-2,4-dinitrophenyl group with maxima at 260 nm and 350 nm. The specific absorption, $A_1^{1\%}{}_{cm}$, measured at 350 nm, was found to be 674. Assuming the derivative to be tobramycin-(2,4-dinitrophenyl)$_5$, the molar absorptivity, ϵ, was calculated from the observed specific absorption and the assumed molecular

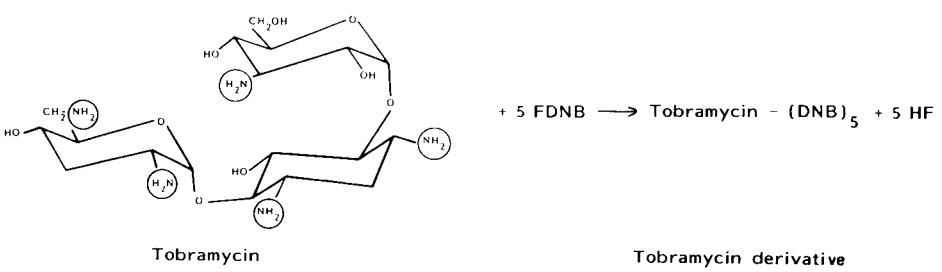

Fig. 3. Derivatisation of tobramycin with 1-fluoro-2,4-dinitrobenzene (FDNB).

weight. ϵ was found to be 8.74×10^4 l/mol · cm. The value of ϵ for the N-2,4-dinitrophenyl group is 1.7×10^4 to 1.8×10^4 l/mol · cm [16], thus 4.9—5.1 dinitrophenyl groups are present in the derivative, which is in good accordance with the expected number of 5. Other reports [1, 17] are also in support of a structure consisting of the five-fold derivatised tobramycin (see Fig. 3).

Recovery

By non-aqueous titration, the tobramycin content of lot OEJ 90 was found to be 86.7% (by weight); the moisture content was found to be 10.3%. From these data, the potency, calculated on the anhydrous basis, is 964 μg/mg [12]; this is in accordance with the stated potency (975 μg/mg). No impurities related to tobramycin could be detected by thin-layer chromatography. It was concluded that lot OEJ90 contained 867 μg (by weight)/mg of the chemical substance tobramycin. The absolute recovery of tobramycin was measured at 4 mg/l and found to be 41% (S.D. = 1%, n = 4). The recovery of tobramycin, added after the protein precipitation was 53% (S.D. = 1%, n = 4). From these data, the recovery of tobramycin from the deproteinisation can be calculated to be 77% (S.D. = 2%). By direct measurement, this recovery from the protein precipitation was found to be 75% (S.D. = 1%, n = 4), which is in accordance with the calculated value.

The recovery of tobramycin in the derivatisation reaction is rather low. Whether this is due to less-favourable derivatisation conditions, or to the difficulty of derivatising all five amino groups of the tobramycin molecule is not yet clear. Up to now, however, we have not been successful in finding other derivatisation conditions which give higher yields (see below).

The low over-all absolute recovery does not affect the reliability of the determination as this recovery is sufficiently reproducible. Until now, no results on the absolute recovery have been published in reports dealing with the chromatographic determination of aminoglycoside antibiotics in serum.

Internal standardisation

We investigated the extent to which the derivatisation conditions could be varied without affecting the peak height ratios of derivatised tobramycin with respect to derivatised gentamicin C_{1a}, and with respect to gentamicin $C_1 + C_2$. In general, we found that under derivatisation conditions far less favourable than those used in the determination, i.e. lower Tris and FDNB concentrations, lower reaction temperature and shorter reaction time, the peak heights are lower for all derivatives, but that the derivatisation of the gentamicin components is more affected than the derivatisation of tobramycin. It is concluded that tobramycin is more easily derivatised than the gentamicin components. Despite internal standardisation, it is advisable, therefore, to do the complete sample preparation for standards and unknown samples together in one run. However, the proposed internal standardisation was found to be effective in compensating for the increase in peak height observed after the storage of the derivatised samples [1]. After storage for 16 h at room temperature we found that peak height ratios were increased by less than 10%.

Side-products of the derivatisation reaction

A solution of 100 mg of FDNB and 30 mg of Tris in 3 ml of a water—acetonitrile mixture (1:2) was heated at 80°C for 45 min. A chromatogram of the derivatisation mixture is shown in Fig. 4. The four substances giving rise to the peaks I, II, III and IV were isolated. Peak I was identified as 1-hydroxy-2,4-dinitrobenzene. The isolated substance and the reference substance have identical UV spectra — which show a pH shift — and identical chromatographic properties in various HPLC systems. 1-Hydroxy-2,4-dinitrobenzene is known to be formed by hydrolysis of FDNB in alkaline solution [3].

Peak II was identified as 2-[N-(2,4-dinitrobenzene)amino]-2-hydroxymethyl-1,3-propanediol, the product of the reaction between Tris and FDNB. Its structure was confirmed by mass spectrometry; the UV spectra and the IR spectrum were in accordance with this structure.

Peak III was identified as the excess FDNB in the reaction mixture.

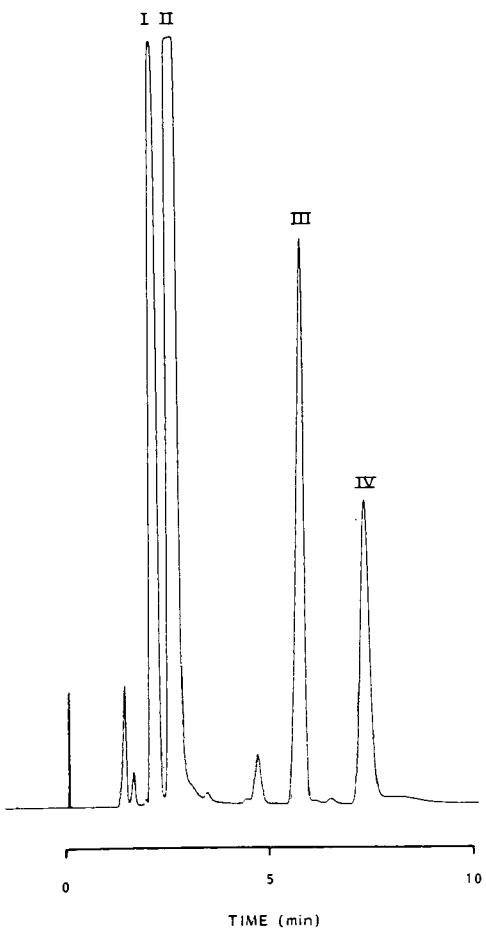

Fig. 4. HPLC of the derivatisation mixture. The detector setting was 2.0 a.u.f.s. Experimental details: mobile phase, 500 ml/l acetonitrile and 500 ml/l water; flow-rate, 1.5 ml/min; injection volume, 7 μl. Other conditions as described in Fig. 1. For identity of the peaks see Results (side-products of the derivatisation reaction).

Substance IV gave the UV spectrum of the N-2,4-dinitrophenyl group, but its structure could not be elucidated with certainty.

As Tris was expected to react with FDNB, we made a search for other substances or buffers that could be useful to alkalinise serum prior to deproteinisation. Tested were solutions in water of 10 g/l sodium carbonate, sodium acetate, triethanolamine and ammonia; 1.5 g/l triethylamine and N,N-diisopropylethylamine; 0.1 *N* sodium hydroxide and 0.1 *N* tetrabutylammonium hydroxide in a propan-2-ol—methanol mixture. Aqueous solutions of 10 g/l dibasic sodium phosphate, sodium bicarbonate and borax were incompatible with an acetonitrile—water mixture (2:1). We were unable, however, to find a suitable substitute for Tris. The best results were obtained with N,N-diisopropylethylamine which, being a tertiary amine, is not attacked by FDNB. After optimising the derivatisation parameters for this amine, we found essentially the same absolute recovery for tobramycin as with the Tris procedure. However, the recovery for gentamicin with N,N-diisopropylethylamine was far less than with the Tris procedure.

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CHROMBIO. 950

RAPID THIN-LAYER CHROMATOGRAPHY OF VARIOUS WEAK ANALGESICS IN SALIVA

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SUMMARY

A thin-layer chromatographic method was developed to analyze phenacetin, 4-aminophenazone and N-methyl-4-aminophenazone (5 and 1.25 μg/ml); bucetin (4-ethoxy-β-hydroxybutyranilide), propyphenazone and N-acetyl-4-aminophenazone (2.5 and 1.25 μg/ml); phenazone (15 and 1.25 μg/ml); and N-acetylsalicylamide (0.25 μg/ml). The method was designed to study the bioavailability of different commercial tablets from salivary concentration data.

INTRODUCTION

Recently pharmacokinetic parameters of drugs have been established from saliva and have either proved to be similar to those obtained from plasma or a good linear relationship between saliva and plasma concentration curves has been demonstrated [1]. For a number of drugs (for example, salicylic acid, acetaminophen, digoxin, phenazone, phenytoin and phenobarbital) the saliva/plasma ratios have been determined [1, 2]. Salivary concentration data should also provide valuable knowledge on the bioavailability of drugs. Thus we developed a rapid analytical method which allows routine determination of a number of drugs over wide concentration ranges.

Several chromatographic methods have been applied to the determination of analgesics in biological samples. Table I provides a comparison of recent work on phenacetin and phenazone with our results. Thin-layer chromatography (TLC)-densitometry as described in this paper is more precise and faster than earlier TLC procedures [5, 6].

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TABLE I

COMPARISON OF CHROMATOGRAPHIC METHODS FOR PHENACETIN AND PHENAZONE

Ref.	Drug	Method*	Sample	Sensitivity (μg/ml)	Reproducibility (%)
3	Phenacetin	HPLC	Plasma	250	4.85
4	Phenazone	GC	Saliva	15—2	5.6—20
5	Phenazone	TLC—densitometry	Saliva	400—50 (ng/spot)	—
This paper	Phenacetin	TLC—densitometry	Saliva	5—1.25	6.3—16.0
This paper	Phenazone	TLC—densitometry	Saliva	15—1.25	5.0—25.2

*HPLC = high-performance liquid chromatography; GC = gas chromatography.

EXPERIMENTAL

Apparatus and materials

The reference substances — phenacetin, bucetin (4-ethoxy-β-hydroxybutyranilide), phenazone, propyphenazone and N-acetylsalicylamide — were purchased from Merck (Darmstadt, G.F.R.), 4-aminophenazone was purchased from Fluka (Neu-Ulm, G.F.R.), and N-acetyl- and N-methyl-4-aminophenazone were obtained from Hoechst (Frankfurt, G.F.R.). All these compounds were of analytical grade, as were the reagents (chloroform, acetone, ammonium sulfate, sodium tetraborate, formic acid and dichloroaethane), which were obtained from Merck.

Extraction procedure

The extraction of phenacetin, bucetin, phenazone, propyphenazone and N-acetylsalicylamide from saliva was performed as described previously [7]. The extraction procedure was essentially the same for 4-aminophenazone and N-methyl-4-aminophenazone apart from using 10 mg of sodium tetraborate instead of ammonium sulfate and sulfuric acid. N-Acetyl-4-aminophenazone was extracted with sodium tetraborate and additionally with ammonium sulfate and sulfuric acid.

Chromatography

Chromatography was performed on 10 × 20 cm HPTLC plates coated with silica gel 60 (Merck). Standard and test solutions (2 μl) were applied to the HPTLC plate using a Mikroliter Applicator and 2-μl capillaries (Merck).

A stock solution and three dilutions in acetone were prepared and kept cool in the dark. The dilutions were used as standard curves and had to be chromatographed on each plate (see Table II).

Nineteen spots were applied 1 cm apart in the sequence standard—test solution—standard, with three spots for each standard concentration and ten spots for test solutions. The spot diameter was less than 2 mm. The starting point was kept constant at 1 cm from the edge of the HPTLC plate by means of the Mikroliter Applicator.

TABLE II

CONCENTRATION RANGES FOR STOCK AND STANDARD SOLUTIONS

Compound	Stock solution (mg/ml)	Standard solution (μg/ml)	Preparation
Phenacetin	1	200, 100, 25	Weekly
Bucetin	1	200, 100, 25	Weekly
Propyphenazone	1	200, 100, 25	Weekly
Phenazone	1	200, 100, 25	Weekly
N-Acetylsalicylamide	0.1	20, 10, 2.5	Weekly
N-Acetyl-4-aminophenazone	1	200, 100, 25	Daily
N-Methyl-4-aminophenazone	1	200, 100, 25	Daily
4-Aminophenazone	1	200, 100, 25	Daily

Table III shows the different solvent systems, the R_F values and the spectrophotometric parameters for the drugs. The inside of a tank for ascending chromatography (Camag, Muttenz, Switzerland) was lined with filter paper to accelerate saturation, which was reached after 45 min. The solvent systems could be used for two plates on the same day at room temperature. The plates were developed for 9 cm, which corresponds to 20 min, and were allowed to dry in the air in a protected area for 15 min. The chromatograms were scanned in situ with a PMQ3 densitometer (Zeiss, Oberkochen, G.F.R.). The spectrophotometer parameters were slit length 7 mm, slit width 0.7 mm, scanning speed 120 mm/min. The scans were recorded on a Metrawatt RE 647 and integration of spot areas (by product of peak height and width at one-half the peak height) was performed using a Spectra-Physics Minigrator (Spectra-

TABLE III

CHROMATOGRAPHY SOLVENT SYSTEMS, R_F VALUES AND SPECTROPHOTOMETRIC DETECTION

Substance	Mobile phase	R_F value	Detection	Wavelength (nm)
Phenacetin	Chloroform—	0.40	UV remission	247
Bucetin	acetone (9 : 1, v/v)	0.56	UV remission	250
Propyphenazone		0.65	UV remission	273
Blank saliva		0.30	UV remission	247, 250, 273
4-Aminophenazone	Chloroform—	0.31	UV remission	275
N-Methyl-4-amino-phenazone	ethanol (9 : 1, v/v)	0.44	UV remission	275
N-Acetyl-4-amino-phenazone		0.20	UV remission	275
Blank saliva		0.60	UV remission	275
Phenazone	Dichlorethane—	0.12	UV remission	255
N-Acetylsalicylamide	formic acid (10 : 1, v/v)	0.43	Fluorescence	excitation 314 emission 390
Blank saliva		0.06	UV-remission	255

Physics, Santa Clara, CA, U.S.A.). Calculation of the concentration in each saliva sample was made from the standard curve obtained for each plate. The standard curves for all substances were linear, the correlation coefficient being at least $r = 0.998$. The spectrophotometric parameters are listed in Table III.

Blank saliva from different subjects was chromatographed on the same plate with each substance. By UV remission one could detect a small peak which was well separated from the compounds of interest (see also Table III). While fluorescence was being measured no spot from blank saliva was detectable over the whole plate under our photometric conditions.

RESULTS AND DISCUSSION

Precision

The concentrations of the standard curves for phenacetin, bucetin, propyphenazone, phenazone, N-acetyl- and N-methyl-4-aminophenazone and 4-aminophenazone were 400, 200 and 50 ng per spot, 50 ng being the limit of

TABLE IV

COMPARISON OF THE ERROR OF THE SCANNING MEASUREMENT TO THE TOTAL INSTRUMENT ERROR

Drug	Spot concentration (ng)	Instrument error (R.S.D., %)	Total error (R.S.D., %)
Phenacetin	400	0.8	1.6
	200	2.0	2.6
	50	4.0	9.0
Bucetin	400	1.2	1.9
	200	2.3	5.9
	50	4.0	5.5
Propyphenazone	400	1.3	2.5
	200	1.2	4.4
	50	6.0	6.2
Phenazone	400	1.3	2.3
	200	3.0	2.0
	50	5.0	9.0
4-Aminophenazone	400	0.9	2.1
	200	1.2	3.4
	50	2.5	6.7
N-Methyl-4-aminophenazone	400	1.0	2.3
	200	0.9	4.9
	50	4.0	5.3
N-Acetyl-4-aminophenazone	400	1.0	3.2
	200	1.3	4.0
	50	5.0	5.8
N-Acetylsalicylamide	40	1.7	2.5
	20	3.6	6.9
	5	4.0	15.2

practical sensitivity. The standard curve for N-acetylsalicylamide was in the concentration range of 40, 20 and 5 ng per spot, 5 ng being the limit of practical sensitivity. The precision of the scanning measurement was determined (A) as instrument error (one spot was measured three times), and (B) as total error (one standard concentration was measured on six different spots, three times). In Table IV the methodological error for all drugs is expressed as the relative standard deviation.

Recovery

Preliminary experiments had indicated the concentration ranges which were to be expected after the intake of a particular commercial tablet. The recovery experiments have been described previously [7]. The recovery data from 12 saliva samples are given in Table V [the extracted concentration (μg/ml), the mean (%) and the relative standard deviation (R.S.D., %)].

Hitherto neither bucetin, propyphenazone nor N-acetylsalicylamide have been extracted from saliva. Phenacetin was extracted from saliva in a way similar to ours and analyzed by GC in the range 0—6 μg/ml, but neither recovery nor the error of the method were given [8].

Several publications are available on the assessment of phenazone in saliva, ranging from direct spotting of saliva on a TLC plate [5] to extraction procedures similar to ours which were followed by GC [4, 9]. The TLC method [5] was done in our concentration range of 50—400 ng/spot with an extraction yield of 97—103%, which is in excellent agreement with our results, but more

TABLE V

MEAN AND STANDARD DEVIATION OF THE EXTRACTION PROCEDURE FROM SALIVA

$n = 12$.

Drug	μg/ml	Recovery	
		Mean (%)	R.S.D. (%)
Phenacetin	5.0	100.2	6.3
	1.25	103.3	16.0
Bucetin	2.5	104.5	5.9
	1.25	95.8	16.2
Propyphenazone	2.5	85.7	10.6
	1.25	90.3	14.9
Phenazone	15.0	101.4	5.0
	1.25	88.5	25.2
N-Acetylsalicylamide	0.25	55.6	9.4
4-Aminophenazone	5.0	92.0	9.3
	1.25	89.1	13.1
N-Methyl-4-aminophenazone	5.0	94.1	5.4
	1.25	89.1	8.4
N-Acetyl-4-aminophenazone Extraction with sodium tetraborate	1.25	40.0	15.4
N-acetyl-4-aminophenazone Extraction with ammonium sulfate and sulfuric acid	1.25	73.0	8.7

precise data are missing. The results after GC assay are in good agreement with our high-performance TLC method: in the range of 15—2 μg/ml the error was 5.6—20% [4], at 15—1 μg/ml the error was 4.1—22% [9], while our results showed an error of 5% at 15 μg/ml and of 25.2% at 1.25 μg/ml.

As can be understood from Table V the extraction of 4-aminophenazone and N-methyl-4-aminophenazone has to be performed at alkaline pH, whereas the extraction of N-acetyl-4-aminophenazone should be done at acidic pH. The extraction of these three pyrazole derivatives from saliva has been reported after the intake of aminopyrine [10] but without any recovery and accuracy data.

The data presented in this paper show that low drug concentrations can readily be determined in saliva. The authors believe that the method described here is convenient for bioavailability studies of commercial products.

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Note

Improved assay for α-tocopherol in the picogram range, using gas chromatography—mass spectrometry

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Vitamin E is the generic name for the biologically active members of the fat-soluble tocopherol and tocotrienol families; α-tocopherol is the most biologically active form. Two theories of the role of vitamin E in the lung have been promoted. According to the antioxidant theory, vitamin E acts as a lipid antioxidant by inhibiting the free-radical-mediated peroxidation of unsaturated lipids by acting as a free-radical scavenger [1, 2]. Other investigators, however, disagree with the primacy of an antioxidant role and suggest a specific metabolic role for vitamin E [3, 4].

Three factors were paramount in our resolve to develop a simple, direct, quantitative microtechnique for determining vitamin E in lung tissue. First, we recognized some years ago from our structural studies [5—7] that the lung does not react homogeneously to oxidant gases. Therefore, meaningful results from the analysis of the whole tissue could be masked by including large areas of the lung that appeared unaffected. Second, vitamin E is very sensitive, and is oxidized readily when exposed to air or other oxidizing agents. Thus extreme care must be exercised to obtain accurate data from tissue samples subjected to complicated extraction procedures. Third, a major deficiency in studies of vitamin E in the lung has been that the tissue levels of vitamin E were rarely determined quantitatively; the actual amount in the lung was presumed to be proportional to the amount fed to the animals.

To overcome the above difficulties we developed a procedure that uses freeze-dried cryostat sections, as well as microdissections of similar freeze-dried sections, as source materials for vitamin E analyses with gas chromatography—mass spectrometry (GC—MS). This procedure is direct, quantitative, and reproducible. Analyses can be conducted on a very small amount of dry tissue; thus the procedure permits us to analyze individually tissues such as

the airways, alveolar parenchyma, focal areas of response (juncture of the terminal bronchiole and proximal alveolar duct region), blood vessels, and connective tissue obtained by microdissection of freeze-dried sections of the lung.

Until now, available methods for α-tocopherol determination (refs. 8—10 and references cited therein) have lacked the sensitivity and specificity needed in our research, and all methods require multi-step extraction—purification procedures that are not only time-consuming but cause significant losses of this very labile compound, particularly when working with sub-nanogram quantities. Major features of our method include: (1) A stable-isotope labeled α-tocopherol, used as an internal standard. (2) Increased stability of standards and endogenous tocopherols by working exclusively with their trimethylsilyl (TMS) derivatives. (3) A rapid extraction procedure that requires no further purification before the GC—MS analysis. (4) The possibility of analyzing other tocopherols simultaneously by monitoring their molecular ions. (5) The precise selection of any tissue or area thereof, as well as blood, may be used as source material without any modification of the procedure.

EXPERIMENTAL

Materials

The silylation reagent [bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane], and the solvent (pyridine) were obtained from Regis Chemical (Chicago, IL, U.S.A.). The GC column packing (3% Silar-10C on 100—120 mesh Gas-Chrom Q) and the cholestane standard were obtained from Applied Science Labs. (State College, PA, U.S.A.). The α-tocopherol standard was obtained from Sigma (St. Louis, MO, U.S.A.).

The deuterated α-tocopherol standard was prepared by reducing α-tocotrienol (from Hoffmann-LaRoche, Basel, Switzerland) with deuterium at room temperature and pressure in ethanol-*d*, using Pd/C catalyst. The product, a mixture of deuterated α-tocopherols resulting from both hydrogenation and exchange, was purified by thin-layer chromatography (TLC) on silica gel using cyclohexane—diethylether (4:1, v/v) as the solvent. The d_{13} species was the most abundant of the many labeled variants in this mixture.

Instrumentation

Analyses were performed on an LKB 9000 combination gas chromatograph—mass spectrometer equipped with a data system [11] based on a DEC PDP-12 computer. Masses 502 [molecular ion of tocopherol trimethylsilyl (TMS) ether] and 515 (molecular ion of tocopherol-d_{13} TMS ether) were monitored at 20 eV with a computer-controlled variable accelerating voltage system [12]. GC was performed with a 2 m × 2 mm glass column of 3% Silar-10C on 100—120 mesh Gas-Chrom Q at 230°C, with an injector temperature of 300°C and a helium carrier gas flow-rate of 30 ml min^{-1}.

Tocopherol standard solutions were assayed on a Hewlett-Packard Model 5710A gas chromatograph with a flame ionization detector (FID). The column had the specifications given above, but was operated at 190°C.

Tissue preparation

Blocks of fresh tissue, approximately 5 mm^3 each, were obtained from each lung to be studied. These were immersed in liquid nitrogen and stored at −80°C. The blocks were mounted on a cryomicrotome chuck, trimmed, and sectioned at 10 to 50 μm, at −20°C. The sections were freeze-dried and then weighed on a microbalance (accurate to 0.1 μg). To determine tissue-specific levels of the vitamin, specific tissues (e.g. airway, alveolar parenchyma, vascular, and connective tissue) were isolated by microdissecting the freeze-dried sections under a stereomicroscope. When necessary, homologous micro-sections were pooled before weighing.

Extraction

Dry samples weighing between 5 and 50 μg were placed in a 0.5-dram vial equipped with a PTFE-lined screw cap. Silylating reagent (15 μl), 15 μl of pyridine, and approximately 40 ng of deuterated α-tocopherol in 2 μl of silylation reagent—pyridine were added, and the vial was capped tightly. After at least 10 min at room temperature, extraction and derivatization were complete. Then the sample was injected, in 2-μl aliquots, directly into the GC—MS system for analysis.

Calibration

Mixtures of known weights of α-tocopherol and deuterated α-tocopherol (both from stock solutions in silylation reagent—pyridine, i.e. TMS-derivatized) were prepared and analyzed. A calibration curve was prepared by plotting the ratio of peak areas in the MS response to α-tocopherol and its deuterated analogue as a function of the weight ratio of these compounds in each sample mixture. The equation for this curve was determined by linear regression analysis.

Standards

Stock solutions (10 mg ml^{-1}) of α-tocopherol (for calibration) and deuterated α-tocopherol (for the internal standard) were maintained in silylating reagent—pyridine (1:1). Before each use, these were assayed by GC with FID by combining an aliquot with a cholestane solution of known concentration. Cholestane was selected as a primary standard because of its known purity, stability, and suitable GC retention time. A relative molar response ratio of 0.99 was determined for α-tocopherol TMS ether versus cholestane, using the labeled tocopherol which was purified by TLC and shown by GC to be 96% pure. The concentration of an aliquot of the labeled stock solution was verified (re-established if necessary), and then the aliquot was diluted to approximately 20 μg ml^{-1}, which was found to be a level suitable for an internal standard.

RESULTS AND DISCUSSION

The main difficulty with all analyses for the tocopherols is that the compounds are unstable. Reference solutions cannot be prepared, stored, transferred, or otherwise manipulated without substantial risk of decomposition.

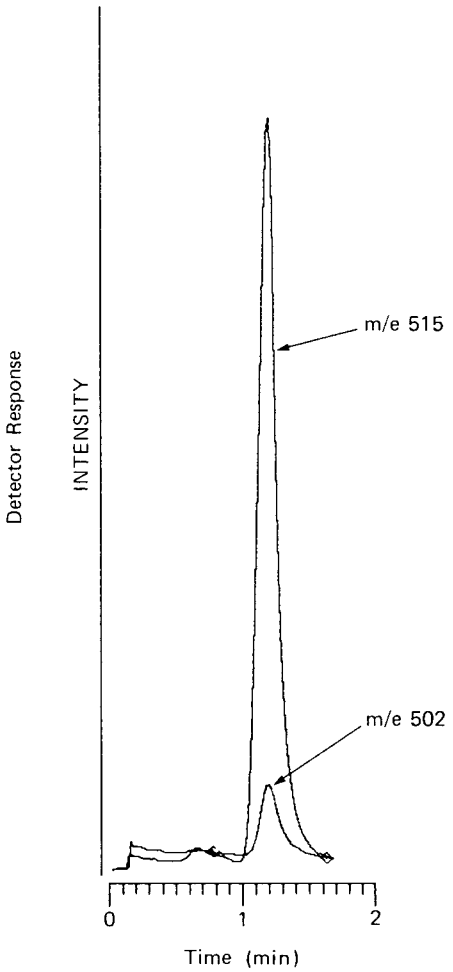

Fig. 1. GC—MS data from a 44-μg dry weight tissue section of rat lung. The *m/e* 502 trace represents 170 pg of α-tocopherol as its TMS derivative. The *m/e* 515 trace is the deuterium-labeled α-tocopherol TMS ether standard.

Likewise, vitamin E cannot be extracted from tissue or other substrates without risking significant, and often variable, losses of the vitamin.

To reduce these problems, we had previously added an internal standard (α-tocopherol labeled with deuterium) to the lung tissue before extraction. A chemically equivalent internal standard is used primarily because losses of the endogenous material should be compensated for by equivalent losses of the standard. However, one point which may be critical in the case of such labile compounds is seldom considered: the standard is added in solution form to a tissue (or blood) sample, while the endogenous material is within a cellular matrix, a distinctly different chemical and physical environment. The two compounds may decompose or be lost at different rates during extraction, particularly with multi-step, harsh extraction procedures. The variability in our early results was evidence of this problem. It soon became apparent that

TABLE I

α-TOCOPHEROL CONTENT OF LUNG TISSUE FROM ONE ADULT CONTROL RAT ON A NORMAL MAINTENANCE DIET

Each number is from a separate tissue section, microdissection, or pooled homologous microdissections.

Whole tissue section (μg/g dry weight)	Parenchyma	Connective tissue	Blood vessel walls	Airway
122	96	162	67	23
144	93	166	65	21
153	110	158	60	
	113			

we needed methods to stabilize both the endogenous and standard tocopherols, to evaluate routinely changes in concentration of our standard solutions, and to reduce the harshness of the extraction procedure while maximizing recovery yields.

Instability of the tocopherols may be significantly reduced by chemically blocking the free hydroxyl function. We have chosen to use a TMS derivative because it is easy to form and has excellent GC properties. All standard solutions are prepared as the TMS derivative, and their concentration may be routinely monitored by conventional GC methods versus a stable primary standard (e.g. cholestane). Special care must be taken, however, that the GC response is linear over the concentration range used, to avoid selective loss of either the tocopherol or cholestane in the GC system. This can be checked by injecting several different volumes and verifying peak ratio consistency.

Complications associated with conventional extraction procedures could be eliminated by changing the method of tissue preparation. For example, previous work [13] on measuring myoinositol in single cells showed the value of direct analysis of tissue constituents, using GC—MS ion monitoring and techniques of quantitative histochemistry. We believed that similar methods could be successfully applied to our lung research if enough vitamin E was present relative to potential interfering substances. Thus using weighed freeze-dried tissue sections or microdissections has several advantages. The departure from standard analyses of tissue homogenates allows, for the first time, the study of vitamin E in specific types of tissue within an organ. Also, of great significance is the fact that there is no need for any laboratory extraction or purification procedures. Extraction is accomplished by adding a silylating reagent directly to the dried tissue, together with the internal standard. Extraction is complete within a few minutes at room temperature. Furthermore, the vitamin E is converted rapidly in this process to its stable TMS derivative, which minimizes losses by decomposition.

Fig. 1 shows a typical chromatogram from the analysis of one tissue section weighing 44 μg (10% of the total extract was injected). This was ob-

tained from a rat that had been maintained on a diet deficient in vitamin E. The small peak (*m/e* 502) is due to endogenous α-tocopherol as the TMS ether, 170 pg in this aliquot, and indicates the excellent sensitivity of the technique in the picogram range. The large peak (*m/e* 515) is from the internal standard, α-tocopherol-d_{13} TMS ether. (Details of how the GC—MS technique of ion monitoring can be applied to similar assays using labeled internal standards have been thoroughly described [13, 14].) To emphasize the specificity of this method, one should note that a very large quantity of cholesterol (TMS ether) coelutes with the α-tocopherol TMS ether, but does not contribute any signal at the mass values chosen for this assay.

The ratio of peak areas observed in Fig. 1 is converted to a weight ratio of endogenous α-tocopherol to internal standard with a calibration curve prepared from known mixtures. A typical calibration had the equation $y = 8.098x + 0.028$ with a correlation coefficient of 0.9998, where y is the peak area ratio (*m/e* 502:*m/e* 515) and x is the weight ratio (α-tocopherol:α-tocopherol-d_{13}). Because there is no contribution at *m/e* 515 from unlabeled α-tocopherol TMS ether alone, the curve is linear [14]. The small positive y intercept results from a trace of unlabeled α-tocopherol in the internal standard, and a slope of considerably greater than 1 results because the d_{13} species chosen is only one of several deuterated variants of α-tocopherol in the standard. The calibration may be extended for other ranges of tocopherol concentration, but it is best to adjust the amount of internal standard added to the sample according to the anticipated endogenous α-tocopherol to obtain peak area ratios close to 1.

Table I presents some preliminary data resulting from the application of these methods to study α-tocopherol distribution in five different types of tissue in rat lung obtained through microdissection. Of special interest is the very remarkable difference in α-tocopherol content from one structural area to another; it is particularly low in the walls of both airways and blood vessels.

Much biochemical research on lung tissue exposed to oxidant gases is hampered because the lung tissue does not react homogeneously to the oxidant. The direct tissue analysis technique, using microdissection, will permit analysis both of the individual foci of injury at selected times during oxidant gas exposure, and of sections of airways, blood vessels, peripheral parenchyma, and even the epithelial layer of the upper airways. This selectivity gives us a means of studying the dynamics of vitamin E in specific lung locations during exposure to oxidant gases.

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Note

High-performance liquid chromatographic determination of homovanillic acid in urine using Sephadex G-10 for isolation

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It is well known that an increased amount of homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA) in urine is usually associated with neuroblastoma. Even if the excretion of vanillomandelic acid (3-methoxy-4-hydroxymandelic acid, VMA) is also elevated in urine from such patients, HVA is found to be more consistently increased [1, 2].

Numerous techniques have been developed for the determination of HVA in urine. Colorimetric measurement is preceded by laborious solvent extractions [1]. Gas chromatographic procedures result in sensitive assays but necessitate tedious extractions and derivatization steps prior to analysis [2, 3]. A specific and highly sensitive method comprised solvent extractions, thin-layer chromatography and high-performance liquid chromatography (HPLC) with electrochemical detection [4]. However, gross substance losses (reported recovery: 63 ± 5% R.S.D.) are probably inevitable consequences of such multi-step procedures and especially the solvent extraction steps.

Recently, simple two-step chromatographic procedures were developed for the determination of 5-hydroxyindole-3-acetic acid in urine [5, 6]. The present paper describes a modification of the latter method [6] for the measurement of urinary HVA. The compound was isolated from acidified urine by elution on a small Sephadex G-10 column and was then separated and determined by reversed-phase HPLC with UV-absorbance detection. This technique was used to measure the HVA excretion from patients with neuroblastoma as well as from healthy adults and children.

EXPERIMENTAL

Apparatus

The previously described [6] chromatographic isolation equipment was

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used with some modification. To make the columns (12 cm × 4 mm I.D.) graduated 2-ml pipettes [5] were cut off leaving 17 cm and were marked at a height of 12 cm. The same HPLC—UV equipment [5, 6] was utilized, although provided with another LDC pump, ConstaMetric I (LDC, Riviera Beach, FL, U.S.A.). Furthermore, instead of PXS 1025, Partisil-10 ODS, a column (25 cm × 4.6 mm I.D. with ZDV fittings) was prepacked with Partisil-10 ODS-2 (Whatman, Clifton, NJ, U.S.A.). The same operation conditions as in the earlier studies [5, 6] were used.

Chemicals and reagents

The mobile phase (pH 2) consisted of 220 ml of acetonitrile, 780 ml of glass-distilled and degassed water, 0.4 ml of concentrated sulfuric acid and 100 mg of sodium lauryl sulfate. Check the accuracy of the stock solution [5, 6] of HVA (Sigma, St. Louis, MO, U.S.A.) in 0.1 *M* ammonium formate buffer (pH 3) (100 μg/ml) as described (diluting to 25 μg/ml resulted in A_{280} = 0.345 ± 0.010 S.D., n = 5, for each of the HVA lots 85C-5043 and 87C-5065). Store the stock solution at 5°C (stable for 6 months). Prepare the working standards, 25, 12.5, 6.25 and 3.125 μg/ml, by serial dilution [5, 6] from the stock solution and a 37.5 μg/ml standard from the 50 and 25 μg/ml standards. Pack the isolation columns with Sephadex G-10 (Pharmacia, Uppsala, Sweden) and equilibrate with 30 ml of 0.1 *M* ammonium formate buffer (pH 3) (flow-rate 3 ml/h). Other chemicals (Sigma) were VMA, 3-hydroxyphenylacetic acid (3-HPAA), 4-hydroxyphenylacetic acid (4-HPAA), acetylsalicylic acid (ASA), salicyluric acid (SUA), 1,3,7-trimethylxantine (caffeine) and bovine serum albumin.

Procedure

Calibration process. Place sets of three equilibrated G-10 columns on the fraction collector over sets of 20 small test tubes, marked for 0.5 ml. Run 200 μl of a 25 μg/ml standard solution as described [6]. Inject 20 μl from each 0.5-ml fraction on to the reversed-phase column (detector sensitivity: 0.02 a.u.f.s.) and establish the elution range as well as the elution volume.

Sample preparation. Obtain morning urine (accumulated from midnight) voided after fasting overnight. Acidify with glacial acetic acid (0.03 ml per ml of urine), centrifuge and analyze.

Determination of HVA in urine. Carry out the determination as described in Calibration process with the following modifications. Place a set of one to ten calibrated columns over one to ten sets of two test tubes, graduated for 10 ml. Run 200 μl of the sample. Collect 4 ml and discard. Collect 3 ml in the second test tube and keep for separation and quantitative determination on the HPLC—UV system, checked daily with an HVA stock solution (average peak height for a 100 μg/ml standard at 0.64 a.u.f.s. is 46 ± 2 mm). Obtain the concentration by means of a calibration curve (peak height in mm versus concentration in μg/ml) constructed using working standards, run in the same way as the samples.

Column maintenance. After using, regenerate the G-10 columns by elution with about 30 ml of 0.1 *M* ammonium formate buffer (pH 3) and store tightly capped at 5°C (see also ref. 6, p. 462). Flush the ODS-2 column daily

with about 30 ml of methanol and store in methanol at 5°C. It may be added that the following test for albumin was performed. Proteins, if present in large amounts in urine, may be carried over into the second test tube and cause contamination of the ODS-2 column, especially with the mobile-phase composition used here. A 5 mg/ml solution in the buffer was chromatographed on G-10 as described in Calibration process and the UV absorbance (A_{280}) of the individual fraction was monitored on the spectrophotometer. The compound was eluted in the first half of the 4-ml fraction to be discarded, indicating the probability of protein-free injections all through.

RESULTS AND DISCUSSION

HVA was determined in morning-urine specimens obtained from 23 healthy individuals, ranging from 1 to 63 years of age. The HVA content ranged, without any age dependency, from 2 to 17 μg/ml, corresponding to a total output of 0.2—5 mg. An amount of 37.5 μg/ml was found to be excreted in urine from a neuroblastoma patient (Fig. 1B). No reports concerning normal HVA levels in morning urine could be found in the literature. As normal values for 24-h urine, the following data were available (mg per 24 h): 5.4 ± 1.4 [7], 9.4 ± 2.5 [8] and 4.9 ± 1.3 [9]. Otherwise, the amount of HVA in urine was generally reported [1, 4, 10] as related to the creatinine content (μg/mg or mg/g) only. Expressed in this way, an age dependency was observed [1, 10], which could be misleading. As was earlier suggested [11], the excretion

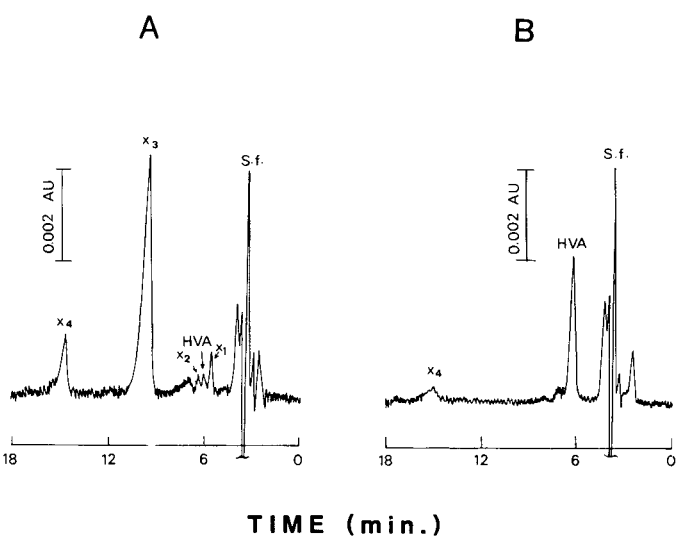

Fig. 1. Typical chromatograms of HVA in urine obtained by the present HPLC method. (A) A morning urine specimen from a healthy subject (18 months old) containing 4.1 μg/ml; and (B) from a neuroblastoma patient (1 year old) containing 37.5 μg/ml. Referring to Tables I and II and Fig. 2, the peaks x_1—x_3 were identified as follows: x_1 (t_R = 5.6 min) = 4-HPAA; x_2 (t_R = 6.6 min) = 3-HPAA; and x_3 (t_R = 9.1 min) = SUA. (t_R of x_4 was 14.1 min.)

TABLE I

RETENTION ON SEPHADEX G-10 OF SOME AROMATIC REFERENCE SUBSTANCES

Volumes of 200 μl of a 25 μg/ml* solution of each respective compound in 0.1 *M* ammonium formate buffer (pH 3)** were chromatographed on a 12 cm × 4 mm I.D. column and fractions of 0.5 ml monitored by HPLC—UV (see Calibration process). V_e = elution volume, and V_t = total volume of the mobile phase.

Compound	Elution range as Nos. of UV-positive fractions	Total volume of UV-positive fractions (ml)	V_e/V_t
HVA	9—14	3.0	3.40—3.66
VMA	6— 7	1.0	1.73—2.00
3-HPAA	12—18	3.5	3.73—4.00
4-HPAA	11—17	3.5	3.73—4.00
ASA	5—10	3.0	2.06—2.33
SUA	11—15	2.5	4.40—4.66
Caffeine***	2— 4	1.5	0.73—1.00

*ASA: 100 μg/ml.
**ASA, respective SUA, in distilled water.
***When 200 μl of a 10 mg/ml solution was chromatographed, the caffeine was eluted in fraction Nos. 1—5, corresponding to 2.5 ml of UV-positive eluate.

of most metabolites relative to creatinine appears to be very high in the first few years of life, owing to the fact that the creatinine output in that period is much lower than in later life.

In order to investigate any possible interference with HVA by some structurally related urine compounds and other aromatic compounds frequently found in urine, the corresponding reference solutions were run on G-10 and on ODS-2, respectively (see Table I and Fig. 2). The elution range on G-10 of some of the tested compounds was found to overlap the elution range of HVA. However, none of these proved to be any interference risk with regard to the analysis procedure as a whole, since those compounds from the G-10

TABLE II

RETENTION ON SEPHADEX G-10 OF THE COMPOUNDS x_1—x_4 IN FIG. 1

A 200-μl aliquot of morning urine* from a healthy subject was chromatographed on a 12 cm × 4 mm I.D. column and fractions of 0.5 ml were collected (see Calibration process). Fractions between Nos. 5 and 20 were monitored by HPLC—UV (operation as in Apparatus).

Compound	V_e/V_t **
x_1	3.73—4.00
x_2	3.73—4.00
x_3	4.40—4.66
x_4	4.40—4.66

*Drug-free (x_1, x_2 and x_4) and after intake of aspirin (x_1—x_4).
** V_e/V_t of the endogenous HVA was 3.40—3.66.

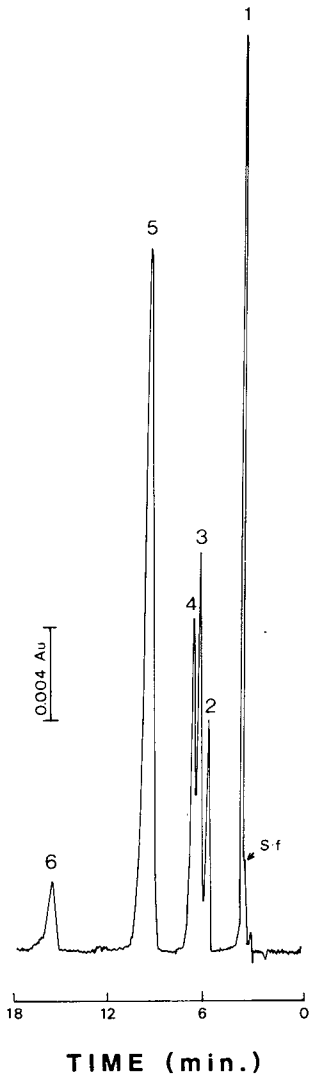

Fig. 2. Chromatogram of a pool of equal volumes of 100 μg/ml reference-substance solutions. Respective peaks were identified by injection of each stock solution separately (operating as under Apparatus): 1 (t_R = 3.5 min) = VMA; 2 (t_R = 5.6 min) = 4-HPAA; 3 (t_R = 6.1 min) = HVA; 4 (t_R = 6.6 min) = 3-HPAA; 5 (t_R = 9.2 min) = SUA and caffeine; and 6 (t_R = 15.1 min) = ASA.

column would be adequately resolved from the HVA by separation on the ODS-2 column with the mobile phase used. Moreover, the retention data obtained in this way (see also Table II) could be utilized for identification purposes also.

The HVA calibration curve is linear from 3.125 to 37.5 μg/ml, passing through the origin by extrapolation. Higher concentrations will require dilution of the urine samples. For quantities less than about 3 μg/ml the second

fractions are concentrated [6]. The lowest detectable amount of HVA in urine is 0.5 μg/ml, corresponding to 10 ng injected.

The same set of G-10 columns was used repetitively for hundreds of urine analyses required for the present work, without any reduction in capacity. By checking with a 25 μg/ml standard, 99.1% (0.6% R.S.D., n = 5) of HVA was recovered. The ODS-2 column retained its performance after being utilized extensively for this and other investigations during a two-year period.

The specificity, the simplicity and the quantitative recovery make the new method useful for diagnostic purposes. Furthermore, the data presented show that the general approach [5, 6] may also be utilized as a basis for simple methods for the determination of some other aromatic urine components.

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Note

Determination of pyridine dinucleotides in cell extracts by high-performance liquid chromatography

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During recent studies of the effects of various respiratory inhibitors and hypoxia on cellular adenylates and pyridine dinucleotides [1], we have found that the high-performance liquid chromatographic method of Schweinsberg and Loo [2] for adenylate determination can be modified in a very simple fashion to obtain simultaneous determination of NAD^+ and $NADP^+$.

Currently, most widely used methods for determination of pyridine dinucleotide concentrations in cell and tissue extracts rely upon changes of the absorbance or fluorescence of the reduced forms following incubation with selected enzymes [3, 4]. High-performance liquid chromatography (HPLC) has also been used in studies of these compounds [5, 6] but has not been optimized for tissue determinations. The following method provides such an application. NADH and NADPH are determined similarly but require alkaline extraction because they are labile in acid.

EXPERIMENTAL

Chemicals used for buffer solutions were at least of reagent grade. Buffer A was 100 m*M* potassium phosphate, pH 6.0, and Buffer B was 100 m*M* potassium phosphate, pH 6.0, containing 5% methanol (v/v). Buffers were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to use. Distilled water was used for buffer preparation. β-NADH, β-NAD^+, NADPH, $NADP^+$, ATP, ADP, AMP and collagenase (Type IV) were purchased from Sigma (St. Louis, MO, U.S.A.). Calibration of standards was done using the millimolar extinction values as follows: NADH, 6.2 (340 nm); NAD^+, 18.0 (260 nm); NADPH, 6.2 (340 nm); $NADP^+$, 18.0 (340 nm); ATP, 15.4 (259 nm); ADP, 15.4 (260 nm); AMP, 15.4 (260 nm). Isolated liver cells were pre-

pared by the recirculating perfusion method of Moldeus et al. [7], and extractions were performed as described by Tischler et al. [8]. For NADH and NADPH determination, a 1-ml cell suspension (10^6 cells/ml) was treated with 0.1 ml of 0.5 *M* potassium hydroxide containing 50% (v/v) ethanol and 35% (w/v) cesium chloride, immediately cooled on ice, and centrifuged to remove insoluble material. For NAD^+ and $NADP^+$ determinations, cell suspensions (10^6 cells/ml) were treated with 3 *M* perchloric acid (0.5 ml per 1.0-ml incubation), immediately cooled on ice, and centrifuged to remove insoluble material. The oxidized forms were stable at −20°C for several days, but the reduced forms showed about 30% loss after 24 h. Pyridine nucleotides were separated on a μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) with either a two-pump Waters system with Model 720 system controller and Data Module or a Beckman Model 334 gradient chromatograph. The Waters chromatograph was equipped with a variable-wavelength detector employing an 8-μl flow cell, and the Beckman chromatograph was equipped with a Model 155 variable-wavelength detector employing a 20-μl flow cell. General operating conditions were as follows: flow-rate, 1 ml/min; recycling time between runs, 10 min; temperature, 18—22°C.

RESULTS AND DISCUSSION

Standard mixtures of NADH, NAD^+, NADPH and $NADP^+$ could be readily separated using 3.75% methanol in 100 m*M* phosphate, pH 6.0, and measuring absorbance at 260 nm. Retention times were: $NADP^+$, 4.6 min; NADPH, 6.6 min; NAD^+, 8.0 min; and NADH, 12.4 min. Decreased retention times occur as the C_{18} columns are continually exposed to phosphate buffers. Consequently, new columns show somewhat longer retention times and columns aged for several months show shorter retention times under the conditions illustrated here. Compensation for these changes can be made by increasing or decreasing the methanol content, respectively. Calibration of standards by peak integration was linear over the range of 10—2000 pmol for absorbance at 260 nm (injection volumes 10 μl or 80 μl). Similar calibration of NADH and NADPH at 340 nm was linear over the range of 30—2000 pmol. Calibration by peak height was acceptable above 100 pmol; below this there was an apparent peak broadening that required either integration or a complete standard curve for accurate quantitation.

Simultaneous separation of the adenylates from NAD^+ and $NADP^+$ in the perchloric acid extract of liver cell suspension is shown in Fig. 1. Peaks identified as ATP, ADP and AMP in cell extracts have retention times the same as the corresponding standards and can be selectively manipulated by incubation of cell under anaerobic conditions or with antimycin A, ethionine or carbonyl-cyanide-P-trifluoromethoxyphenylhydrazone (FCCP). The absolute concentrations as well as the ratios of ATP/ADP and ATP/AMP are the same as those obtained by other approaches [9]. Peaks identified as NAD^+ and $NADP^+$ in cell extracts have retention times corresponding to standards and can be eliminated by alkaline treatment or reduction with dithionite. Recoveries of NAD^+ and $NADP^+$ added to cell extracts were 96 ± 3% (n = 5) and 101 ± 4% (n = 5), respectively, for 0.14 nmol per 80-μl additions. Measured values

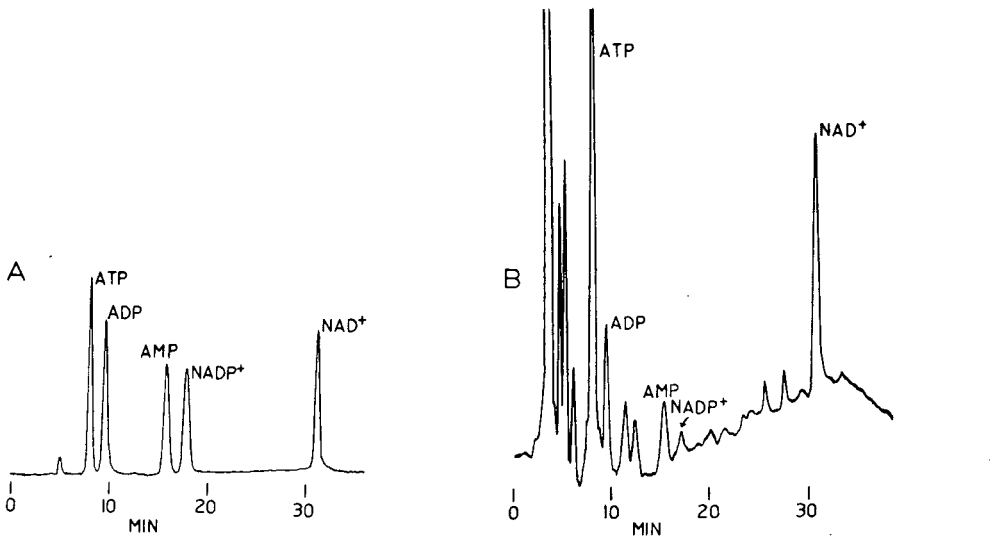

Fig. 1. Separation of $NADP^+$, NAD^+, ATP, ADP and AMP in extracts of isolated liver cells. (A) Standard mixture (2 nmol each: NAD^+, $NADP^+$; 3 nmol each: ATP, ADP, AMP) was injected on a column run on the Beckman system with the program as follows: 8.5 min at 100% Buffer A, 0.5 min gradient from 100% Buffer A to 80% Buffer A, hold 6 min at 80% Buffer A, 5 min gradient to 0% Buffer A, hold 20 min at 0% Buffer A. Flow-rate was maintained at 1 ml/min; recycle time was 0.5 min to 100% Buffer A and 10 min before next injection. Injection volume was 7 μl. Detector was at 0.1 sensitivity. (B) 20 μl of the perchloric acid extract of 10^6 liver cells per ml (approx. 1.6 mg cell protein per 10^6 cells; 0.5 ml 3 *N* perchloric acid added per ml of cells) was injected and chromatographed as above. Perchloric acid was removed by neutralization of sample with potassium hydroxide and centrifugation immediately before injection. Detector was at 0.01 sensitivity.

TABLE I

COMPARISON OF DETERMINATIONS BY HPLC METHOD TO REPORTED VALUES FOR FLUOROMETRIC METHOD

	HPLC* (nmol/10^6 cells)	*n*	Fluorometric** (nmol/10^6 cells)
$NADP^+$	1.47 ± 0.27	8	1.03
NAD^+	11.8 ± 2.2	8	8.7
NADPH	4.12 ± 0.45	7	3.24
NADH	1.49 ± 0.24	7	1.05

*Data obtained by methods as shown in Figs. 1 and 2 for isolated rat liver cells.
**Data from Williamson and Corkey [3] converted from perfused liver data (μmol/g dry weight) using 3.7 g wet weight per g dry weight and 10^6 cells per 10 mg wet weight. Sums of values, NAD^+ plus NADH, and $NADP^+$ plus NADPH, measured by the fluorometric method [8] for isolated liver cells were higher than those shown above for perfused liver, presumably due to the elimination of the vascular space in the cell preparation.

for NAD^+ and $NADP^+$ contents in isolated liver cells by this method (Table I) correspond well with previously reported values.

Separation of NADH and NADPH in alkaline extract of cell incubations is most readily visualized at 340 nm (Fig. 2) due to lack of interference by

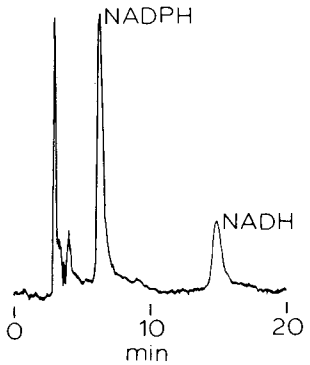

Fig. 2. Separation of NADPH and NADH in extracts of isolated liver cells. An 80-μl aliquot of ethanolic potassium hydroxide extract of cells (10^6 cells/ml) was injected on a column run with the following program: 5 min at 50% Buffer A, 10 min gradient to 25% Buffer A, hold 4 min at 25% Buffer A. Recycle time was 1 min to 50% Buffer A and 10 min before next injection. Detector sensitivity was 0.01.

other chromophores. Detection is more sensitive at 260 nm due to the greater extinction coefficient; however, interference by other chromophores is pronounced so that measurement at 340 nm is preferred. Retention times for these peaks correspond to those for standards, and the peaks are destroyed by acid treatment. Values obtained with this method are comparable to reported values for the fluorometric method (Table I).

The current approach to pyridine nucleotide quantitation in cell extracts offers a simple alternative to the enzyme-coupled fluorometric assays. Total run time for determination of NAD^+, NADH, $NADP^+$, NADPH, ATP, ADP and AMP is slightly more than 1 h, and thus, for large numbers of samples probably offers no time advantage. However, for smaller numbers of samples or under conditions in which compounds may be present that interfere with the fluorometric assays, this HPLC method offers a reliable and sensitive approach.

ACKNOWLEDGEMENTS

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Note

Nano-scale densitometric quantitation of phospholipids

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(Received March 26th, 1981)

In our investigations of the Leydig cells from essential fatty acid deficient rats we needed to determine the phospholipid composition of purified Leydig cells. For this purpose we had to solve the problems posed by the small sample size, and by the desire to separate six phospholipid classes in a one-dimensional system.

Leydig cells constitute about 2.7% of the total testicular weight [1], and the lipids constitute about 1.8% of the testicular weight [2]. Thus, very limited amounts of lipid were available for analysis. A procedure which allowed determination of as little as 40 ng of phospholipid was developed, using a thin-layer densitometric method in which sample application was carried out on high-performance thin-layer chromatography (HPTLC) plates by means of a micrometer-adjustable precision syringe. One-dimensional separation of five diacylglycerophospholipids, sphingomyelin, and cerebrosides was achieved through a modification of previously reported solvent systems [3].

EXPERIMENTAL

Extraction

Leydig cells from rat testes were isolated according to the description of Janszen et al. [4], except that the raw cell suspension was purified by centrifugation in a Ficoll—metrizoate gradient [5]. The cells were extracted twice, first in 20 volumes of chloroform—methanol (2:1, v/v), and then in 10 volumes of chloroform—methanol (1 : 2, v/v). For the extractions an IKA Ultra Turrax T18/10 disperser (Ika-Werk, Staufen, G.F.R.) with the 10N shaft was used. The two extracts were centrifuged for 5 min at 1250 *g*. During extraction and centrifugation the samples were protected by low temperature (0—2°C) and a nitrogen atmosphere. The supernatants were filtered through a glass

filter, and the supernatants from the two extractions were then combined. The solvents were evaporated under vacuum, which was broken by introducing nitrogen. The residue was dissolved in 2 ml of chloroform.

Neutral and polar lipids were separated on a column packed with 1.0 g of silicic acid (Unisil, Clarkson Chemical Co., Williamsport, PA, U.S.A.) in chloroform. The neutral lipids were eluted with 25 ml of chloroform and the polar lipids with 15 ml of methanol [6]. The column was regenerated with 15 ml of chloroform. The eluates were concentrated on a rotatory evaporator. The neutral lipids were redissolved in chloroform, and the polar lipids in chloroform—methanol (1:1). All solvents used for extraction and chromatography procedures were of analytical grade.

Thin-layer densitometry

HPTLC plates (10 × 10 cm; silica gel 60; Merck, Darmstadt, G.F.R.) were washed by developing the plates with methanol for 1 h. The washed plates were dried for 1 h at 110°C. Phospholipid standards (Serdary Research Lab., London, Canada) were from natural sources. A composite standard phospholipid mixture was prepared in accordance with the composition of the samples.

Samples and standards were applied by means of a micro-applicator with micrometer-adjustment (Micro-Applicator; Camag, Muttenz, Switzerland). Normally, from 0.3 to 2.2 μl were applied. The distance between applied spots was 6 mm, i.e. the minimum distance allowed by the size of the densitometer light spot. Each plate was used for the analysis of one extract which was applied in two amounts, each in duplicate. The standard solution was applied to the same plate in five amounts, each in duplicate.

The plates were developed in chloroform—methanol—2-propanol—0.25% aqueous potassium chloride—ethyl acetate (30:9:25:6:18, v/v). The development was carried out in a horizontal chamber (Camag HPTLC Linear Developing Chamber) saturated with solvent vapours. For optimum separation the plate was developed twice, being dried in a stream of nitrogen between the two developments. After 15 min of drying at room temperature the plate was sprayed with 3% (w/v) copper(II) acetate in 8% (w/v) aqueous phosphoric acid [7]. The plate was sprayed to transparency, and charred at 180°C for 10 min. Densitometry was carried out on a Vitatron TLD 100 densitometer (Vitatron, Dieren, The Netherlands), the light source of which had been partially masked to narrow the light spot projected on to the plate. Because of the small size of the sample spots, relative to the width of the light spot, the densitometer was run in the slit scanning mode (i.e. flying-spot oscillations were switched off). The scanning speed was 5 mm/min. Integration was performed by an electronic integrator (Model 3390A; Hewlett-Packard, Avondale, PA, U.S.A.) with pre-programmed background correction, adjusted for the plate being analyzed.

Calculations of the nominal mass of the phospholipids in the samples were done manually, using standard curves based on integration values from the standards measured on the same plate. The standard curves were used in their full range (0.035—3.4 μg), whether the relationship between integration value and mass was linear or not.

RESULTS AND DISCUSSION

The mobile phase presented in this note is a modification of phases described by Touchstone et al. [3]. It was constructed in order to achieve a non-tailing, simultaneous separation of sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylethanolamine (PE). This modified phase has been used for the analysis of Leydig cell lipids, as presented here (Figs. 1 and 2). The mobile phase has also been used for separations of polar lipids from intestine and brain [8], as well as from liver and kidney [9].

The combination of our modified solvent with HPTLC plates and microapplication equipment has been used for measurements of phospholipid amounts ranging from 40 ng to 3.4 μg. The overall reproducibility of the method was estimated from quadruplicate measurements of the standards, each measured in two amounts. The relative reproducibilities varied between classes. The coefficients of variation were as follows (the ranges of the normally used standard curves are given in parentheses): SPH, 2% (80—1000 ng); PC, 4% (260—3400 ng); PS, 9% (110—1500 ng); PI, 12% (35—470 ng); and PE, 8% (120—1600 ng).

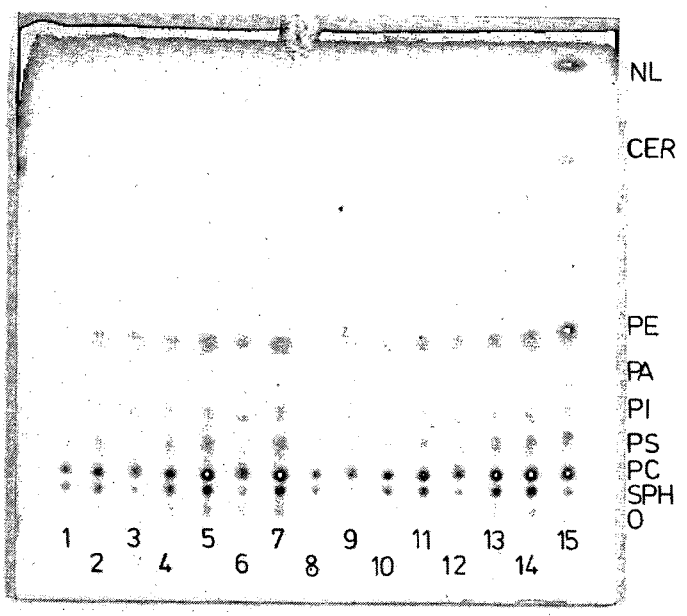

Fig. 1. Leydig cell phospholipids were separated on silica gel 60 HPTLC plates in two developments in chloroform—methanol—2-propanol—0.25% aqueous potassium chloride—ethyl acetate (30:9:25:6:18), sprayed with 3% copper(II) acetate in 8% phosphoric acid, and charred at 180°C for 10 min. O = origin; SPH = sphingomyelin; PC = phosphatidylcholine; PS = phosphatidylserine; PI = phosphatidylinositol; PE = phosphatidylethanolamine. The chromatogram shows standard phospholipids (lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, and 14), Leydig cell phospholipids (lanes 3, 6, 9, and 12), and brain cortex lipids (lane 15). The latter lane shows the positions of phosphatidic acid (PA), cerebrosides (CER), and neutral lipids (NL).

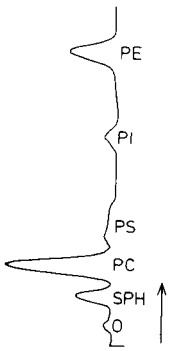

Fig. 2. Integrator tracing of a densitometric scan of Leydig cell phospholipids which were separated and charred as described (cf. Fig. 1 and Methods). The densitometer scanning speed was 5 mm/min; the integrator chart speed was 10 mm/min. The arrow indicates the direction of scanning. The names of the phospholipids are abbreviated as in Fig. 1. The peaks were calculated to correspond to the following amounts: SPH, 54 ng; PC, 362 ng; PS, 57 ng; PI, 97 ng; PE, 228 ng.

Utilization of HPTLC plates in a commercially available, horizontal developing chamber has the advantages of minimal solvent use (4—5 ml/run), and reproducible solvent composition as fresh solvent is used for each development. Further advantages inherent in the described method are a fast charring procedure (ca. 30 min), and a direct quantitation without further manipulation of plate or spots. The micro-application equipment and the horizontal developing chamber are not prerequisites for the separation achieved by the use of the described mobile phase, but they do add certain benefits, as indicated.

The densitometric principle of quantitation as applied in this method is dependent on the availability of suitable phospholipid standards. It permits a relative quantitation of sample lipids, relating the absorption of the charred spots to that of the standards. Since the degree of charring is related to fatty acid unsaturation [10], the method does not give absolute values. To approximate the charring of the standards to that of the samples, the standards should preferably be derived from natural sources, rather than be synthetic saturated standards. However, Chapelle [11] has demonstrated that for erythrocyte phospholipids, the percentage distribution based on densitometry is virtually unaffected by hydrogenation of the samples. This suggests that at least minor variations in degree of unsaturation between samples and standards can be tolerated.

The wide span of amounts analyzed by the present method (0.04—3.4 μg) necessitates the use of non-linear standard curves. However, Downing and Stranieri [12] find the use of non-linear standard curves permissible, provided extensive use is made of reference compounds for each lipid.

We find the problems posed by the varying degrees of unsaturation of the samples, and by the non-linear standard curves to be minor compared to the advantages of the present method, namely its sensitivity and its efficiency of separation.

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Note

Determination of pyruvate, lactate, acetoacetate, and 3-hydroxybutyrate in serum by capillary isotachophoresis

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In numerous pathological states the concentration of organic acids in the blood increases enormously and the resulting acidosis can lead to acidotic coma and even to death. Prompt determination of organic acids in the blood is therefore of diagnostic and prognostic importance. Pyruvate and lactate concentrations provide information on the state of tissue oxidation [1, 2], acetoacetate and 3-hydroxybutyrate are significant for the monitoring of ketoacidosis, usually as a consequence of diabetes mellitus. The last three anions are the most important for disorders of the acid—base balance [3].

At present, the so-called anion gap ($[Na^+] + [K^+] - [Cl^-] - [HCO_3^-]$) is usually used to evaluate metabolic acidosis; however, it provides only rough information on the content of organic acids.

The photometric determination of these acids in blood is not very sensitive [4] and thus enzymatic [4—8] methods are usually recommended [4] despite the fact that they require pure enzyme preparations, and some other organic acids present in blood (for example, 2-hydroxybutyrate) affect the determination. Chromatographic methods [9] are laborious and time-consuming [4].

In the present paper we show that isotachophoresis may serve as a method suitable for the quantitation of the organic acids mentioned by fast, direct analysis of blood serum.

EXPERIMENTAL

All the chemicals used were of analytical grade, provided by Lachema, Brno, Czechoslovakia, with the exception of sodium 3-hydroxybutyrate (BDH Chemicals, Poole, Great Britain), sodium pyruvate, lithium lactate (both from E. Merck, Darmstadt, G.F.R.), 2-hydroxybutyric acid (Fluka, Buchs, Switzer-

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land), β-alanine (Loba Chemie, Vienna, Austria), and acetoacetic acid (prepared by hydrolysis of the ethyl ester of acetoacetic acid [10]).

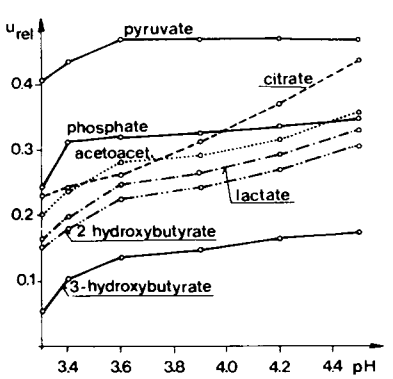
The blood samples were clotted and centrifuged in the cold for 10 min at 1000 *g*. The supernatants were immediately frozen and stored at −20°C until analysis.

Analyses were performed at room temperature in a capillary isotachophoretic column (0.2 × 1 × 200 mm). Zones were detected by a potential gradient detector with two platinum contacts placed in the capillary ca. 0.05 mm apart. A power supply, with a stabilized d.c. current up to 400 μA and with a maximum voltage of 16 kV, was connected to the column. A detailed description of the instrumentation can be found elsewhere [11, 12]. A Perkin-Elmer Model 196 line recorder was used. Leading electrolytes were prepared by adding β-alanine to 10 mmole/l HCl containing 0.3% of polyethylene glycol until the pH of the solution reached the required value.

Relative mobilities were determined as the ratio of the step height of the leading ion (Cl^-) to the step height of the compound under investigation.

RESULTS AND DISCUSSION

When selecting working conditions for the separation of the acids mentioned above, it is necessary to take into consideration some other blood anions that could interfere with the analysis. Preliminary experiments indicated phosphate, citrate, and 2-hydroxybutyrate. The dependence of the relative mobilities of the respective substances on the pH of the leading electrolyte is presented in Fig. 1. Analyses of model mixtures showed poor separation of lactate and acetoacetate at pH higher than 4. At pH 3.5—4, the pair of lactate and 2-



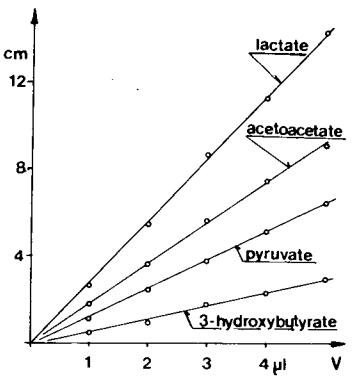


Fig. 1. Dependence of the relative effective mobilities of the investigated acids on the pH of the leading electrolyte. Leading electrolyte: 10 mmole/l HCl, 0.3% polyethylene glycol + β-alanine. Terminating electrolyte: 10 mmole/l propionic acid.

Fig. 2. Dependence of step length (cm) in the trace on the amount analysed. Sample injected: 1—5 μl of the standard mixture containing 5.6, 10, 15 and 19.3 mmole/l 3-hydroxybutyrate, pyruvate, acetoacetate and lactate, respectively. Leading electrolyte: 10 mmole/l HCl, 0.3% polyethylene glycol + β-alanine (pH 3.3). Terminating electrolyte: 10 mmole/l propionic acid. Driving current: 200 μA. Chart speed: 8 cm · min^{-1}.

hydroxybutyrate and that of acetoacetate and citrate were poorly separated from one another. At pH 3.3 all anions shown in Fig. 1 were well separated and the time required for the analysis was acceptable. In the pH range 3.0—3.3 it was possible to separate even some other anions (for example, malate and 2-ketoglutarate); however, the analysis time was longer than 25 min. More detailed experiments showed that these last anions do not interfere with the analysis, and thus pH 3.3 was selected as the optimum.

For quantitation the dependences of the step lengths on the trace upon the amounts injected were measured and are presented in Fig. 2. The dependences were linear, with linear correlation coefficients of 0.9994, 0.9993, 0.9998 and 0.9989 and standard deviations of the regression line of $6.50 \cdot 10^{-10}$, $10.41 \cdot 10^{-10}$, $8.23 \cdot 10^{-10}$, and $4.87 \cdot 10^{-10}$ mole for pyruvate, acetoacetate, lactate and 3-hydroxybutyrate, respectively. The relative standard deviations for the mean of the calibration range were 2.57, 2.77, 1.70, and 3.63%, respectively.

TABLE I

CONCENTRATIONS OF ANALYSED ORGANIC ACIDS IN SELECTED SERA

Results are expressed in mmole/l.

Serum No.	Pyruvate	Acetoacetate	Lactate	3-Hydroxybutyrate
1	0.08	0.09	1.67	<0.03
2	0.11	0.99	3.24	1.18
3	0.06	0.33	2.57	0.73
4	0.05	0.11	3.16	<0.03
5	0.08	0.08	3.76	0.12
6	0.04	0.32	3.53	0.54
7	0.05	0.59	2.75	7.19

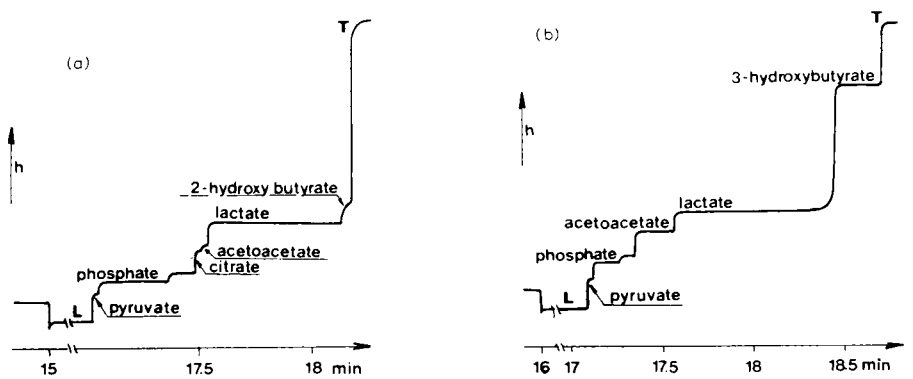


Fig. 3. Analysis of human serum. Sample injected: 15 μl of human serum. (a) Serum of a healthy man (sample No. 1 from Table I). (b) Serum of a patient with diabetes mellitus (sample No. 2 from Table I). For conditions of separation see Fig. 2. The separation proceeded for the first 15 min with a driving current of 300 μA, then it was switched to 200 μA.

For a rough indication of the content of organic acids in normal serum, we analysed sera from three healthy individuals and found values in the range 0.06—0.09 mmole/l, 0.08—0.11 mmole/l, 1.19—1.67 mmole/l, and less than 0.03 mmole/l for pyruvate, acetoacetate, lactate, and 3-hydroxybutyrate, respectively. The concentrations of these acids in selected sera are given in Table I. Serum No. 1 is from a healthy man, the other sera are from acidotic patients. All acidotic sera had elevated lactate levels, and some of them elevated acetoacetate and/or 3-hydroxybutyrate levels. We can thus distinguish between lactic acidosis (serum No. 4) and ketoacidosis (serum No. 7) and their mixed forms. Analysis of sera Nos. 1 and 2 is shown in Figs. 3a and b, respectively.

The isotachophoretic analysis of blood acids is very fast; it needs neither deproteinization, nor derivatization of the sample, and the separation time does not exceed 20 min. Quantitation is easy by simple measurement of the step lengths. Moreover, other pathological blood acids can be analysed simultaneously in one separation run. All these advantages predetermine isotachophoresis to be a useful diagnostic and prognostic tool in intensive medicine.

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Note

Rapid gas chromatographic determination of valproic acid in serum

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(First received February 10th, 1981; revised manuscript received April 17th, 1981)

The anticonvulsant effect of valproic acid (VPA) has been known for some time. Determination of VPA in serum is of most importance in epilepsy therapy for efficient control of seizure.

VPA is a simple fatty acid chemically different from other commonly used anticonvulsants. Most of the methods for its determination in serum are gas chromatographic (GC), and have been based on procedures for analysis of short-chain fatty acids. Some problems need to be solved in its determination. In order to prevent severe tailing of the free acid peak, the column packing must be conveniently deactivated and, after extraction of acidified serum, VPA is assayed by GC in the underivatized form [1—5]. On-column and pre-column derivatization have also been used to improve the characteristics of the VPA separation [6—11]. The volatility of VPA is another problem to be tackled. In some methods the organic extract must be concentrated before chromatography is carried out, but the addition of isoamyl acetate can overcome this disadvantage [12].

In this report a new packing, 2% SP-2110/1% SP-2510 DA from Supelco, is used for the analysis of VPA; it is deactivated for acidic compounds allowing VPA analysis without derivatization. After extraction of acidified serum, the VPA is injected in free form avoiding any evaporation of the solvent.

MATERIALS AND METHOD

Reagents

Methylene chloride and sulphuric acid were pro analysis grade from E. Merck (Darmstadt, G.F.R.).

The internal standard solution was cyclohexane carboxylic acid, 100 μg/ml in methylene chloride (I.C.N. Pharmaceuticals, Cleveland, OH, U.S.A.). For the

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VPA solution, lyophilized sodium valproate is dissolved in water in a 1 mg/ml concentration (Labaz, Maassluis, The Netherlands). For the working VPA control, a 100 μg/ml solution is prepared in pooled drug-free serum, divided into aliquots and stored at −20°C.

Gas chromatographic conditions

A Perkin-Elmer 3920B gas chromatograph was used with a flame ionization detector, fitted with a 1 m × 2 mm I.D. glass column. The column packing is 2% SP-2110/1% SP-2510 DA on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The injector and detector temperatures were both 250°C, the oven temperature was 100°C, and the nitrogen flow-rate was 50 ml/min, the hydrogen flow-rate 34 ml/min and the air flow-rate 300 ml/min. A Minigrator M2 (Perkin-Elmer) was used to measure the area of the peaks.

Procedure

To 0.5 ml of serum sample poured into a PTFE-lined screw-capped extraction tube (Kimax 13 × 100 mm, 45066) is added 0.04 ml of 1 *N* sulphuric acid. After mixing, the sample is extracted with 0.5 ml of internal standard solution by placing it for 5 min at 25 rpm in a Vortex mixer followed by 5 min centrifugation at 1400 *g*. The aqueous layer is aspirated and discarded, and the organic layer is transferred to an 8-ml tube (Kimax 13 × 100 mm, 45066). A 2-μl aliquot of this methylene chloride extract is injected into the gas chromatograph. Calibration is made with the VPA aqueous solution. To 0.5 ml of drug-free serum are added 50 μl of aqueous solution and treated as described above. The working VPA control was used to estimate the between-days precision; 0.5 ml is processed following the described procedure.

VPA concentration is calculated by dividing the VPA area by the internal standard area, taking into account the standard quantity given above, and multiplied by the response factor obtained from the calibration.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of an extract of human drug-free serum and of a patient receiving sodium valproate. The retention times of VPA and cyclohexane carboxylic acid are 1.5 and 2 min, respectively. The chromatogram of drug-free serum does not show interfering peaks from serum constituents.

The range of linearity of the results obtained with this method of analysis was determined by analysing in duplicate serum spiked with 10, 20, 40, 60, 80, 100 and 160 μg/ml VPA and a constant quantity of internal standard (50 μg) by plotting the quotient of the peaks areas against VPA concentration, a straight line is obtained whose equation of linear regression is $y = 0.019\ x - 0.03$, with a linear correlation coefficient of 1.0 and a standard error of 0.015.

The within-run precision (C.V.) of the procedure for two concentrations of VPA ($n = 12$) was 3% for 60.4 μg per 0.5 ml (S.D. = 1.8) and 3.9% for 25.9 μg per 0.5 ml (S.D. = 1.0). The between-days precision ($n = 18$) was 5.6% (mean concentration 55.1 μg per 0.5 ml, S.D. = 3.1).

The range studied is sufficient to cover all clinical situations. Analysis of

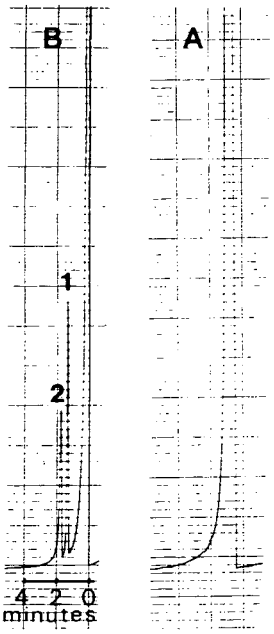

Fig. 1. Gas chromatogram of (A) blank serum and (B) serum from a patient receiving sodium valproate. Serum level = 104 μg/ml. Peaks: 1 = valproic acid; 2 = internal standard.

serum samples from the Antiepileptic Drug Level Control Program was performed. Serum samples from patients who were receiving ethosuximide, phenobarbital, carbamazepine, primidone and phenytoin were analyzed and no interference was found with either the VPA or the internal standard; they have longer retention times.

The detector sensitivity limit was studied in six replicates from a serum pool to which VPA was added. The chromatographic peak corresponding to 5 μg of VPA was perfectly measurable by the integrator, being the mean of the results of the division of the VPA peak area by the internal standard peak area equal to 0.085 (n = 6, S.D. 0.0077, C.V. 9.06%). This limit is correct to the clinic because it is clearly under therapeutic values, and on the other hand it also allows a reduction in the volume of pediatric serum samples.

Recently several techniques have been published for the determination of VPA in serum without derivatization. Different packing materials have been used [1—5], but with these packings nothing has been published on the analysis of the other anticonvulsant drugs in underivatized form. Routine work to determine all common anticonvulsant drugs is more rapid and easy if a change of column is not required in the analysis since many patients are treated with a diversity of anticonvulsant drugs.

The described method is simple and does not require derivatization or evaporation of solvent, and it could be a valuable tool in those laboratories having just one chromatograph. At the same time, the use of the same solvent for the extraction and chromatography avoids the problem of loss by evaporation.

This technique for VPA can be used in the presence of other anticonvulsant

drugs such as ethosuximide, phenobarbital, carbamazepine, primidone and phenytoin, and, conversely, the determination of these drugs can be realized in isothermic conditions with no derivatization and with a simple extraction method [13] using the same column as the one used for VPA.

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CHROMBIO. 957

Note

Determination of oxeladin in human sera by gas—liquid chromatography with thermionic detection

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(First received February 26th, 1981; revised manuscript received April 27th, 1981)

Oxeladin citrate [2-(2-diethylaminoethoxy)ethyl-2′-ethyl-2′-phenyl butyrate] (Pectamol®, Tussimol®) is a cough suppressant which depresses the cough reflex. It does not act on the higher centres and has no hypnotic effect in therapeutic doses. It has local anaesthetic properties, so a combined local and central depressant effect might be possible [1, 2]. So far, no method has been described for the determination of oxeladin in biological materials. Therefore blood levels have not as yet been correlated to cough suppressant activity.

This paper describes a gas—liquid chromatographic (GLC) method developed for the determination of oxeladin in serum samples in bioavailability tests of oxeladin from tablet and linctus formulations. Pharmacokinetic results are presented elsewhere [3].

EXPERIMENTAL

Standards and reagents

Oxeladin citrate was a gift from Apothekernes Laboratorium for Specialpræparater A/S (Oslo, Norway). Oxeladin base (OX) was supplied by E. Merck (Darmstadt, G.F.R.).

An aqueous stock solution of OX hydrochloride (1.0 mg/ml) was prepared by dissolving the base in 2 ml of ethanol, adding 0.1 mol/l hydrochloric acid to a pH of 2.5—3.0 and diluting to correct volume (100 ml) with redistilled water. Working standards (1 μg/ml) were made by diluting aliquots of the stock solution with redistilled water.

Tripelennamine hydrochloride (Pyribenzamine®, pharmacopoeial quality) was used as internal standard (I.S.); aqueous working standards of 1 μg/ml were prepared from an aqueous stock solution (1.0 mg/ml).

The stock solutions were stable for at least two months kept at 4°C. The working standards were prepared weekly.

All reagents were analytical grade. *n*-Hexane, chromatography grade, was supplied by Rathburn Chemicals (Walkerburn, Great Britain). Sodium hydroxide (1 *N*) was ether-washed.

Glassware

Conical glass-stoppered centrifuge tubes (15 ml) were silanized with 5% dimethyldichlorosilane in toluene for 4 h, then washed with methanol and acetone. Used extraction tubes were washed in hot soapy water, rinsed with tap water twenty times and then three times with ethanol.

The evaporating tubes were 15-ml Quickfit tubes finely tapered at the bottom (100 µl). After use they were kept overnight in 2 mol/l hydrochloric acid, rinsed with water twenty times, and then three times with ethanol. During the washing procedure the solvents were withdrawn from the bottom tip with a Pasteur pipette by suction with water vacuum. In order to prevent adsorption of OX to the glass walls, they were rinsed with acetone just before use. The injection syringes (Hamilton 10 µl) were rinsed with methanol which was a better solvent for OX than ethyl acetate. Three 200-µl volumes of methanol were washed through the syringe using a larger syringe (Hamilton 250 µl) as a supply.

Chromatography

The instrument used was a Varian Aerograph Series 1400 equipped with a nitrogen-sensitive Varian TSD detector (Basle, Switzerland) and a W + W recorder 1011 (Basle, Switzerland). The column, 180 cm × 2 mm I.D., was made of Pyrex glass and hand-packed with 3% SP-2100 (Supelco, Bellefonte, PA, U.S.A.) to which was added 0.1% HIEFF-8 BP (Applied Science Labs., State College, PA, U.S.A.). The support was Chromosorb G AW DMCS 70—80 mesh (Johns-Manville, Denver, CO, U.S.A.). Temperatures were injector 265°C, column 211°C and detector 280°C. The detector was used in the nitrogen mode under the following conditions: hydrogen 4.5 ml/min, air 162 ml/min, bias voltage —4.0 V, bead current adjust 5.20. A nitrogen carrier flow-rate of 25.2 ml/min was used.

Retention times were OX 110 sec, and I.S. 60 sec.

Extraction procedure

Aliquots (1.0 ml) of the serum samples were placed in 15-ml centrifuge tubes to which were added 100 µl of the internal standard solution (100 ng), two drops of 2 mol/l sodium hydroxide to a pH of 12, and 6 ml of *n*-hexane. The tubes were shaken horizontally for 5 min in a mechanical shaker (90 times/min), centrifuged for 5 min at 1000 *g*, and 5.5 ml of the hexane layer were then withdrawn. The serum samples were extracted a second time with 6.0 ml of *n*-hexane and the hexane layers were collected in the tapered evaporation tubes. Hexane was evaporated at 60—65°C in a water-bath. A stream of nitrogen was blown from above on to the hexane layer. The residual dry extracts were dissolved in 600 µl of methanol, the tube walls being thoroughly rinsed. After a renewed cautious evaporation, the residual extract was dissolved

in 25 μl of ethyl acetate by whirlmixing for 10 sec. A 1-μl aliquot was injected.

Calibration curve

Pooled blank serum samples (Red Cross Bloodcentre, Oslo, Norway) were spiked with fixed amounts of OX. Five concentrations (10, 50, 100, 200, and 250 ng/ml) were chosen and ten spiked samples of each concentration prepared. To each sample were added 100 ng of internal standard (100 μl of the working internal standard solution) and the samples were treated as above.

RESULTS AND DISCUSSION

Chromatograms of extracts from 1 ml of serum after single-dose administration of oxeladin linctus to a volunteer are shown in Fig. 1a and b. Ap-

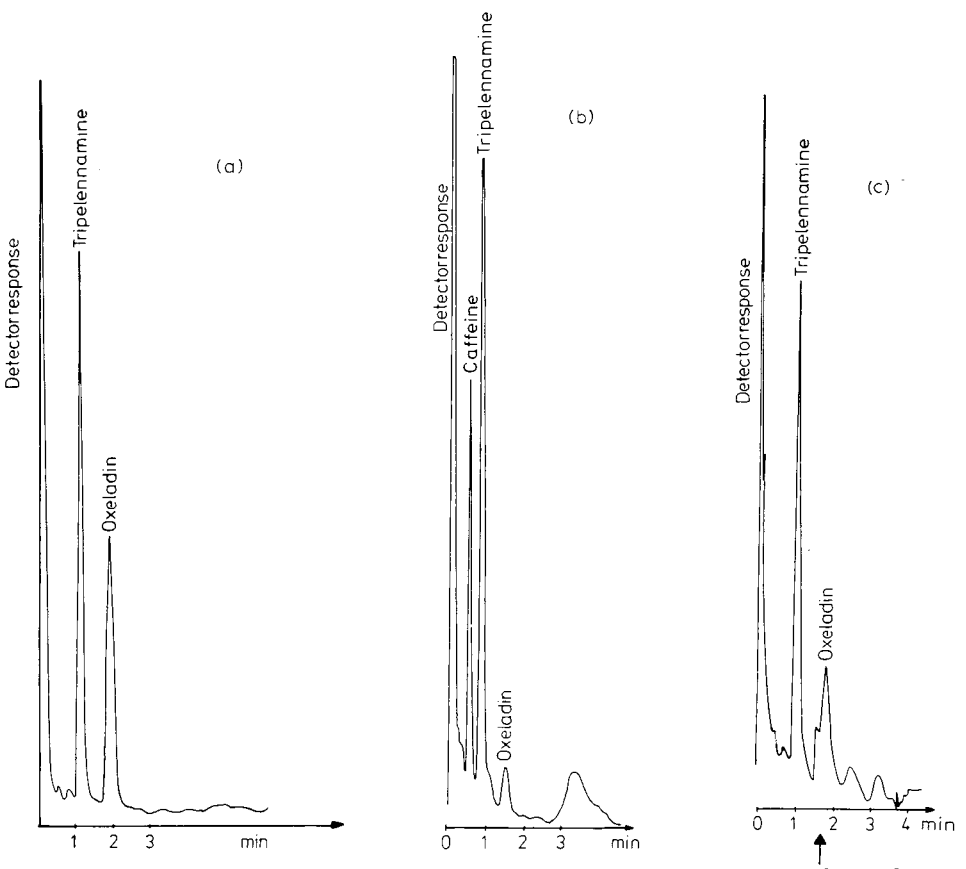

Fig. 1. Chromatograms of serum extracts from volunteers given a single dose of a cough linctus: (a) 1 h after administration and without coffee intake; (b) 6 h after administration and following coffee drinking. (c) A blank serum sample spiked with 10 ng/ml oxeladin base. Arrow marks attenuator shift.

parently caffeine does not interfere in the analysis. However, the caffeine peak interferes with the baseline when very small amounts of OX are being analyzed. In Fig. 1c is shown a chromatogram obtained from a serum spiked with 10 ng/ml OX. (Injected 1 μl = 0.4 ng.) After attenuator shift the OX peak is well above twice the noise.

Based upon the pooled blank serum samples spiked with OX and internal standard, a calibration curve was constructed, where 100 times peak height ratios of OX/IS were plotted as ordinates versus concentration of OX (in ng/ml) as abscissa. The calibration graph was linear over the concentration range 10—250 ng/ml with a regression line $y = ax + b$, where $a = 0.219$ and $b = -0.0846$. The correlation coefficient was 1.00. $S.D._{rel}$ at each concentration is shown in Table I.

TABLE I

RELATIVE STANDARD DEVIATION ($S.D._{rel}$) FOR EACH POINT OF THE CALIBRATION GRAPH

$n = 10$.

Concentration (ng/ml)	$S.D._{rel}$ (%)
10	7.9
50	6.2
100	6.5
200	5.2
250	5.0

Stability of solutions

Stock solutions with a pH of 2.5—3.0 were stable for at least two months at 4°C. The aqueous working standards with a pH of 6.5 were stable for at least two months, kept in a refrigerator at 4°C.

The stability of serum samples containing oxeladin was checked. A blank serum sample was spiked with 167 ng/ml OX and kept at −20°C for four months. Aliquots were analyzed at different times. A patient serum sample was kept for several weeks at −20°C. Replicate analyses were performed. In spite of repeated thawing and freezing of the samples, all analytical data were within the standard deviation range of the method.

Recovery

Recovery of oxeladin and tripelennamine was approximately 100%. About 95% of OX and 99% of internal standard was extracted into the first 6-ml hexane layer.

Injection precision

Ten injections of a given serum extract (spiked with 250 ng/ml OX and 100 ng/ml of internal standard were performed. $S.D._{rel}$ was 2.0%.

Applications

During bioavailability experiments blood was sampled into Venoject tubes without heparin, centrifuged after cooling and kept at −20°C until analysis. The quality of the serum samples varied and some samples were not easily mixed with the hexane layer in a rotating mixer. So a more heavy, horizontal shaking was necessary.

Although caffeine did not interfere in a normal chromatogram, the presence of a large caffeine peak during the analysis of very small amounts of oxeladin presented difficulties in drawing a correct baseline.

The flameless nitrogen-selective thermionic detector proved to be very stable and selective towards nitrogen under the operating conditions given.

In Fig. 2 are presented serum concentration—time data obtained from volunteers given a single dose of Pectamol® linctus.

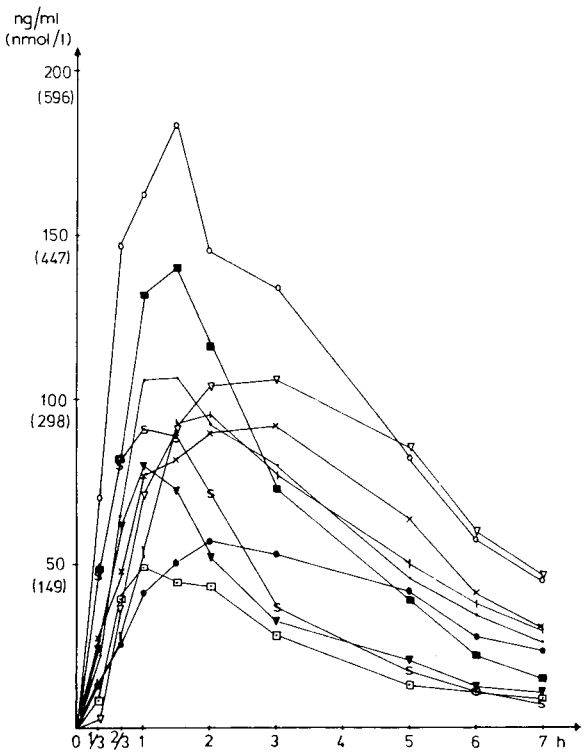

Fig. 2. Typical serum level—time profiles from healthy volunteers after oral administration of 80 mg of oxeladin citrate. Abscissa values represent hours after administration, ordinate is oxeladin concentration.

CONCLUSIONS

The GLC method described above for the determination of oxeladin in 1.0-ml serum samples using a flameless nitrogen-selective thermionic detector

was precise and sensitive. Mean $S.D._{rel}$ in the concentration range 10—250 ng/ml was better than 6.2%. Detection limit was 5 ng/ml of serum or 0.2 ng (1 μl) injected. Twenty serum samples can be analysed in duplicate a day.

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Note

Simple method for the measurement of tocainide and lignocaine in blood plasma or serum using gas—liquid chromatography with flame ionisation detection

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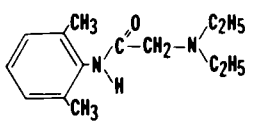
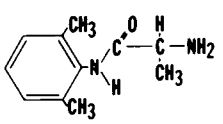
Tocainide [2-amino-N-(2,6-dimethylphenyl)propanamide; Fig. 1] is an orally-effective analogue of lignocaine, and may have advantages over this latter compound in the chronic treatment of ventricular arrhythmias [1—3]. Published high-performance liquid chromatographic methods for the measurement of plasma tocainide concentrations require long extraction times (5—15 min) [4—7], solvent evaporation [5—7], derivatisation following a multiple extraction [6] or relatively large (1 ml) sample volumes [4,5,7]. The gas-liquid chromatographic (GLC) methods described [1,8,9] also require derivatisation following solvent extraction and evaporation steps.



Lignocaine



Tocainide

Fig. 1. Structural formulae of tocainide and lignocaine.

The method described here involves the extraction of only 100 μl of plasma or serum at an alkaline pH with 50 μl of chloroform containing an internal standard, followed by the direct analysis of a portion of the resulting extract using GLC with flame ionisation detection [10]. This technique can be used to measure plasma concentrations of both lignocaine and tocainide simultaneously and has been found to be suitable for the measurement of these drugs at the concentrations attained during therapy.

EXPERIMENTAL

Materials and reagents

Lignocaine and tocainide hydrochlorides were supplied by Astra (Watford, Great Britain) and were used as aqueous solutions containing 1.00 g/l free base of each drug.

The internal standard, *n*-eicosane (C_{20}), was obtained from Koch-Light (Colnbrook, Great Britain) and was used as a 10 mg/l solution in chloroform (analytical reagent grade). γ-Glycidoxypropyltrimethoxysilane (A-187) was obtained from H.S. Chromatography Packings (Bourne End, Great Britain). Tris(hydroxymethyl)aminomethane (analytical reagent grade) was used as a 2 mol/l aqueous solution (Tris solution).

TABLE I

TABLE OF RETENTION TIMES RELATIVE TO *n*-EICOSANE

Compound	Relative retention time
Norpseudoephedrine	0.10
Brompheniramine	0.12
Nicotine	0.12
Pseudoephedrine	0.12
Mexiletine	0.13
Ephedrine	0.14
2,7-Dimethylquinoline [cf. ref. 10]	0.16
Diethylpropion	0.20
Nikethamide	0.20
Chlorphentermine	0.25
Pethidine	0.40
Tocainide	0.45
Monoethylglycinexylidide	0.62
Diphenhydramine	0.63
Ethoheptazine	0.63
Caffeine	0.65
Lignocaine	0.71
Oxprenolol	0.71
n-Eicosane	1.00
Procainamide	2.18
Disopyramide	5.21
Quinidine*	11.3—12.9

*Tailing peak, retention times measured at 1.0 g/l and 10 mg/l, respectively.

Gas—liquid chromatography

A Pye Series 204 dual-column gas chromatograph equipped with flame ionisation detectors was used and integration of peak areas was performed using a Hewlett-Packard 3352 data system. The detector oven temperature was 250°C and the column oven temperature was 230°C. Injection block heaters were not used. The nitrogen (carrier gas) flow-rate was 40 ml/min, and the oxygen and hydrogen inlet pressures were 1.03 and 1.38 bar, respectively, giving flow-rates of approximately 400 and 40 ml/min. The column, a glass tube 2.1 m × 4 mm I.D., was silanised by immersion in 2% dichlorodimethylsilane in toluene for 1 h, rinsed with methanol and dried at 100°C. The column was packed with 3% (w/w) OV-101 on 80—100 mesh Supelcoport purchased ready-prepared from Chromatography Services (Merseyside, Great Britain), and was conditioned at 250°C with nitrogen flow (40 ml/min) for 15 h. Subsequently, daily injections of 5—10 μl of A-187 were performed to maintain the packing in a deactivated form [10].

The retention times of lignocaine, tocainide and some other compounds on this system, measured relative to the internal standard, are given in Table I.

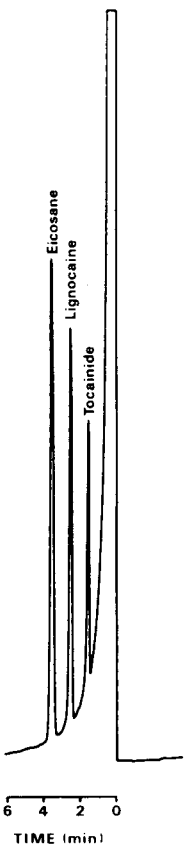


Fig. 2. Chromatogram obtained on analysis of standard solution in chloroform containing lignocaine and tocainide (both 10 mg/l); 5-μl injection. The *n*-eicosane concentration was 10 mg/l. Chromatographic conditions as given in text, except that a 1.5 m × 4 mm I.D. column was used.

The chromatogram of a standard chloroform solution containing tocainide and lignocaine is illustrated in Fig. 2.

Sample preparation

Plasma or serum (100 μl), Tris solution (20 μl) and internal standard solution (50 μl) were added to a small Dreyer test tube (Poulten, Selfe and Lee, Wickford, Great Britain). Hamilton gas-tight luer-fitting glass syringes (1.0 and 2.5 ml, respectively) fitted with Hamilton repeating mechanisms and stainless-steel needles were used for the addition of these latter volumes. The contents of the tube were vortex-mixed for 30 sec and the tube was centrifuged for 2 min at 9950 *g* in an Eppendorf centrifuge 5412 (Anderman, East Molesey, Great Britain). Subsequently, a portion of the extract was obtained by drawing 5 μl of air into a gas chromatographic syringe and passing the syringe needle through the aqueous layer into the chloroform. The air was expelled, and a 2—5 μl portion of the organic phase was taken up into the syringe and injected onto the gas chromatographic column using a syringe fitted with an 11.5-cm needle.

The extraction was performed in duplicate and the mean results taken.

Instrument calibration

Standard solutions containing lignocaine or tocainide at concentrations of 2.0, 5.0, 10 and 20 mg/l free base were prepared in heparinised human plasma by dilution of the appropriate 1 g/l aqueous solution. On analysis of these solutions, the calibration graphs of peak area ratio (drug/*n*-eicosane) against drug concentration were linear, with zero intercept, across the range of the standards. The calibration gradient (peak area ratio/plasma drug concentration) was normally 0.126 l/mg (lignocaine) or 0.065 l/mg (tocainide).

In practice, the instrument was calibrated using a 5.0 mg/l plasma standard, and the calibration confirmed by the analysis of an internal quality control sample containing lignocaine or tocainide (10.0 mg/l) prepared from an independent stock solution.

RESULTS AND DISCUSSION

Recovery studies

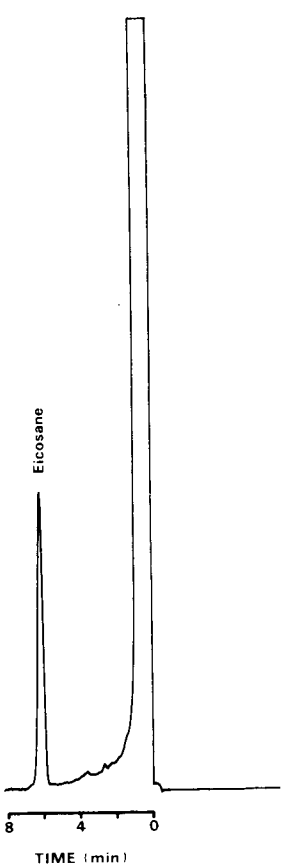
Standard solutions containing either lignocaine or tocainide hydrochlorides at a concentration equivalent to 1.00 g/l free base were prepared in chloroform or chloroform—ethanol (95:5, v/v), respectively. Standard solutions in chloroform were prepared by dilution from the stock solution, each containing the appropriate drug at concentrations equivalent to 2.0, 5.0 and 10.0 mg/l together with *n*-eicosane (10 mg/l). Calibration graphs of peak area ratio drug/*n*-eicosane against drug concentration were prepared using these solutions and the mean apparent recoveries of drug from the heparinised human plasma solutions were found to be 98.7 ± 6.8% (S.D.) and 99.7 ± 7.3% (S.D.) for tocainide and lignocaine, respectively (n = 4 at each concentration) after taking into account the 2:1 concentration inherent in the extraction.

The effect of a high concentration of lignocaine on the recovery of tocainide from plasma standards and vice versa was investigated by the preparation of

appropriate standard solutions. The presence of lignocaine at a concentration of 20 mg/l did not significantly affect the tocainide concentration measured at 25 mg/l [without lignocaine 25.62 ± 1.21 (S.D.) mg/l; with lignocaine 24.97 ± 1.09 mg/l] or at 2 mg/l (without lignocaine 2.40 ± 0.08 mg/l; with lignocaine 2.31 ± 0.11 mg/l). Analogous results were obtained when tocainide (20 mg/l) was added to solutions containing lignocaine (18 and 2 mg/l, respectively).

Selectivity

No endogenous sources of interference have been observed [10] and a chromatogram obtained on analysis of an extract of drug-free human plasma



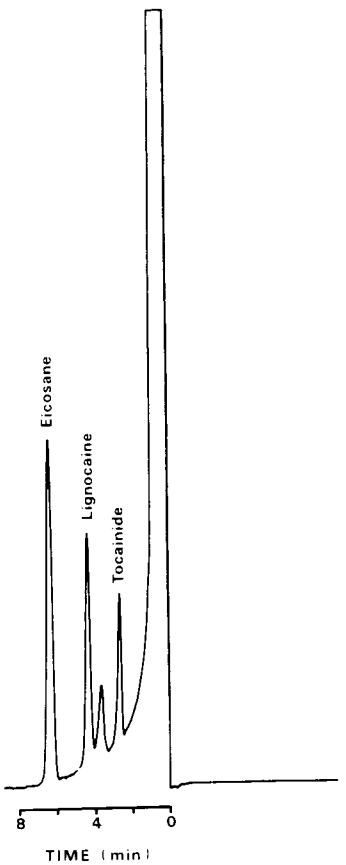


Fig. 3. Chromatogram obtained on analysis of an extract of a plasma sample from a patient receiving neither lignocaine nor tocainide; 5-μl injection. The *n*-eicosane concentration was 10 mg/l. Chromatographic conditions as given in text.

Fig. 4. Chromatogram obtained on analysis of an extract of a plasma sample from a patient receiving both lignocaine and tocainide (1 mg/min intravenously and 600 mg twice daily orally, respectively); 5-μl injection. The sample was drawn during lignocaine infusion and 2 h after the last dose of tocainide. The *n*-eicosane concentration was 10 mg/l, and the tocainide and lignocaine concentrations were found to be 8.8 and 4.9 mg/l, respectively. The peak eluting at a retention time of 0.65 relative to *n*-eicosane was caffeine. Chromatographic conditions as given in text.

is shown in Fig. 3. The chromatogram obtained on analysis of a plasma specimen from a patient under treatment with both tocainide and lignocaine is illustrated in Fig. 4. No interfering peaks were found when this and other specimens from patients receiving tocainide alone were analysed without the addition of the internal standard.

Of other drugs studied which were both extracted and chromatographed under the conditions of the assay (Table I), only the β-blocker drug oxprenolol presents a potential source of interference. However, the plasma concentrations attained during therapy of this latter drug are very low (less than 0.5 mg/l) [11], and interference from this source is unlikely to prove serious. Other compounds, including propranolol, tricyclic antidepressants and benzodiazepine drugs, all elute after the internal standard and, during normal therapeutic administration, are not present in sufficient concentration to be detected by this method. Monoethylglycinexylidide, a metabolite of lignocaine, is extracted under the conditions of the assay but co-elutes with caffeine and cannot, therefore, be measured.

Reproducibility

For tocainide, the intra-assay coefficient of variation (C.V.) was 5.40% at 2.0 mg/l (n = 10) and 3.12% at 10.0 mg/l (n = 10). The inter-assay C.V. was 3.87% at 10.0 mg/l (n = 10).

For lignocaine, the intra-assay C.V. was 5.23% at 2.0 mg/l (n = 10) and 3.20% at 10.0 mg/l (n = 10). The inter-assay C.V. was 3.81% at 10.0 mg/l (n = 10).

Limit of sensitivity

The limit of accurate measurement of the method was 0.2 mg/l for both drugs. A 0.2 mg/l plasma standard gave a mean value of 0.22 ± 0.03 mg/l (S.D.) (lignocaine) and 0.17 ± 0.01 mg/l (S.D.) (tocainide) (n = 5 in both instances). The concentrations of both drugs attained during normal therapy are well above this limit [10,12].

External quality control

Tocainide solutions prepared in heparinised human plasma and with weighed-in tocainide free-base values in the range 0.56—8.48 mg/l were supplied by Astra Chemicals and analysed by the present method. There was good correlation between the results obtained [mean = 4.12 ± 3.39 (S.D.) mg/l] and the weighed-in tocainide value [mean = 4.35 ± 3.45 (S.D.) mg/l] (r = 0.997; n = 5). Linear regression analysis using the weighed-in value as the independent variable revealed a gradient of 0.98 and an intercept on the Y-axis of —0.15 mg/l.

A quality control specimen prepared internally from an external stock-solution was used in the lignocaine assay [10].

CONCLUSIONS

The method described here has been found to be suitable for the simultaneous measurement of the plasma concentrations of both tocainide and lignocaine attained during therapy. Neither extract concentration nor derivatisa-

tion steps are required, and no sources of interference have been identified. Only 200 μl of specimen are required for an analysis, in duplicate, which can be completed together with the analysis of a quality control specimen within 30 min.

ACKNOWLEDGEMENTS

We are grateful to Dr. Nick Boyes, Astra Clinical Research, Edinburgh, Great Britain, for supplying the pure drugs and for the preparation of the quality control samples and to Dr. Brian Widdop, Poisons Unit, Guy's Hospital, for criticism of the manuscript.

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Note

Liquid chromatographic determination of drugs in urine by direct injection on to a reversed-phase column

Fluorescence versus UV detection

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In the routine analysis of drugs in biological samples by liquid chromatography (LC) and spectroscopic detection the sample work-up procedure is often the most time-consuming step. Direct injection of the samples without any prior purification (extraction) will of course simplify the analyses and shorten the analysis time. However, the following prerequisites generally must be valid. (1) The compound possesses such properties that it can be selectively detected, such as absorbance at a wavelength where few other compounds absorb or high fluorescence. (2) The concentration of the compound in the sample is relatively high, because in most cases only a relatively small sample volume can be injected (20—50 μl). (3) The column can withstand injection of a large number of samples without any degeneration; otherwise there is little to gain compared to a regular work-up procedure.

In biopharmaceutical studies for the development of more efficient drug preparations there is a need to measure the amount of drug excreted in urine during a certain time interval. Compounds that we have analysed in connection with biopharmaceutical studies by direct injection of urine samples on to columns with a chemically bonded non-polar phase are quinidine (dihydroquinidine), furosemide, salicylic, salicyluric and gentisic acid, salicylazosulfapyridine and N-acetyl-5-aminosalicylic acid.

During the last few years simplified methods for drug determination, mostly in plasma but also in urine, have become increasingly popular. After protein precipitation, plasma samples have been injected direct on to an LC column. Methods for analysis of quinidine [1], furosemide [2—4], salicylic acid [5] and metabolites [6] and N-acetyl-4-aminosalicylic acid [7] in plasma have been published. Furosemide [3, 4] and salicylic acid and metabolites [8] have also

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been determined in urine samples. Fluorescence [1, 2, 4, 7] and UV detection [3, 5, 6, 8] were used.

In this study UV and fluorescence detection have been compared for sensitivity and selectivity. The signal-to-noise ratio for fluorescent compounds is often not dependent on the type of detector (UV or fluorescence), while the selectivity in most cases is much better with the fluorescence detector, which increases the overall sensitivity of the analytical procedure.

EXPERIMENTAL

Chromatographic apparatus

The liquid chromatograph consisted of an Altex 110A (Berkeley, CA, U.S.A.) pump, an LDC spectromonitor III (Riviera Beach, FL, U.S.A.) UV detector and a Perkin Elmer LC 1000 (Norwalk, CT, U.S.A.) fluorescence detector. The injector was from Rheodyne (Berkeley, CA, U.S.A.; 70-10) with a 20-μl loop. The separation column of stainless steel (length 150 mm, O.D. 6.35 mm, I.D. 4.5 mm) had end fittings of modified Swagelok® connections. Operations were carried out at room temperature.

Chemicals and packing material

Acetonitrile, dichloromethane and hexane (pro analysi; E. Merck, Darmstadt, G.F.R.) were used.

Tetrabutylammonium hydrogen sulphate ($TBAHSO_4$) obtained from the Department of Organic Chemistry, AB Haessle, Mölndal, Sweden, was neutralized with sodium hydroxide to pH 5—10 and purified by shaking three times with dichloromethane (one-tenth volume) and two times with hexane.

All reagent and buffer solutions were prepared with analytical-reagent grade chemicals.

The drug compounds fulfilled the quality requirements of the Pharmacopoeia Nordica.

The packing materials used in the separation columns were LiChrosorb RP-8 (average diameter 5 or 10 μm) and RP-18 (5 μm) (E. Merck). The performance of the columns was maintained by exchange of the particles at the top of the column every day.

Analytical procedure

The general scheme for the analytical procedures is as follows: The thawed urine samples are shaken and centrifuged. (Filtration is an alternative to centrifugation but there is a risk of adsorption losses in the filter.) The volume injected on to the LC column is 20 μl.

The chromatographic conditions for each compound are summarized in Table I. Chromatograms for each compound are shown in Figs. 1—5. Comparisons are made between detection by UV and fluorescence.

Quantitative evaluation

In the routine analysis of drug levels in urine samples, peak heights were measured and the concentrations were obtained by comparison with analysed standard urine samples. No internal standard was used owing to the very simple procedure.

RESULTS AND DISCUSSION

In the selection of experimental conditions for determination of drug levels in urine by direct injection of the sample, the characteristics of the analytes, for example fluorescence or absorbance at a wavelength where other sample components do not interfere, were utilized. Furosemide, quinidine, salicylic, salicyluric, gentisic and N-acetyl-5-aminosalicylic acid are all fluorescent and are often excreted in relatively high concentrations in urine. By injection of 20 μl of authentic samples on to a reversed-phase column, using acetonitrile—buffer mixtures as mobile phases, each of these six compounds could easily be separated from other urine components and detected with a fluorescence detector (Figs. 1—4). Furosemide, being a diuretic drug and thus producing a rather diluted urine sample, could also be selectively detected with a UV detector (Fig. 1). This was not possible for the others (Figs. 2—4) since other UV-absorbing compounds interfered in the chromatograms. Salicylazosulfapyridine is non-fluorescent but absorbs at a rather high wavelength (360 nm, Fig. 5), a valuable property in terms of selectivity.

Since a large number of unpurified urine samples are injected, the column will sooner or later degenerate. Column performance will be maintained over a longer period, however, if the bonded silica particles at the top of the column are exchanged daily or if an exchangeable guard column is used.

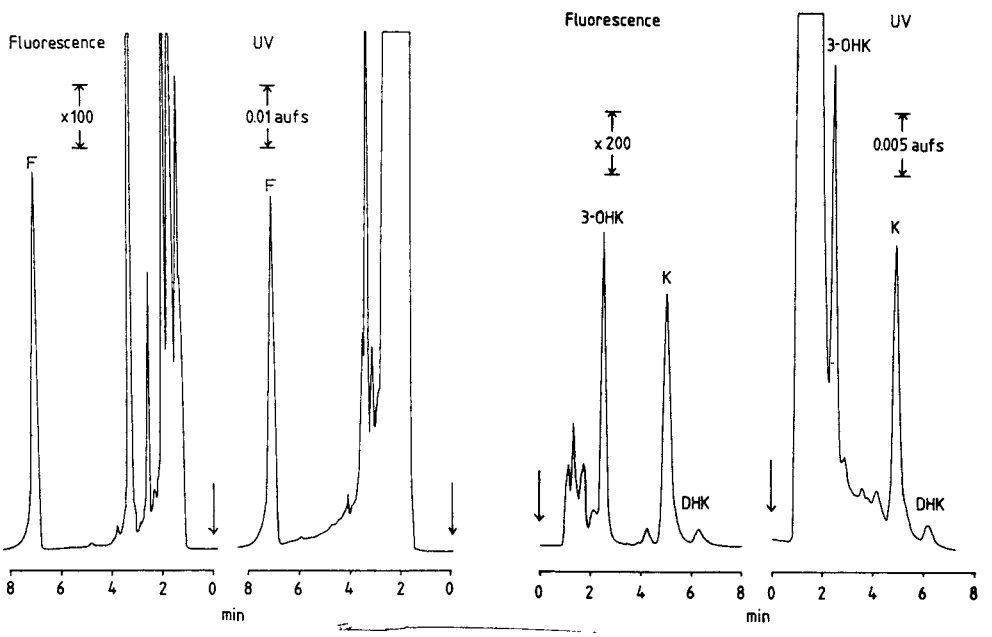

Fig. 1. Furosemide (F) in 20 μl of an authentic urine sample containing 20 μmol/l furosemide injected directly on to an LC column. Detection by UV and fluorescence.

Fig. 2. Quinidine in 20 μl of an authentic urine sample containing 40 μmol/l quinidine (K), 3 μmol/l dihydroquinidine (DHK) and 25 μmol/l 3-hydroxiquinidine (3-OHK) injected directly on to an LC column. Detection by UV and fluorescence.

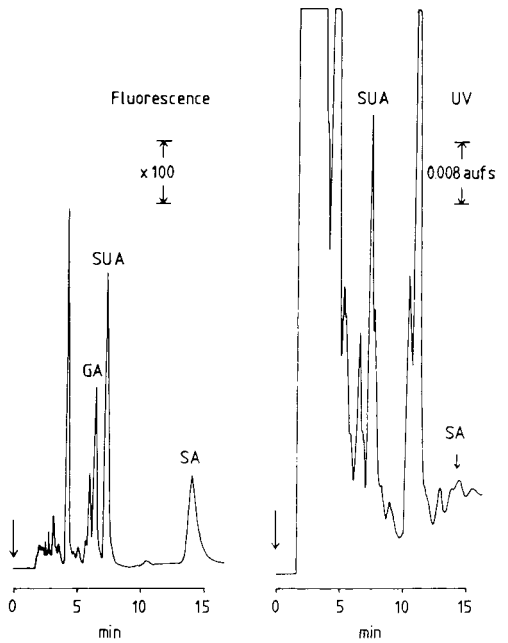

Fig. 3. Salicylic (SA), salicyluric (SUA) and gentisic (GA) acid in 20 μl of an authentic urine sample containing 80 μmol/l SA, 400 μmol/l SUA and 60 μmol/l GA injected directly on to an LC column.

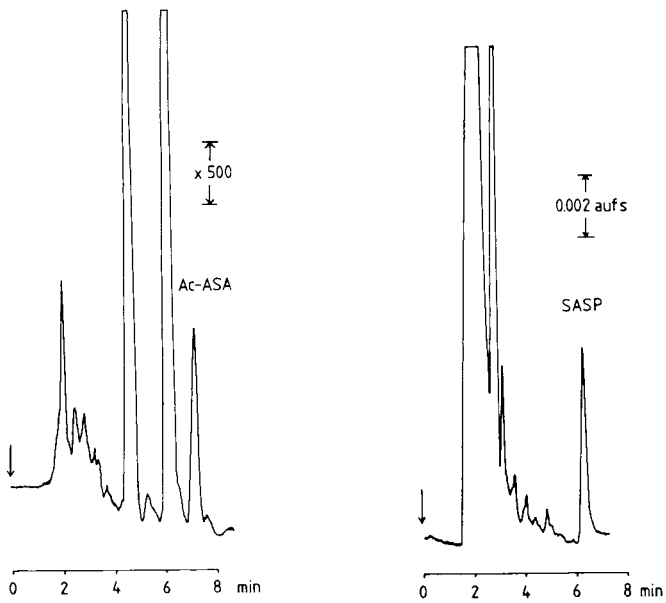

Fig. 4. N-Acetyl-5-aminosalicylic acid (Ac-ASA) in 20 μl of an authentic urine sample containing 8 μmol/l N-acetyl-5-aminosalicylic acid injected directly on to an LC column, with fluorescence detection.

Fig. 5. Salicylazosulfapyridine (SASP) in 20 μl of an authentic urine sample containing 3 μmol/l of salicylazosulfapyridine injected directly on to an LC-column, with photometric detection.

TABLE I

ANALYTICAL PROCEDURES AND CHROMATOGRAPHIC CONDITIONS

Sample: urine. pH of sample is not adjusted. Injection volume: 20 μl. Column: LiChrosorb RP-8, 150 × 4.5 mm.

Drug	Aqueous mobile phase	Flow-rate (ml/min)	Wavelength (nm)		MDC* (μmol/l)	Chromatogram shown in Fig. No.
Furosemide	Acetonitrile 33% H_3PO_4 0.1 mol/l	1.0	270/>389 274	(F)** (UV)	0.2 (UV, F)	1
Quinidine (dihydroquinidine	Acetonitrile 25% $NaClO_4$ + $HClO_4$ (0.095 + 0.005 mol/l)	1.4	363/440 254	(F) (UV)	0.3 (UV, F)	2
Salicylic acid, salicyluric acid, gentisic acid	Acetonitrile 22% TBA 0.01 mol/l phosphate buffer (pH 7, I = 0.05)	1.0	315/420 280	(F) (UV)	5—10	3
N-Acetyl-5-aminosalicylic acid	Acetonitrile 20% TBA 0.01 mol/l phosphate buffer (pH 6.5, I = 0.075)	1.0	315/430	(F)	1	4
Salicylazosulfapyridine***	Acetonitrile 22.5% phosphate buffer (pH 7.6, I = 0.007)	1.0	360	(UV)	0.2	5

*Minimum determinable concentration, S.D. ⩽ 10% (n = 10).
**F = fluorescence.
***Column: LiChrosorb RP-18.

The minimum determinable concentration for each compound is given in Table I, and is defined as the concentration giving a relative standard deviation of ⩽ 10% (n = 10). At concentration levels higher than 5—10 times the minimum determinable concentration the relative standard deviation for each compound was ⩽ 2%.

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Note

Determination of 6,11-dihydro-11-dibenz[*b*,*e*]oxepin-2-acetic acid (isoxepac) in plasma by high-performance liquid chromatography

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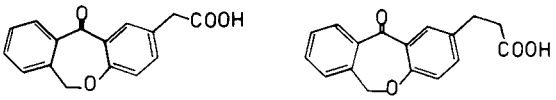
6,11-Dihydro-11-oxodibenz[*b*,*e*]oxepin-2-acetic acid (HP 549, isoxepac, Hoechst, Frankfurt/M, G.F.R.) is an anti-inflammatory and analgesic agent presently undergoing extensive clinical trials in man. In our department the possibility of pharmacokinetic interaction between acetylsalicylic acid (aspirin) and isoxepac during acute and long-term therapy of both agents, is being investigated. The gas—liquid chromatographic method described by Bryce and Burrows [1] was used by us initially, but found to be too time consuming. The large number of samples which had to be analysed for salicylic acid and isoxepac required the development of an analytical method for isoxepac which would be sensitive, accurate, precise and simple from the point of view of time consumption. This paper describes a high-performance liquid chromatographic (HPLC) method, using native fluorescence detection, for the determination of isoxepac in plasma. The assay is sufficiently sensitive to follow reliably plasma levels of isoxepac for a period of five half-lives after a therapeutic dose of 100 mg.

EXPERIMENTAL

Reagents

The chemical structures of isoxepac and the internal standard, 6,11-dihydro-11-oxodibenz[*b*,*e*]oxepin-3-propionic acid are shown in Fig. 1. Both compounds were obtained from Hoechst.

Methanol, methylene chloride and acetic acid were guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used as received.



1 2

Fig. 1. Chemical structures of isoxepac (1) and 6,11-dihydro-11-oxodibenz[*b,e*]oxepin-3-propionic acid (2).

Apparatus

An M6000A pump and a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.) were coupled to a Radial-Pak C_{18} (10 μm) cartridge (10 cm × 8 mm high-density polyethylene column packed with octadecylsilane-bonded porous silica) held in an RCM-100 Radial compression unit (Waters Assoc.). A Fluorichrome fluorescence detector (Varian, Walnut Creek, CA, U.S.A.) was used to measure fluorescence of the eluate. Alternatively, a Model 450 variable-wavelength detector (Waters Assoc.) was used. The results were processed on a Waters 730 data module in the peak-height mode.

Other apparatus used consisted of glass centrifuge tubes with B19 ground glass joints and stoppers, a variable-speed multi-purpose rotator (Scientific Instruments, Springfield, MA, U.S.A.), a Clements Model B universal centrifuge, (H.I. Clements, Sydney, Australia), 1-, 2- and 5-ml glass ampoules, high-purity nitrogen and a 100-μl Hamilton syringe.

Stock solutions

A stock solution of the internal standard was prepared by dissolving 2.6 mg of the internal standard in 10 ml 0.02 *M* sodium carbonate solution. Aliquots (0.5 ml) of stock solution were kept frozen (−20°C) in sealed glass ampoules.

Plasma standards

An accurately weighed amount of isoxepac was dissolved in a weighed amount of fresh human plasma by shaking for 4 h. By using an average density of 1.027 for plasma [2] isoxepac concentration can be calculated. Weighed amounts of this plasma stock solution were further diluted with weighed amounts of plasma to obtain standards with lower concentrations of isoxepac. Aliquots (1.5 ml) of these standard plasmas were stored frozen (−20°C) in 2-ml sealed glass ampoules.

Extraction

To 1 ml plasma (standard or unknown) in a 10-ml B19 ground glass centrifuge tube was added 20 μl internal standard solution followed by 0.25 ml 1 *M* hydrochloric acid and 5 ml methylene chloride. The stoppered tubes were rotated for 5 min at a speed of 10 rpm on a rotator and were then centrifuged at 900 *g* for 10 min at room temperature. Emulsions which formed very easily during the extraction procedure were found to be broken more readily by centrifuging at room temperature than by centrifuging in a refrigerated centrifuge. The supernatant aqueous layer was aspirated off and the organic phase transferred to 5-ml glass ampoules in which the solvent was evaporated at 40°C under a gentle stream of high-purity nitrogen.

The residue in the ampoules was dissolved in 100 μl of the mobile phase used for chromatography and 60 μl of this solution were injected for analysis.

Chromatography

The mobile phase consisted of methanol—double distilled water—glacial acetic acid (550:450:2). A constant flow-rate of 2 ml/min was maintained with a pressure of about 60 bar at ambient temperature (24°C) through a Radial-Pak C18 (10 μm) column. Excitation energy was obtained through glass band filters 7-54 with 7-60 giving an excitation band maximum at 340—380 nm while emission energy was monitored above 400 nm by using glass cutoff emission filters 3-73 which cut off energy below 400 nm, combined with a wide-band filter 4-76 which prevents transmission of long-wavelength red leakage above 600 nm.

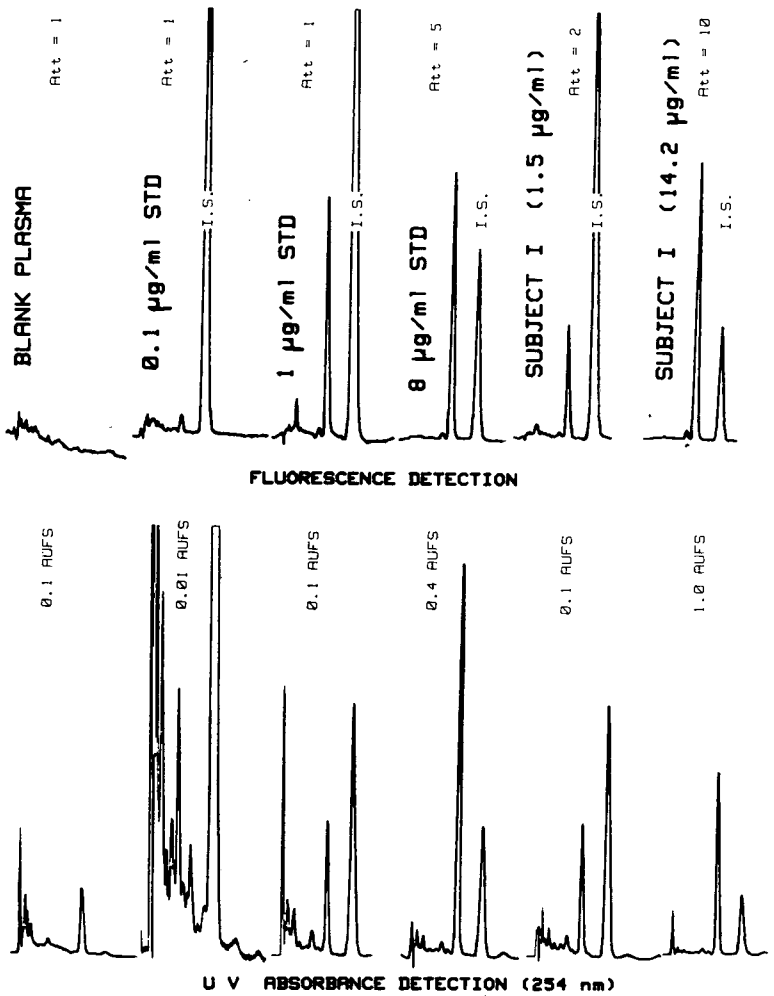


Fig. 2. Typical chromatograms of plasma samples.

Retention times of isoxepac and the internal standard were approximately 7 and 11 min respectively.

RESULTS AND DISCUSSION

Fig. 2 shows representative chromatograms obtained and demonstrates the lack of interfering endogenous compounds.

For comparison representative chromatograms obtained when monitoring absorbance of the same samples at 254 nm instead of fluorescence are shown. Although good results were obtained an interfering UV-absorbing peak with retention time very similar to the internal standard favours the use of the fluorescence detector.

Linear calibration curves of isoxepac peak height/internal standard peak height versus plasma concentrations were obtained with plasma standards containing 2—32 μg/ml. The lines passed close to the origin and the slopes remained relatively constant as can be seen from the following equation which represents the average of twelve calibration curves (obtained by linear regression analysis) constructed during a period of six weeks while the assays of isoxepac in actual plasma samples of the interaction study were being carried out: $y = (9.179 \pm 0.602)x - (0.262 \pm 0.342)$. Correlation coefficients for these linear regressions were consistently greater than 0.998 ($n = 4$) making one-point calibration feasible.

A summary of the results with spiked plasma samples using the fluorescence detector during the validation period of this assay method and with samples processed as quality controls during the period of the interaction study over a period of six weeks is presented in Table I. For comparison, results obtained

TABLE I

RECOVERY OF ISOXEPAC IN SPIKED PLASMA SAMPLES

Concentration spiked (μg/ml)		Mean concentration found (μg/ml)	C.V. (%)	*N*
a*	39.07	37.65	3.1	4
	30.00	32.13	4.3	3
	14.86	14.83	4.9	4
	5.29	5.08	3.0	4
	1.20	1.26	4.5	4
	0.60	0.64	7.5	4
	0.12	0.14	6.5	4
b**	22.11	21.05	1.4	4
	14.34	13.53	8.6	4
	7.16	7.16	0.6	4
	2.77	2.83	0.9	4
c***	29.01	27.25	6.1	11
	17.41	17.68	10.5	11
	5.80	5.73	5.1	9
	2.90	2.97	9.4	9

*(a) Fluorescence detection during validation period.
**(b) UV absorbance detection (254 nm) during validation period.
***(c) Fluorescence detection: quality controls during clinical trial.

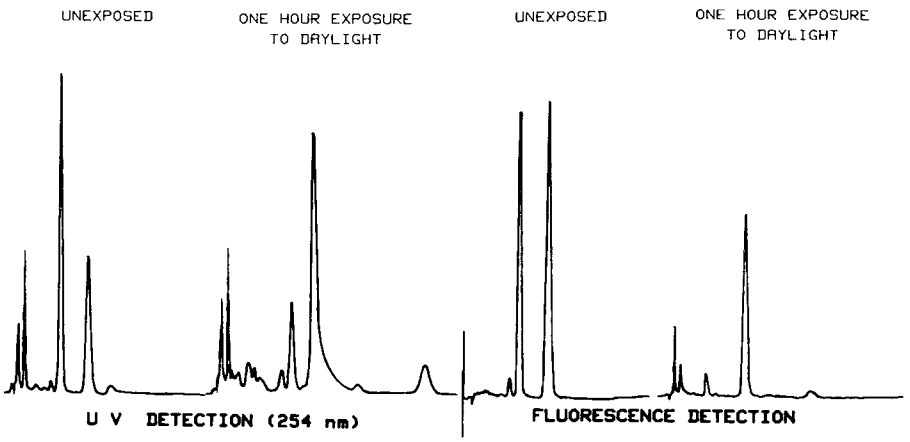


Fig. 3. Effect of exposing plasma extracts, dissolved in mobile phase, to daylight.

with the UV absorbance detector during the validation period are also included in Table I. Although excellent results were obtained with UV (254 nm) detection it was felt that the accuracy and precision could be adversely influenced by the component in normal plasma which eluted with almost the same retention time as the internal standard. The good accuracy and precision obtained during the validation period could be ascribed to the fact that the spiked plasma samples and plasma standards were made up from a homogeneous plasma pool.

The extraction procedure with methylene chloride is that described by Bryce and Burrows [1] and no other solvents were evaluated. The absolute extraction yield of isoxepac carried through the extraction procedure with spiked plasma samples containing 10 μg/ml isoxepac and using 6,11-dihydro-11-oxodibenz-[*b*,*e*]oxepin-3-propionic acid as an external standard was 82%. That of the internal standard at 10 μg/ml, using isoxepac as external standard, was 70%.

The stipulation by Bryce and Burrows [1] that all operations be carried out in subdued light was found to be very important. Fig. 3 shows chromatograms of extracts which, after dissolution in mobile phase, were injected after minimum exposure to strong daylight conditions compared with the same extracts after exposure of the dissolved extract for 1 h to daylight prevailing on a window sill in the laboratory. This effect was not observed with dissolved extracts kept in the dark for several hours.

Specificity

Since this method was used only during a study of the possible pharmacokinetic interaction of isoxepac with acetylsalicylic acid, only salicylic acid was tested for interference with this assay. No interference by salicylic acid was observed up to concentrations of 400 μg/ml, in fact, salicylic acid can be quantitated simultaneously with isoxepac in the same plasma samples provided care is taken to prevent sublimation of the salicylic acid during the solvent evaporation stage.

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CHROMBIO. 955

Note

Liquid chromatographic analysis of an antimicrobial 5-nitroimidazolyl-2-sulphide derivative in biological fluids

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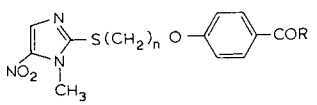
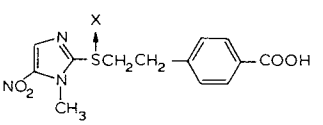
(First received March 16th, 1981; revised manuscript received April 28th, 1981)

SC-28538 [Fig. 1 (1a); sodium 4-[2-(1-methyl-5-nitro imidazolylthio)ethoxy] benzoate] is a novel antimicrobial nitroimidazole. The compound has high activity against obligatory anaerobic bacteria, including several strains of Bacillus fragilis [1, 2]. SC-28538 is also active against several Trichomonads, and penicillin resistant Neisseria gonorrhoeae [2].

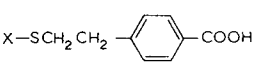
Further study of this compound required an assay for the unchanged drug in biological fluids. Preliminary investigations indicated poor gas chromatographic properties of the methyl esters of SC-28538 and some analogues selected as possible internal standards. High-performance liquid chromatography (HPLC) was therefore selected as an alternative method.



1. (a) R = ONa, n = 2
   (b) R = ONa, n = 3
   (c) R = NH.CH_2CO_2H, n = 2
   (d) R = NH.$CH.CO_2H$, n = 2
   $CH_2.CONH_2$



2. (a) X = O
   (b) X = O_2



3. (a) X = CN
   (b) X = $CONH_2$

Fig. 1. Structures of SC-28538 and related compounds.

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EXPERIMENTAL

Apparatus

Chromatography was performed with a system comprised of an Applied Chromatography (Luton, Great Britain) Model 750/03 pump, Deci-Linear gradient programmer, solvent composition optimising unit and a Model 750/11 UV detector (254 nm). The analysis was carried out in a stainless-steel column (100 mm × 5 mm I.D.) fitted with a syringe injector (Shandon Southern Products, Runcorn, Great Britain), and slurry packed with a C_{18} alkyl silylated silica (5-μm diameter, ODS-Hypersil, Shandon Southern Products). The output from the UV detector was linked to a 10-mV potentiometric recorder (Servoscribe IS, Smiths Industries, London, Great Britain).

Materials

SC-28538 (1a) was obtained from G.D. Searle, and the homologue (1b) was synthesized by a modification of the method for SC-28538 as described by Tweit and co-workers [2, 3], and purified by column chromatography on silica gel in chloroform—methanol (97 : 3, v/v). All other reagents, and solvents which were redistilled before use, were purchased from Hopkin & Williams (Chadwell Heath, Great Britain).

Metabolite synthesis

The glycine and glutamine conjugates (1c and 1d) were synthesised by the reaction of the acid chloride derivative of SC-28538 (0.500 g, 1.46 mmole) with the corresponding amino acid (1.40 mmole) in dioxan (30 ml) and 1 *M* sodium hydroxide solution (4 ml). The crude product obtained by acidifying the reaction mixture was purified by column chromatography on silica gel in chloroform—methanol (96 : 4, v/v), to give the pure conjugates (0.63 mmole).

The sulphone (2a) and sulphoxide (2b) were synthesised by a modification of the method used by Tweit et al. [3], and compound (3a) was prepared by the reaction of potassium thiocyanate (0.396 g, 4.082 mmole) with 4-(2′-bromoethoxy)-benzoic acid (1.00 g, 4.082 mmole) in dimethylformamide (12 ml) at 90°C for 18 h. The crude product (0.898 g) was crystallised from chloroform (30 ml) to give white crystals of 3a (0.505 g, 2.25 mmole).

Compound 3a (0.400 g, 1.79 mmole) was converted to 3b by hydrolysis in concentrated sulphuric acid (5 ml) at 5°C for 1.25 h. The crude product (0.391 g) obtained by dilution with water (25 ml) was chromatographed on a silica gel column in chloroform—methanol (90 : 10, v/v), to give the purified product (0.050 g, 0.21 mmole).

The structures of these compounds were confirmed by NMR, IR and mass spectrometry and their purity was established by thin-layer chromatography (TLC) on silica gel plates ($60F_{254}$, E. Merck, Darmstadt, G.F.R.) in the solvent systems ethylene dichloride—methanol (9 : 2, v/v), *n*-butyl acetate and ethyl acetate—toluene (1 : 1, v/v) with detection of components under UV light (254 nm).

Selection of HPLC conditions

The separation of SC-28538 and its homologue (1b) from endogenous components of urine and plasma was optimised by using different proportions of 0.01 *M* potassium dihydrogen phosphate buffer, at different pH values, and methanol monitoring absorbance at 254 nm. The optimal solvent mixture of 50% methanol—0.01 *M* potassium dihydrogen phosphate buffer adjusted to pH 3.5 with phosphoric acid was used.

Assay procedures

The internal standard (1b; 1.5 μg) was added in aqueous methanol (15 μl) to plasma or urine (1 ml) in acid-washed glass test tubes. Saturated aqueous potassium dihydrogen phosphate solution (1 ml), adjusted to pH 4 with phosphoric acid, was added to plasma samples and mixed on a Whirlimixer. The urine and acidified plasma were extracted for 10 min with 1 ml or 2 × 2 ml of ethyl acetate respectively on a partitioning extractor and centrifuged. The separated organic phases were evaporated to dryness under a nitrogen stream, the residues dissolved in 0.01 *M* sodium hydroxide solution (20 μl) and methanol—water (80 μl, 40:60, v/v) and 10-μl aliquots were analysed on ODS-Hypersil (5 μm, 100 mm × 5 mm I.D.) in 50% methanol—0.01 *M* potassium dihydrogen phosphate (pH 3.5) at 2 ml/min, pressure 70—80 bar with UV detection at 254 nm. The peak height ratio of SC-28538 to internal standard was measured and concentrations were calculated from a calibration curve.

The standard curves and the accuracy and precision of the assay methods were obtained by the analysis of plasma and urine samples containing added SC-28538.

RESULTS AND DISCUSSION

The chromatographic conditions efficiently resolved SC-28538 and the internal standard from the co-extracted components of urine and plasma (Fig. 2). The extraction procedures recovered 96.4 ± 2.60% (S.D.) and 88.2 ± 3.17% of SC-28538 and the internal standard respectively from the biological fluids.

There was a linear correlation between SC-28538 concentration and SC-28538/internal standard peak height ratio for urine or plasma samples over the concentration range 0—2.5 μg/ml. Analysis of quality control samples showed an acceptable level of accuracy and precision (Table I) with a detection limit of about 0.05 μg/ml. The HPLC retention times of several potential SC-28538 metabolites (Table II) showed that they or closely related compounds would not interfere in the assay.

The assay was used to analyse plasma and urine samples from a monkey that had received a single intravenous dose of SC-28538. The plasma results (Fig. 3) showed a fast initial decline in SC-28538 concentrations over the first 1.5 h. They declined more slowly over the next 8 h with an apparent elimination half-life of 3.75 h. Some 14% of the administered SC-28538 was excreted unchanged in urine during the first 24 h after dosing, and a metabolite with a retention time similar to that of the glutamine conjugate (1d) was detected in the urine.

These results indicate that the HPLC method will be suitable for the analysis of SC-28538 in biological fluids.

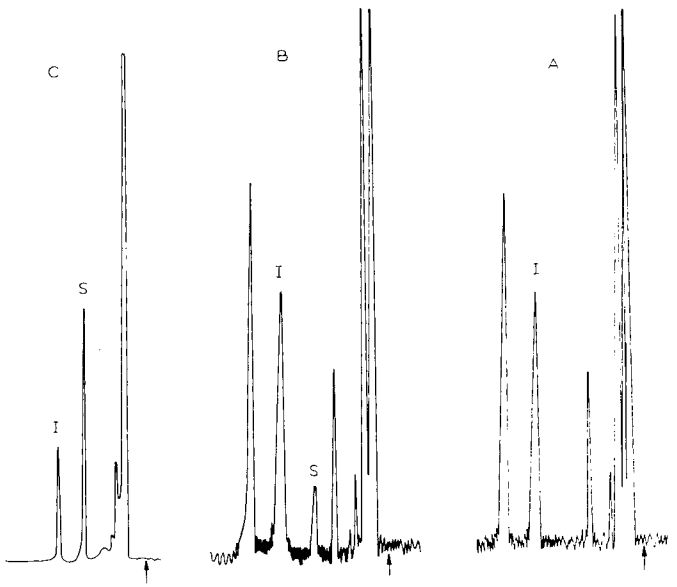

Fig. 2. Chromatograms of extracts from (A) plasma containing 0 μg/ml SC-28538, (B) plasma containing 0.25 μg/ml SC-28538, (C) urine containing 2.5 μg/ml SC-28538. Peaks: S = SC-28538; I = internal standard.

TABLE I

ACCURACY AND PRECISION OF ASSAY FOR SC-28538 IN PLASMA AND URINE

SC-28538 concentration (μg/ml)			Recovery (%)	
Theory	Measured ± S.D. (*n*)		Plasma	Urine
	Plasma	Urine		
2.50	2.56 ± 0.23 (16)	2.67 ± 0.27 (9)	102	107
0.25	0.24 ± 0.03 (15)	0.25 ± 0.01 (9)	96.4	100
0.10	0.09 ± 0.02 (16)	0.10 ± 0.01 (9)	90.0	100
Mean recovery (± S.D.)			96.1 ± 6.0	102 ± 4.0

TABLE II

RETENTION TIMES OF SC-28538 AND POTENTIAL METABOLITES

Compound	Retention time (min)
1a	4.30
1b	6.10
1c	2.00
1d	1.80
2a	1.80
2b	1.30
3a	1.70
3b	0.94

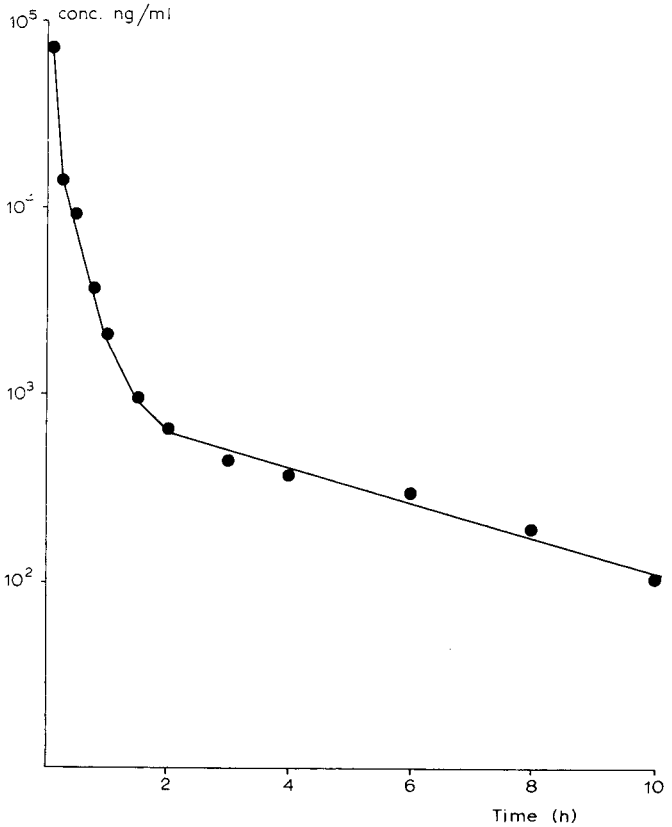

Fig. 3. Plasma levels of SC28538 in a monkey after a single intravenous dose of the compound.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. A. Barrow and Mr. T.J. Forrest for the biological samples from the monkey and Mrs. D. Morris for typing the manuscript.

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CHROMBIO. 962

Note

Determination of Ro12-0068, a new anti-inflammatory and analgesic compound, in plasma by means of high-performance liquid chromatography

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(First received February 23rd, 1981; revised manuscript received April 22nd, 1981)

Ro12-0068 (4-hydroxy-2-methyl-N-2-pyridyl-2H-thieno[2,3-*e*]-1,2-thiazine-3-carboxamide 1,1-dioxide) is a new anti-inflammatory and analgesic compound. Its structure is not related to any anti-inflammatory drug presently on the market. The drug is positive in various acute and chronic experimental inflammation models in laboratory animals, has a marked analgesic effect directed selectively against pain induced by inflammatory or traumatic processes; and inhibits prostaglandin synthetase. Doses of 20 and 40 mg per day in man are clinically and biologically well tolerated [1,2].

A method for the estimation of Ro12-0068 in plasma using thin-layer densitometry has been established [3] but the restricted availability of such instrumentation has necessitated the development of a more convenient assay procedure, namely high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and solvents

Methanol (HPLC grade) was obtained from Rathburn Chemicals (Peebleshire, Great Britain); dichloromethane (AnalaR) and monosodium phosphate from BDH Chemicals (Poole, Great Britain) and disodium phosphate (AnalaR) from Fisons (Loughborough, Great Britain). For the preparation of aqueous solutions, single-distilled water was used.

Standard solutions

A solution containing 1 μg ml^{-1} Ro12-0068 in methanol was used. The internal standard is Ro13-9297 (6-chloro-4-hydroxy-2-methyl-N-2-pyridyl-2H-thieno[2,3-*e*]-1,2-thiazine-3-carboxamide 1,1-dioxide). A solution containing 1 μg ml^{-1} in methanol was used as an internal standard. These solutions were freshly prepared.

Calibration procedure

Spiked plasma samples were prepared in the concentration range 0.05—25 μg ml^{-1} and calibration curves constructed either in the range 0.05—5 μg ml^{-1} (for high sensitivity work) or 5—25 μg ml^{-1}. The appropriate volume of standard solution together with 400 μl internal standard solution was evaporated in a test tube using oxygen-free nitrogen, followed by the addition of 1 ml plasma. The tubes were allowed to stand at room temperature for 20 min with occasional whirlimixing before extraction.

Preparation of samples for assay

Internal standard (400 μl) was blown dry in a test tube and 1 ml of plasma added. The tube was allowed to stand with occasional mixing (as described above) before extraction.

Quantitation of plasma concentration was achieved by reference to the appropriate calibration curve.

Extraction procedure

The plasma was acidified with 1 ml of 1 *N* hydrochloric acid, the mixture whirlimixed and 8 ml dichloromethane added. The tube was shaken gently for 3 min, centrifuged (980 *g*) to break any emulsion formed, and the upper aqueous layer discarded. The organic layer was transferred to another tube, blown dry with nitrogen at a temperature not exceeding 40°C and the residue taken up into 0.5 ml of methanol—buffer mixture (mobile phase). The mixture was whirlimixed (2 min) and centrifuged before injection.

Chromatography

The chromatography system consisted of a Constametric III pump (Laboratory Data Control) and Rheodyne injection system (20-μl loop), LiChrosorb RP-18 column (150 × 3.2 mm I.D., steel), 5-μm particle size (Magnus Scientific). Detection was by means of a Spectromonitor III variable-wavelength dual-cell UV detector operated at 361 nm, coupled to a 308 computing integrator (both from Laboratory Data Control). A guard column (5 cm × 4.6 mm) of Whatman Co:Pell ODS was used between injector and analytical column.

The mobile phase consisted methanol—phosphate buffer, 0.1 mol l^{-1}, pH 5 (60:40) and was used after de-gassing and filtration. The flow-rate used was 0.5 ml min^{-1}. The retention times of Ro12-0068 and internal standard were 2.8 and 4.9 min, respectively.

A methanol—water (60:40) mixture was flushed through after usage to prevent crystallization of the buffer in the system.

RESULTS AND DISCUSSION

Chromatography

In Fig. 1 are illustrated representative chromatograms of blank plasma with internal standard (Fig. 1a), plasma spiked with 0.1 μg ml^{-1} (Fig. 1b), and plasma spiked with 0.8 μg ml^{-1} (Fig. 1c). The extraction and concentration procedure described, necessary in view of the high sensitivity required for assay of samples following single doses of Ro12-0068, did produce two small peaks either side of the drug peak which interfered only at low drug concentrations (Fig. 1a and b). This extraction procedure minimized the interference compared with methods involving methanol precipitation.

Fig. 1. Chromatograms of plasma containing (a) internal standard, Ro13-9297; (b) Ro13-9297 and 0.1 μg ml^{-1} of Ro12-0068; and (c) Ro13-9297 and 0.8 μg ml^{-1} of Ro12-0068.

Linearity and sensitivity

Despite slight peak tailing and interference at low drug concentrations, peak height assessment was used and linear calibration curves were obtained over the concentration ranges studied. The regression line cut the *Y*-axis slightly above the origin probably due to the interference described. The mean correlation coefficient obtained from six successive calibrations carried

out over a period of four weeks was $r = 0.9996$. The detection limit, defined on the basis of the amount injected producing a peak approximately twice that of background, was 0.05 $\mu g\ ml^{-1}$.

Accuracy and reproducibility

Twelve 1-ml aliquots of plasma each spiked with 5 $\mu g\ ml^{-1}$ of Ro12-0068 were assayed consecutively. The mean concentration determined was 5.02 $\mu g\ ml^{-1}$ (S.E.M. = 0.07, C.V. = 5%). At lower concentrations (0.1 and 2 $\mu g\ ml^{-1}$) the C.V. values were 7 and 4%, respectively.

Drug recovery

Drug recovery using a range of initial drug plasma concentrations is shown in Table I. Plasma samples were extracted as described but with the addition of internal standard after the extraction step. The mean recovery was 97.8% with a C.V. of 5%.

TABLE I

RECOVERY OF Ro12-0068 FROM PLASMA (1 ml)

Ro12-0068 added (μg)	Recovery (%)
0.1	96
2.0	89
5.0	101
10.0	102
15.0	99
20.0	100
Mean	97.8

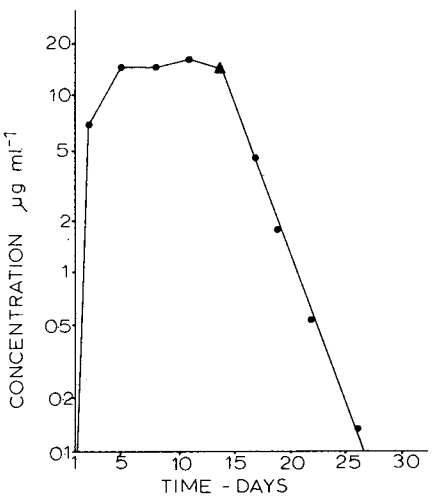

Fig. 2. Plasma concentrations (•——•) of Ro12-0068 following oral dosing of 40 mg daily for two weeks in a volunteer subject. Drug commenced on day 1 and was withdrawn following a dose on day 14. ▲ ≡ computed point.

Application of the assay to biological specimens

The procedure has been used to measure plasma concentrations following a dose of 40 mg day^{-1} for two weeks in eight volunteers. A typical plasma level profile from one such volunteer is shown in Fig. 2.

ACKNOWLEDGEMENTS

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Note

Simplified high-performance liquid chromatographic method for 5-aminosalicylic acid in plasma and urine

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(First received March 9th, 1981; revised manuscript received April 24th, 1981)

During the last few years interest in salicylazosulfapyridine (SASP; Azulfidine®) has been focussed on 5-aminosalicylic acid (5AS) [1—3]. Gut bacteria split the azo bond of SASP forming 5AS and sulfapyridine (SP). Several groups reported the superiority of 5AS and SASP over SP in the therapy of Crohn's disease and ulcerative colitis [1—3], indicating that 5AS may be the therapeutic active moiety of SASP, an established drug for the therapy of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.

SASP has to be taken for long periods of time even after achieving remission phases to prevent a relapse (for review see refs. 4 and 5). Thereby the sulfonamide SP is responsible for the side effects which are reported with an incidence of 20—30% [6—10]. Since 5AS has been proven as the active moiety of SASP, SP could be excluded by the direct administration of 5AS, and thus side-effects might be minimized.

On treating patients with 2—4 g of SASP daily, 5AS and its major acetylated metabolite in plasma and urine show maximal concentrations of about 1 μg/ml [11]. These levels require a method of high sensitivity. We will describe a specific high-performance liquid chromatographic (HPLC) assay with increased sensitivity compared to our previously published measurements [12]. Its application to monitoring plasma and urine of patients receiving SASP or 5AS will be reported.

EXPERIMENTAL

Reagents and material

5-Amino-2-hydroxybenzoic acid (5AS) and 4-amino-2-hydroxybenzoic acid

0378-4347/81/0000—0000/$02.50

(PAS) were purchased from Merck (Darmstadt, G.F.R.). The internal standard AcPAS was synthesized by acetylation of PAS with acetic anhydride and purified by recrystallization. All other reagents were of analytical grade (Merck).

Apparatus

The chromatographic separations were performed on a high-performance liquid chromatograph (Model SP 740, Spectra-Physics, Darmstadt, G.F.R.) equipped with a self-packed 250 mm × 4.6 mm I.D. analytical column packed with reversed-phase material (Nucleosil 10 C-18, 10 μm; Macherey and Nagel, Düren, G.F.R.) and a 120 mm × 4.6 mm I.D. precolumn, prepacked with a reversed-phase material (LiChrosorb C-18, 5 μm, Knauer, Oberursel, G.F.R.). The detection was performed with a fluorescence monitor (Spectra-Physics, Model FS 970 M-A 1), excitation at 300 nm, cut-off filter at 418 nm.

Sample preparation

Table I summarizes the extraction procedure for plasma or urine. All samples were run in duplicate, one with and one without acetylation prior to extraction.

Chromatographic conditions

The mobile phase consisted of deionized water adjusted to pH 3 by concentrated perchloric acid—methanol—acetonitrile (75 : 12.5 : 12.5). A flow-rate of 0.5—0.6 ml/min with a resulting pressure of about 300 bar was established and samples of 100 μl were injected.

TABLE I

EXTRACTION PROCEDURE FOR 5AS AND 5AcAS FROM PLASMA AND URINE

A. Acetylation procedure
500 μl of plasma or urine (diluted 1:100 to 1:1000) in duplicate (one with and one without acetylation prior to extraction)
20 μl of internal standard (0.1 mg AcPAS per ml double-distilled water)
10 μl of acetic acid anhydride in one of the duplicates
Shake for 15 min
B. Deproteinization
Add 50 μl of concentrated perchloric acid
Shake immediately for 5 min
Separate from proteins by centrifugation
C. Extraction
Take 400 μl of supernatant
Add 500 μl of 1 *N* HCl and 9 ml of diethyl ether
Extract by shaking for 10 min
Separate by centrifugation for 5 min
Evaporate the organic phase under nitrogen to dryness
Dissolve residue in 200 μl of the mobile phase

RESULTS

5AS as an amphoteric compound can be extracted into organic phases only

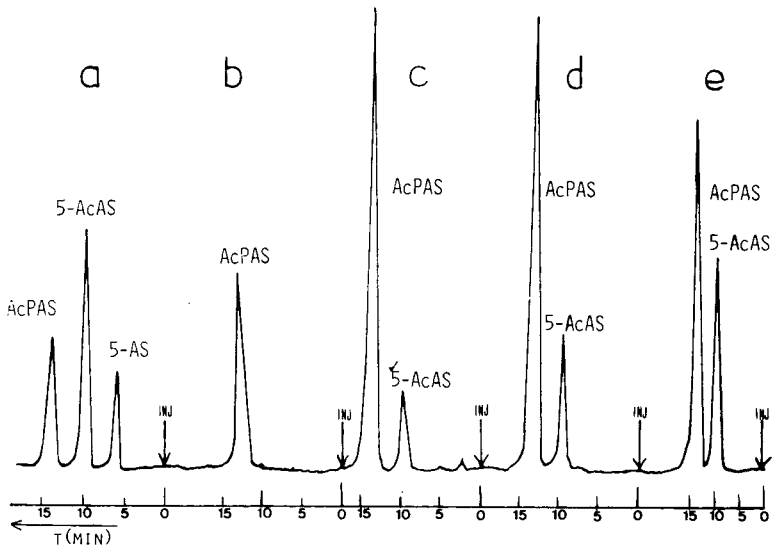

Fig. 1. HPLC separation of (a) 50 ng each of 5AS, 5AcAS and AcPAS, injected directly into the HPLC system; (b) extract of 500 μl of blank plasma spiked with internal standard; (c, d) extract of 500 μl of blank plasma spiked with 1 μg of 5AS, 0.1 μg of 5AcAS and 1 μg of AcPAS, without (c) and with (d) acetylation procedure; (e) extract of plasma of patient treated with 3 g of SASP and spiked with internal standard before acetylation and extraction procedure.

in poor yield by addition of an ion-pair reagent to the aqueous medium [12]. The major metabolite of 5AS, the N-acetylated 5AS, is more lipophilic and can be extracted easily from the acidified aqueous phase into organic solvents such as ethyl acetate, dichloromethane or diethyl ether. In addition, 5AcAS possesses better fluorescence characteristics than 5AS. We used these favourable properties of 5AcAS to obtain a more sensitive detection.

The N-acetylated isomer of 4-aminosalicylic acid (AcPAS) was added as internal standard prior to the acetylation procedure. Since only the 5AcAS has to be extracted no addition of an ion-pair reagent is necessary. In contrast to dichloromethane, no interfering peaks appear after HPLC separation, and the extraction yield is increased by a factor of 2 if diethyl ether is used as solvent.

Comparing peak heights after direct injection into the HPLC system, 5AcAS is extracted in 50% yield into diethyl ether. This result was achieved with two different concentrations (0.1 and 0.4 μg/ml). The recovery is relatively low but the achieved sensitivity (lower limit 0.02 μg/ml) is sufficient for plasma level monitoring.

Some typical HPLC chromatograms are reproduced in Fig. 1. By injecting 50 ng each of 5AS, 5AcAS and AcPAS directly into the HPLC system, three well-separated peaks appear with retention times of 5.8, 9.6 and 13.8 min, respectively. The peak heights indicate about a two-fold higher fluorescence sensitivity of 5AcAS than 5AS. Fig. 1c (without acetylation) and Fig. 1d (with acetylation) show the chromatograms obtained after extraction of 500 μl of blank plasma spiked with 0.1 μg of 5AS, 0.1 μg of 5AcAS and 1 μg of AcPAS. Only the acetylated compounds are extracted. No interfering peaks can be seen (Fig. 1b) if blank plasma is used.

TABLE II

ACCURACY AND PRECISION OF THE MEASUREMENT OF 5AS AND 5AcAS IN PLASMA

Spiked concentration μg/ml		Acetylation procedure	n	Measured concentration (mean ± S.D. μg/ml 5AcAS)	
5AS	5AcAS				
0.4	0.4	—	6	0.40	± 0.02
0.4	0.4	+	6	0.80	± 0.03
0.8	0.8	—	6	0.80	± 0.02
0.8	0.8	+	6	1.59	± 0.05
0.4	—	+	5	0.40	± 0.01
1.6	—	+	6	1.60	± 0.05
0.1	0.1	—	8	0.10	± 0.01
0.1	0.1	+	8	0.20	± 0.01

The HPLC system involves a ternary solvent system (aqueous phase, methanol, acetonitrile). Using an ion-pair reagent like trimethylcetylammonium bromide we observed a decrease in resolution after several injections.

5AcAS is quantified by measuring its peak heights related to those of the internal standard AcPAS. Standard curves are constructed after analysis of plasma or urine samples containing known amounts of 5AS and 5AcAS with and without the acetylation procedure. The standard curve is linear from 0.02 to 8 μg/ml.

Table II demonstrates the accuracy and precision for different concentrations of 5AS and 5AcAS. The lower detection limit depends on several parameters such as the mobile phase and the fluorescence monitor used. In our system we can measure 0.02 μg/ml 5AcAS either in the form of 5AcAS (without acetylation), or 5AS (with acetylation), or the sum of both.

Stability tests of 5AS and 5AcAS in plasma were performed at room temperature or at 4°C. After 7 days neither a decrease in the content of the plasma samples could be detected nor did additional peaks appear.

The application of the method to patients (see Fig. 1e) treated with 3 g of SASP revealed very low plasma levels of both 5AS and 5AcAS. The trough steady-state plasma concentrations of six patients ranged between 0.04 and

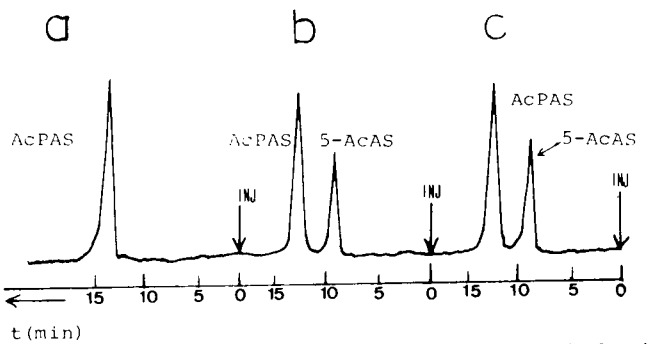

Fig. 2. HPLC chromatograms of urine samples. (a) Blank urine, diluted 1 : 1000, spiked with internal standard; (b, c) urine samples without (b) and with (c) acetylation procedure of a patient receiving 1.5 g of 5AS per day.

0.34 μg/ml 5AS and between 0.2 and 1.4 μg/ml 5AcAS. Plasma levels of patients receiving 1.5 g/day 5AS as suppositories fluctuated between 0.1 and 0.4 μg/ml 5AS and between 0.1 and 1.4 μg/ml 5AcAS.

In the urine of a patient receiving 1.5 g of 5AS in the form of suppositories we could detect only the acetylated metabolite. No difference between the acetylated and non-acetylated urine sample was seen (Fig. 2). No other peaks appeared in the HPLC chromatogram than those of 5AcAS and the internal standard. The extraction of a 100—1000-fold diluted 10-μl aliquot of an 8-h urine collection resulted in good measurable peaks.

DISCUSSION

5AS is determined indirectly in the form of its acetylated derivative 5AcAS to improve the detection limit. Samples run without the acetylation procedure will provide the endogenous 5AcAS concentration, whereas acetylated samples result in the sum of acetylated 5AS and 5AcAS; the difference between the two values will give the 5AS moiety. The plasma levels of patients receiving therapy with SASP or 5AS suppositories can be monitored by the extraction of two samples of 500 μl of plasma, one with and one without the acetylation procedure.

The ternary solvent system offers some advantage over the use of a mobile phase containing the ion-pair reagent trimethylcetylammonium bromide: quicker equilibration of the column, longer lifetime of the column, better separation of the peaks and lower cost of material. The use of a precolumn is recommended to obtain better separation and to prolong the life-span of the analytical column. Decreasing the percentage of both organic solvents from 12.5 to 7.5% in the mobile phase prolongs the retention times. This might be of advantage when interfering peaks are to be expected.

Data from Khan et al. [13] suggest that aspirin interferes with the photometric assay of Hannson and Sandberg [11]. In our more specific method this often used drug was well resolved from 5AS and its acetylated metabolite, indicating no interferences with the described method.

In conclusion, our method can be used for plasma level monitoring of 5AS, which might be helpful in guiding therapy in patients with ulcerative colitis or Crohn's disease.

ACKNOWLEDGEMENTS

We are greatly indebted to Miss P. Kopp and Mr. F. Schönberger for their helpful technical assistance.

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Note

Determination of etozolin and ozolinone in human plasma and tissues by reversed-phase high-performance liquid chromatography

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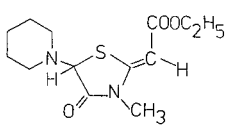
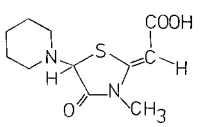
Etozolin [ethyl(*Z*)-(3-methyl-4-oxo-5-piperidino-thiazolidin-2-ylidene)acetate (I) is a novel diuretic drug that is rapidly metabolized by hydrolysis of the ester group to give the free acid ozolinone, *Z*-(3-methyl-4-oxo-5-piperidino-thiazolidin-2-ylidene)acetic acid (II). Ozolinone itself exhibits a high pharmacological activity and has a longer half-life in man than etozolin, so that an estimation of the concentration of both I and II is of interest in pharmacological investigations [1]. A thin-layer chromatographic (TLC) method [2,3] and a high-performance liquid chromatographic (HPLC) method [4] for the estimation of I and II have been reported. The HPLC method was reported to be more sensitive and accurate and less prone to interference than the TLC method [4].



I



II

In the HPLC method, adsorption chromatography on a LiChrosorb Si-100 column using cyclohexane—chloroform mixtures for elution was described. Because of the cost of the elution mixtures, the toxicity of chloroform and the limited life-span of the column used, we have chosen to develop a reversed-phase HPLC method.

EXPERIMENTAL

Compounds I and II, ethyl(*Z*)-(3-ethyl-4-oxo-5-piperidino-thiazoldin-2-ylidene) acetate (internal standard I), and (*Z*)-(3-ethyl-4-oxo-5-piperidino-thiazolidine-2-ylidene)acetic acid (internal standard II), were generously donated by Gödecke, Freiburg, G.F.R. Methanol was LiChrosolv, other chemicals were of analytical grade and obtained from E. Merck (Darmstadt, G.F.R.).

HPLC measurements were made using a Perkin-Elmer Model 2/2 liquid chromatograph equipped with a Model LC 75 UV detector and a Rheodyne 7105 valve. Stainless-steel columns (25 cm × 4 mm, Knauer, Berlin, G.F.R.) were packed with LiChrosorb RP-18, 7 μm (Merck) and fitted with a pre-column (4 cm × 4 mm) filled with the same material.

Plasma (1—2 ml) was selectively extracted with dichloromethane according to the method of Hengy et al. [4], to give two extracts for each plasma sample. Extract I was obtained from alkalinized plasma and contained I and internal standard I. Extract II was obtained from reacidified plasma and contained II and internal standard II.

Tissue was homogenized with four times its own weight of triple-distilled water using a Polytron homogenizer. Five millilitres of homogenate were then extracted in a similar manner to plasma.

Aliquots of 3—10 μl of extract I were injected on to the HPLC column and eluted isocratically with 65% methanol in 20 m*M* phosphate buffer at pH 2.2 (Fig. 1). Aliquots of 3—10 μl of extract II were injected on to the HPLC column and eluted isocratically with 40% methanol in 20 m*M* phosphate buffer at pH 2.2 (Fig. 2). Calibration curves (Fig. 3) were prepared each day by adding I and II to drug-free plasma or tissue homogenates and handling these in the manner outlined above. Peak height ratios were used in all determinations. The UV detector was operated at 282 nm for the analysis of both I and II.

RESULTS AND DISCUSSION

Although I and II may be analyzed by reversed-phase HPLC in one run using gradient elution, such a procedure could not be used for the assay of low drug concentrations in tissues (below 100 ng/g) due to noisy baselines and interfering peaks. By utilizing the selective extraction of I and II and their respective internal standards and analyzing for I and II separately using reversed-phase HPLC and isocratic elution, the sensitivity of the assay could be greatly increased and, even more importantly, such procedures were applicable to the analysis of low drug levels in tissues. Strictly linear calibration curves (Fig. 3) were obtained for the estimation of I and II in the concentration ranges from 30 ng/ml to 10 μg/ml. The lower limit of detection for both I and II was approximately 10 ng/ml. The regression coefficients of the calibration curves always exceeded 0.99 for both I and II. The relative standard deviation of the method, as determined by analysis of multiple serum samples containing 1 μg/ml, was between 4 and 6% for both I and II.

The use of pre-column, which was normally changed after approximately 200 injections, effectively protected the column from damage. A column in everyday use had an average lifetime of more than 4 months.

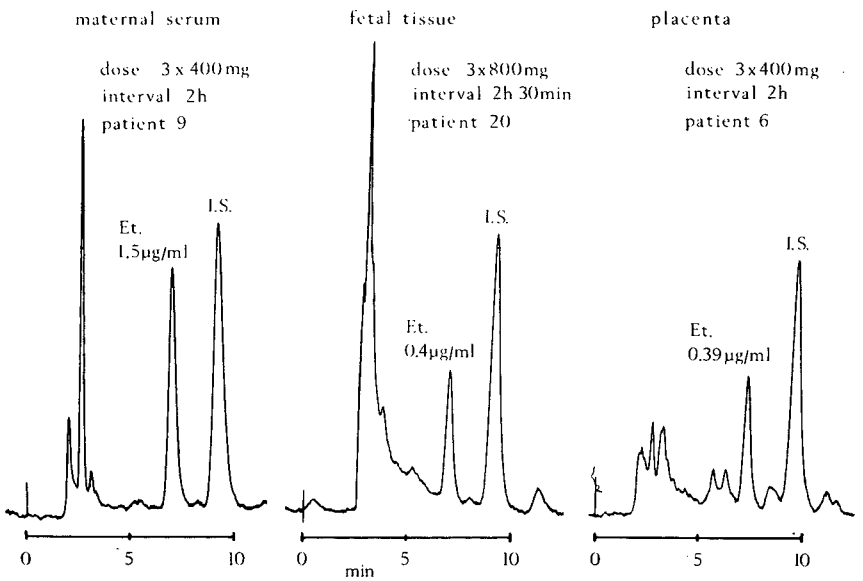


Fig. 1. Analysis of etozolin (Et.) in human serum, placenta and fetal tissue. Solvent: 65% methanol in 20 m*M* phosphate buffer (pH 2.2). I.S. = internal standard I; interval = time between the dose and interruption of pregnancy by curettage (first trimester).

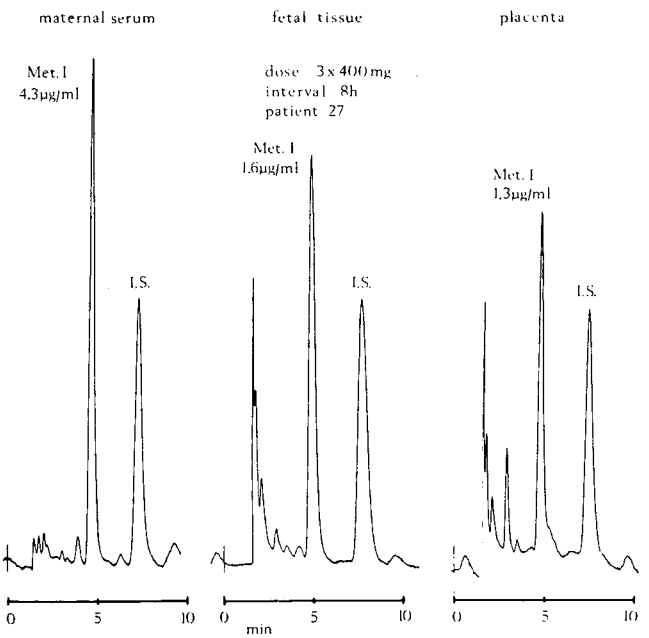


Fig. 2. Analysis of ozolinone (Met. I) in human serum, placenta and fetal tissue. Solvent: 40% methanol in 20 m*M* phosphate buffer (ph 2.2). I.S. = internal standard II; interval = time between the dose and interruption of pregnancy by curettage (first trimester).

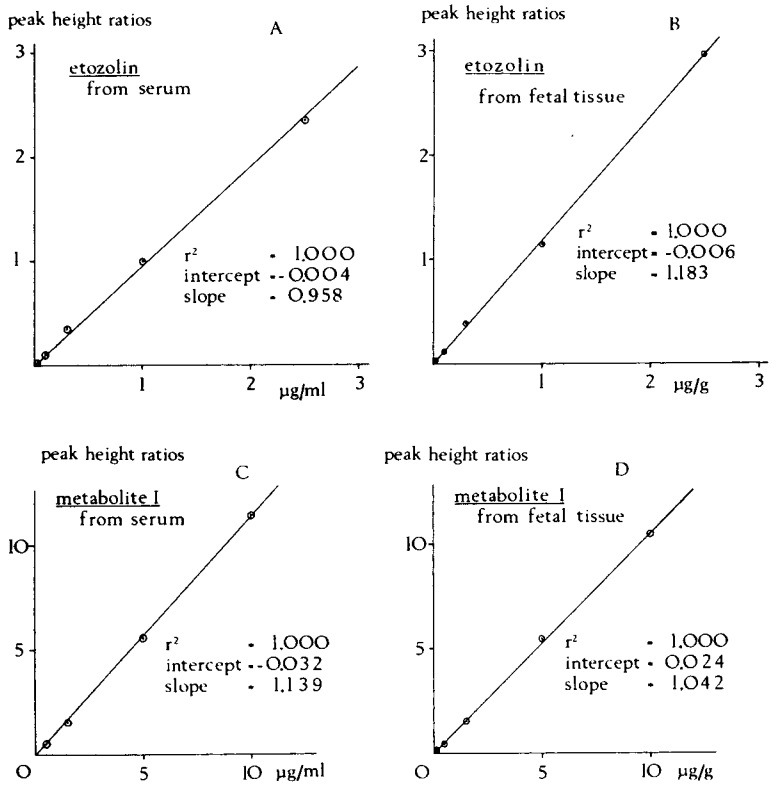


Fig. 3. Plots of peak height ratios (A and B: etozolin/I.S. I; C and D: ozolinone/I.S. II) versus amounts of etozolin and ozolinone added to serum (A and C) and tissue homogenates (B and D).

In spite of the simplicity of the assay procedures developed, I and II could be determined in small samples of blood and, without modification, also in tissue homogenates following therapeutic doses of etozolin (Figs. 1 and 2). Low-cost columns (see Experimental) with theoretical plate numbers of 8000—12,000/m are sufficient for all analytical problems encountered so far.

The method described here has been in continual use in our laboratory for more than one year for the investigation of the placental transfer of I and II during the first and second trimester of human pregnancy. Both I and II were found in fetal and placental tissue in lower concentrations than in maternal serum (fetal tissue/maternal serum ratios of between 0.3 and 0.6). Also, the drugs were distributed rather evenly within the human fetal compartment. Drug concentrations in the blood of pregnant patients were lower than in non-pregnant patients (unpublished results).

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Note

Dosage de l'acide dipropylacétique dans le plasma par chromatographie en phase liquide et détection spectrofluorimétrique

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(Reçu le 29 décembre 1980; manuscrit modifié reçu le 8 mai 1981)

L'acide dipropylacétique (DPA) ou acide valproïque, sous forme de sel de sodium est utilisé en thérapeutique pour ses propriétés anticonvulsivantes, le plus souvent en association avec le phénobarbital. Son dosage dans les milieux biologiques fait appel à des méthodes chromatographiques.

De nombreuses techniques de dosage en chromatographie en phase gazeuse (GLC) ont été décrites. Elles procèdent soit par injection directe, soit par dérivatisation [1—14]. Les principaux problèmes rencontrés et différemment résolus par les auteurs, étant liés aux pertes d'acide dipropylacétique lors de la phase finale de concentration et à l'adsorption de la molécule sur les supports chromatographiques.

Le développement et les facilités d'utilisation de la chromatographie en phase liquide (HPLC) au laboratoire ont été à l'origine de la mise au point d'un certain nombre de dosages.

Cependant, la faible absorbance du DPA dans l'ultra-violet nécessite en HPLC, soit une détection colorimétrique en sortie de colonne, basée sur ses propriétés acides [15], soit une détection dans l'ultra-violet après formation d'esters [16—19]. Afin d'augmenter la sensibilité du dosage par HPLC, nous proposons une méthode rapide et spécifique avec une détection fluorimétrique. Elle consiste en la formation d'un ester fluorescent avec la bromomethyl-4-methoxy-7-coumarine (BrMmC). L'étalon interne utilisé est l'isomère linéaire du DPA: l'acide caprylique. Cette technique est utilisable en routine pour l'évaluation des taux plasmatiques de DPA lors des traitements.

MATÉRIEL ET MÉTHODE

Appareillage

Le chromatographe en phase liquide utilisé est constitué d'une pompe Chromatem 380 (Touzart et Matignon), d'un injecteur à boucle (20 μl) Rhéodyne 7125.

Le détecteur est un spectrofluorimètre Jobin-Yvon JY 3D équipé d'une microcuve à circulation de 20 μl.

La colonne (15 cm × 9 mm I.D.) est remplie de Sphérisorb ODS (5 μm) (Touzart et Matignon, Paris, France) selon la méthode de Coq et al. [20].

Le chromatographe en phase gazeuse est un Girdel 3000 équipé d'un détecteur à ionisation de flamme. La colonne en inox (2 m × 2 mm I.D.) est chauffée à 140°C, l'injecteur à 160°C et le détecteur à 170°C.

Le débit d'azote (gaz vecteur) est de 30 ml/min. La phase stationnaire est constituée de 10% de butanediol succinate et 3% d'acide phosphorique sur GasChrom Q 80—100 mesh (Girdel, Rueil-Malmaison, France).

La chromatographie semi-préparative est réalisée sur des plaques de Kieselgel 60F 254 d'une épaisseur de 2 mm (Merck, Darmstadt, R.F.A.).

Réactifs

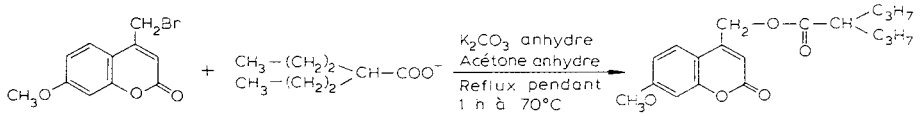
Bromométhyl-4-méthoxy-7-coumarine: Regis Chemical Co. (Morton, Grove, IL, É.U.). 18 Crown-6-éther: Aldrich (Milwaukee, WI, É.U.). Acide caprylique: Applied Science Labs. (State College, PA, É.U.). Ethanol, méthanol, acétone, acétonitrile, éther (Normapur): Prolabo (Paris, France). Acide perchlorique 70%: Prolabo. Dipropylacétate de sodium: Laboratoires Labaz, Bordeaux, France.

Étalons

Solution de bromométhyl-4-méthoxy-7-coumarine à 2 g/l dans l'acétone. Solution d'acide caprylique à 1 g/l dans l'acétonitrile. Solution aqueuse de dipropylacétate de sodium à 1.16 g/l (correspondant à 1 g/l de DPA). Solution de 18 crown-6-éther à 1 g/l dans l'acétone. Acide chlorhydrique 5 *N*. Soude méthanolique 0.05 *N*.

Préparation de l'ester de référence

L'ester de référence a été préparé selon la méthode proposée par Dünges et al. [21]. La schéma de la réaction est le suivant:



Les rapports molaires sont de 1 *M* de DPA pour 3 *M* de BrMmC. La solution acétonique est filtrée. Le filtrat est évaporé à sec et recristallisé plusieurs fois dans l'éthanol. La présence d'impuretés nécessite cependant une purification par chromatographie préparative sur gel de silice. Le solvant de migration est un mélange hexane—chloroforme (3:8, v/v). L'identité des cristaux obtenus a été déterminée par spectrophotométrie infra-rouge et par résonnance magnétique nucléaire.

Méthode d'extraction et de dérivatisation

Après acidification par 100 µl de HCl 5 *N* et addition de 75 µl de la solution d'étalon interne, 0.5 ml de plasma sont extraits par 10 ml d'éther. La phase éthérée est desséchée sur du sulfate de sodium anhydre puis récupérée dans des fioles coniques. Après addition de 0.5 ml de solution de soude méthanolique 0.05 *N* et de 2 mg de carbonate de potassium anhydre, la phase organique est évaporée à sec. La fiole est entourée d'un film de papier aluminium. Au résidu sont ajoutés 420 µl de BrMmC et 100 µl de solution de 18-crown-6-éther. Un réfrigérant à air est adapté sur la fiole. L'ensemble est ensuite porté au bain-marie à 70°C pendant 15 min. La phase organique est récupérée, évaporée à sec. Le résidu est repris par 100 µl de méthanol.

Chromatographie en phase liquide

Vingt µl du résidu sont injectés dans le chromatographe. La phase mobile est constituée d'un mélange méthanol—eau—$HClO_4$ (70:30:0.05, v/v). Le débit est maintenu à 2 ml/min. Les longueurs d'onde d'excitation et d'émission de fluorescence sont respectivement de 336 et 408 nm. Les concentrations sont calculées à partir des rapports de hauteurs de pic en référence à une courbe d'étalonnage. Cet étalonnage est réalisé par extraction et dérivatisation de plasma surchargé par des quantités croissantes de dipropylacétate de sodium (de 1 à 150 mg/l d'acide dipropylacétique) et par une même quantité d'étalon interne.

RÉSULTATS

La Fig. 1A représente le chromatogramme du plasma d'un sujet traité par du DPA sodique. On constate, par rapport au plasma d'un sujet non traité (Fig. 1B), que les constituants normaux n'interfèrent par sur le tracé.

Le DPA n'absorbant pas dans l'ultra-violet, le rendement de l'extraction par l'éther a été étudié par chromatographie en phase gazeuse. Il est de 95.7 ± 9.7%.

La reproductibilité de la méthode déterminée par l'analyse de 10 échantillons surchargés donne des coefficients de variation de 8.2% pour 50 mg/l, 6.4% pour 75 mg/l et 5.8% pour 100 mg/l.

La linéarité de la méthode pour des concentrations de 0 à 1.04 mmol/l (0 à 150 mg/l) est satisfaisante ($y = 0.01x + 0.01$, $r = 0.999$).

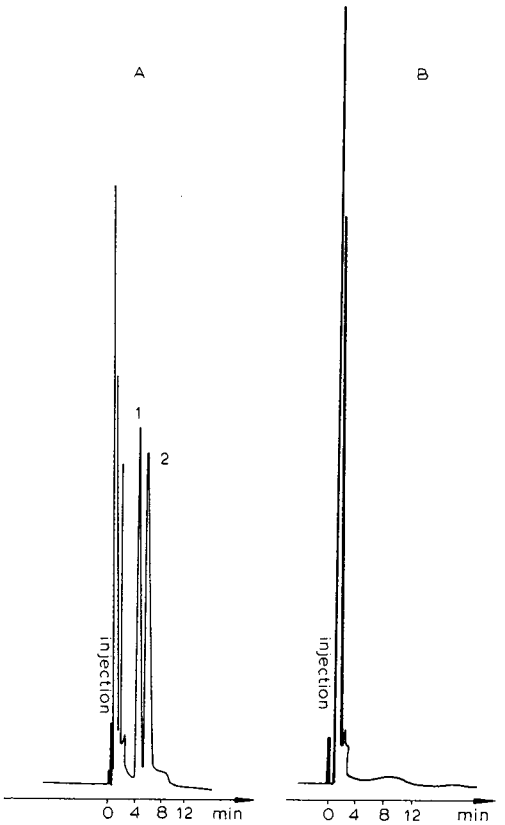


Fig. 1. (A) Chromatogramme d'un plasma humain. (1) DPA-MmC (107 mg/l) en DPA, temps de rétention = 4.6 min. (2) Ester coumarinique de l'acide caprylique, temps de rétention = 6.7 min. (B) Chromatogramme du plasma d'un sujet non traité.

La limite de sensibilité déterminée est de 0.5 mg/l, soit 0.75 μmol injecté dans un volume de 20 μl.

DISCUSSION

Le spectre de fluorescence de l'ester DPA-Mmc a été étudié dans les solvants classiquement utilisés en chromatographie liquide en polarité de phase inversée: l'eau, le méthanol, l'acétonitrile. Quelle que soit la nature du solvant, les maxima d'excitation et d'émission sont peu modifiés (Fig. 2): $\lambda_{exc.}$ = 336 nm, $\lambda_{ém.}$ = 408 nm. Cependant, comme le montre le Tableau I, la fluorescence est inhibée dans l'acétonitrile. Des phases méthanol—eau sont essentiellement retenues et les sensibilités optimales sont atteintes avec un mélange ayant une teneur en eau voisine de 60%. Les variations de pH (1 à 5) et de force ionique (0.04 à 0.5) ont peu d'influence sur l'intensité de la fluorescence. D'autre part, il est observé que celle-ci, pour une solution méthanolique conservée à 4°C à l'obscurité est stable pendant quinze jours.

La cinétique de la réaction d'estérification en présence de 18-crown-6-

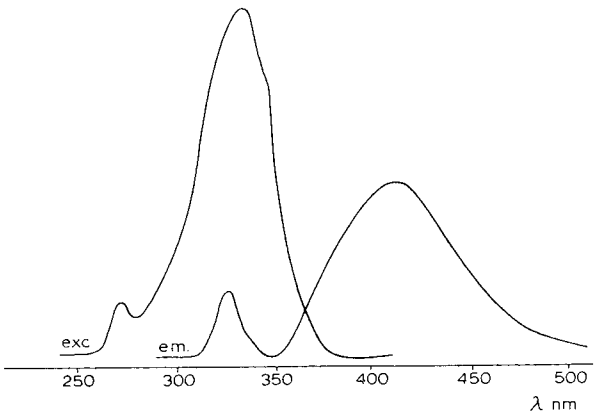


Fig. 2. Spectre de fluorescence du DPA-MmC dans le mélange eau—methanol (30:70, v/v).

TABLEAU I

INTENSITÉ RELATIVE DE FLUORESCENCE DU DPA-MmC DÉTERMINÉE PAR RAPPORT AU DÉRIVÉ DIMÉTHOXY-5,7-COUMARINE-4-ACÉTIQUE (CONCENTRATION $2 \cdot 10^{-6}$ *M*), EN FONCTION DE LA NATURE DU SOLVANT

Solvant	Intensité relative de fluorescence
Eau	0.42
Méthanol	0.13
Acétonitrile	0
Eau—méthanol (20:80)	0.25
Eau—méthanol (40:60)	0.37
Eau—méthanol (60:40)	0.45
Eau—méthanol (80:20)	0.39

éther et de K_2CO_3 a été déterminée pendant une heure à des températures de 30, 50 et 70°C. La Fig. 3 montre que les conditions optimales sont réalisées pour une température de 70°C pendant 15 min. Dans ces conditions, un rapport molaire 1 *M* DPA et 2 *M* BrMmC est suffisant pour l'obtention d'une réaction totale. Par ailleurs, l'évaporation de la phase éthérée avant estérification n'occasionne aucune perte d'acide dipropylacétique puisqu'il se trouve sous la forme de sel de sodium.

Compte-tenu des données de la littérature et de la faible polarité des esters obtenus, la chromatographie en polarité de phase inversée semble être la méthode de choix, qui permet une bonne séparation des deux isomères. Le choix des conditions chromatographiques a été en partie imposé par les résultats de l'étude de fluorescence. Le Tableau II rapporte les valeurs des facteurs de capacité du DPA-MmC et de l'ester coumarinique de l'acide caprylique, en fonction de la polarité de la phase mobile eau—méthanol et du pH.

L'augmentation de la teneur en méthanol diminue les k' ainsi que la sélectivité, alors que la diminution du pH, si elle diminue les k', semble sans effet sur la sélectivité. Compte-tenu de la nécessité d'un temps d'analyse court, le

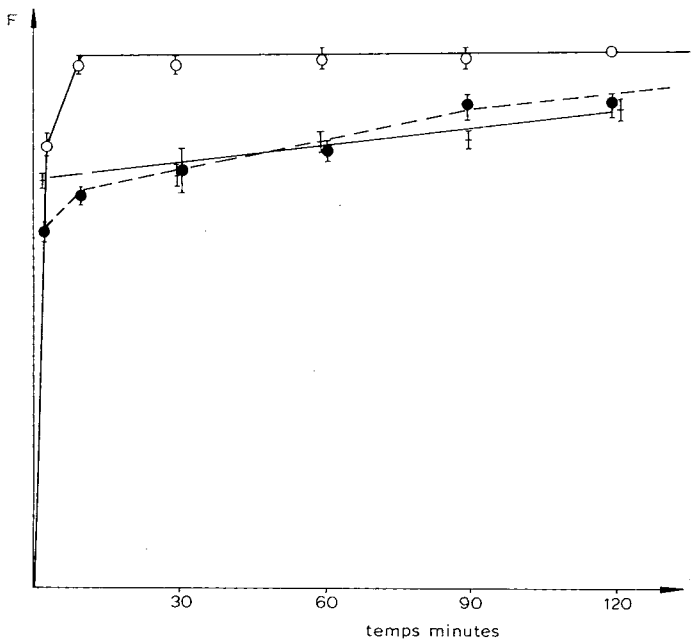


Fig. 3. Cinétique de la réaction d'estérification pour différentes températures: (—) 30°C, (•) 50°C, (○) 70°C. Rapport molaire BrMmC/DPA = 3:1.

TABLEAU II

VARIATIONS DES FACTEURS DE CAPACITÉ k', DES DÉRIVÉS COUMARINIQUES DU DPA ET DE L'ACIDE CAPRYLIQUE EN FONCTION DE LA POLARITÉ DE LA PHASE MOBILE ET DE SON pH

	k'								
	Eau—méthanol (v/v)*					pH apparent**			
	35:65	30:70	25:75	20:80	15:85	1	1.7	2.2	2.8
DPA-MmC	4.9	4.0	3.6	1.9	1.2	1.7	3.1	4.0	5.1
Acide caprylique-MmC	7.1	5.5	4.7	2.5	1.6	3.3	4.2	5.5	6.3

*pH apparent = 2.2.
**Eau—méthanol (30:70, v/v).

mélange eau—méthanol (30:70, v/v) et un pH apparent de 2.2 permet une bonne séparation des deux esters considérés.

CONCLUSION

La méthode proposée s'est révélée simple, rapide, fiable et utilisable en routine. L'emploi de la 18-crown-6-éther comme catalyseur permet l'obtention d'une estérification totale en 15 min. La comparaison des résultats avec ceux obtenus en GLC selon la méthode de Pfaff et Mahuzier [2] donne pour

22 extraits surchargés une corrélation de 0.961. La détection fluorimétrique permet d'atteindre une grande sensibilité (0.5 mg/l) et celle-ci a pu être améliorée (0.1 mg/l) en utilisant un fluorimètre adapté à la HPLC (cellule de 8 μl). La détection des esters coumariniques est également possible, avec une sensibilité moindre, dans l'ultra-violet à 320 nm. De plus, comme l'a montré Dünges et Seiler [18], la bromométhyl-4-méthoxy-7-coumarine est susceptible d'alkyler des barbituriques et nous étudions au laboratoire un dosage simultané de plusieurs anticonvulsivants traités par ce réactif.

REMERCIEMENTS

Nous remercions Monsieur le Professeur Mahuzier pour l'intérêt qu'il a porté à notre travail.

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Note

Determination of a novel fluoropyrimidine, 5′-deoxy-5-fluorouridine, in plasma by high-performance liquid chromatography

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(First received January 6th, 1981; revised manuscript received May 22nd, 1981)

5′-Deoxy-5-fluorouridine (5′-dFUR, Ro21-9738) is a fluoropyrimidine recently synthesized by Cook et al. [1] with antineoplastic activity. The therapeutic potential has been tested both in vitro and in vivo against several rat and murine tumour lines [2—4]. Up to now, only isotopic methods using labelled drugs with high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have been described in the literature [5, 6]. Also, it appeared of interest to develop a new sensitive and specific HPLC method not requiring labelled substances and utilizable in phamacokinetic studies.

EXPERIMENTAL

Apparatus and operating conditions

A high-performance liquid chromatograph (Hewlett-Packard 1084B) was equipped with automatic injector, variable-wavelength spectrophotometer and chromatograph terminal (Hewlett-Packard 79850 ALC). Detection was performed at 269 nm. The column used was LiChrosorb RP-18 (5 μm), 125 mm × 4 mm I.D. (E. Merck, Darmstadt, G.F.R.).

The mobile phase was of water—methanol—acetonitrile (97:1.5:1.5) with a flow-rate of 1 ml/min. After degassing, the mobile phase was maintained at a temperature of 80°C for the water and at 40°C for the mixture methanol—acetonitrile.

A mass spectrometer, Model 5980A, with data system 5934A (Hewlett-Packard) was also used to establish identity and purity of the 5′-dFUR HPLC peak.

Reagents

Methanol (HPLC grade; Merck), acetonitrile (HPLC grade; Carlo Erba, Milan, Italy), diethyl ether (analytical grade; Solvant Documentation Synthèse, Valbonne, France), acetic acid 99.7% (analytical grade; Riedel de Haën, Hannover, G.F.R.) and isopropanol (analytical grade; Prolabo, Paris, France) were used without further purification. Double-distilled water was filtered through a 0.22-μm pore membrane filter (Millipore, Bedford, MA, U.S.A.).

The stock solutions of 5-dFUR (Ro21-9738) and 3-methylxanthine (No. 69772; Fluka, Buchs, Switzerland) were prepared in water at 100 μg per 100 μl and 10 μg per 100 μl, respectively. The same solvent was used for standard solutions.

Operating procedures

The blood samples were collected in oxalated tubes (Venoject T 200 × F 105) and then centrifuged for 15 min at 2400 *g*. The plasma should then be immediately frozen until analysis.

Place 50—100 μl of an internal standard solution (in the range 20—0.2 μg/ml) into a 10-ml cylindro-conical centrifuge tube. The concentration used depends on the level of 5'-dFUR to be analysed in the sample. Add 0.2—1 ml of plasma

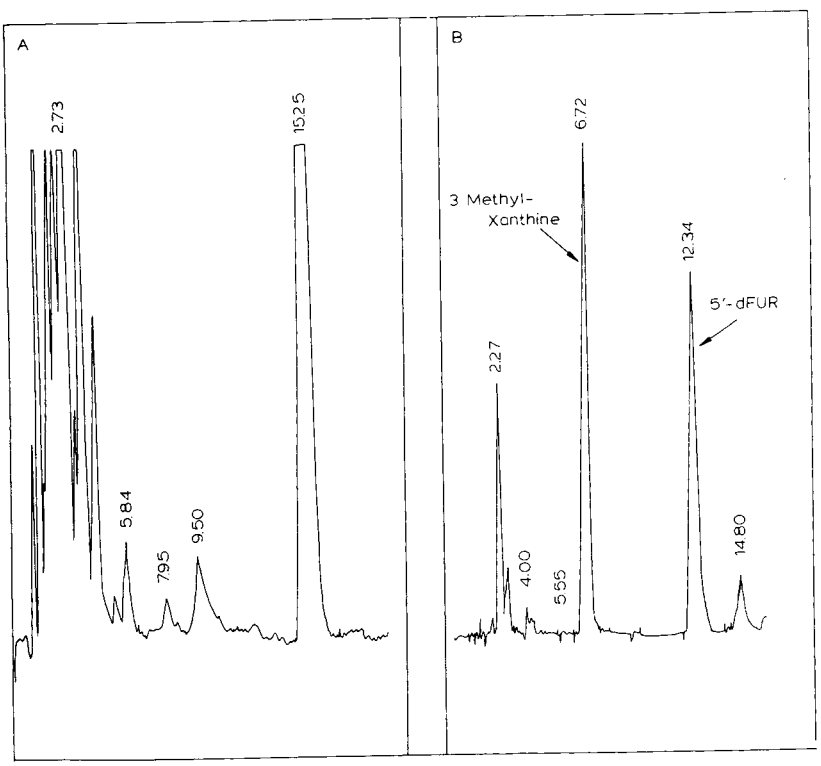

Fig. 1. (A) Plasma control after extraction. (B) Chromatogram of a patient's plasma containing 19 μg/ml of 5'-dFUR with 3-methylxanthine as internal standard (10 μg/ml).

and mix on a vortex-type mixer for a few seconds. Add 1 ml of a 0.3 *M* solution of acetic acid in methanol and mix for about 20 sec to obtain a homogeneous mixture. Place the tube in a water-bath (100°C) for 1 min and during this time mix the contents twice for 2 sec. Cool the tube with ice and centrifuge at 2800 *g* for 20 min. Collect the supernatant, add 20 ml of the diethyl ether—isopropyl alcohol mixture (8:2) and mix the contents vigorously; then agitate mechanically for 30 min. Centrifuge at 2800 *g* for 20 min. Recover the organic phase, evaporate to dryness at 45°C under a stream of nitrogen and re-dissolve the residue in 50—100 μl of water while vortex-mixing for 15 sec. Then, inject 10—25 μl of this solution into the chromatograph for analysis.

Under the conditions defined above, the retention times for internal standard (I) and 5′-dFUR (II) were 6.72 min and 12.34 min, respectively. In Fig. 1 are presented chromatograms of extracts of control plasma (A) and plasma from a patient (B).

Standard calibration curves (ratios of the 5′-dFUR to internal standard peak areas versus concentrations of 5′-dFUR) were obtained after analysis of plasma samples to which increasing quantities of 5′-dFUR [either (a) 0.05—1 μg/ml, or (b) 1—20 μg/ml] were added together with a constant quantity of internal standard [either (a) 0.22 μg/ml, or (b) 2.2 μg/ml]. The following values were obtained for the regression curves: (a) $y = 2.835x - 0.018$, and (b) $y = 0.313x - 0.058$. Both have a correlation coefficient of 0.999.

RESULTS AND DISCUSSION

Internal standard

3-Methylxanthine was chosen as internal standard. This compound is not structurally similar to 5′-dFUR, but its maximum absorption wavelength (272 nm) and its percentage recovery were nearly the same. (The recovery relative of 5′-dFUR to the internal standard was about 90%.) In addition, 3-methylxanthine is neither a drug nor a metabolite of 5′-dFUR. Although 3-methylxanthine is not used as a therapeutic agent, it is a metabolite of both caffeine and theophylline. Therefore, a control plasma sample is examined prior to each pharmacokinetic study to make sure that the retention times corresponding to compounds I and II are free from any possible interference.

Specificity

The maximum absorbance of 5′-dFUR occurred at 269 nm with the instruments used. Each morning the column was conditioned by flushing for 1 h with water—acetonitrile—methanol (90:5:5) at a rate of 1 ml/min followed by the analytical mobile phase at 1 ml/min for 30 min.

No interference from such compounds as uric acid, 5-fluorouracil, 5-fluorouridine, 5-fluoro-2′-deoxyuridine, thymine, thymidine, uracil and uridine was found. However, some samples of plasma, in this study, presented an unknown interference and the complete baseline separation of this peak and 5′-dFUR was only possible by changing the percentage composition of the eluting solvent. Thus, the percentage of methanol—acetonitrile in the mobile phase was decreased from 3% to 2.5% or 2.2%.

Plasma from a patient was checked using mass spectrometry and it was shown that the HPLC peak with a retention time of 12.34 min could be attributed to 5′-dFUR itself. This study was carried out in the chemical ionization mode using 50 eV ionisation energy, an emission current of 200 μA, source temperature of 180°C and pressure of 0.5—1 Torr. Under these conditions, 3 μl of a methanol standard solution containing 10 μg per 100 μl were injected into the mass spectrometer via the direct insertion probe, heating progressively until 250°C. The mass spectrum of 5′-dFUR showed a small amount of $[M^+ + H]$ at m/e = 247 (relative intensity = 1.5%), an abundant m/e = 117 corresponding to the sugar fragment (relative intensity = 100%), and a peak at m/e = 131 corresponding to the base, 5-fluorouracil, plus hydrogen arising from the loss of the sugar fragment from 5′-dFUR (relative intensity = 30%). These results, excluding the relative intensity of the fragments, were in agreement with those obtained for parent compounds, such as adenosine [7].

Thus, the drug-containing fraction of a patient plasma was collected and evaporated completely at 45°C under a stream of nitrogen. The residue was dissolved in 100 μl of methanol and 3 μl of this solution were analysed by direct probe chemical ionization mass spectrometry. Under the analysis conditions defined above, mass spectral fragments identical to those of the standard solution were obtained.

Extraction procedure

The performance of the proposed method was influenced by the extraction pH and the nature of the solvents. The best results were obtained using a mixture of methanol—acetic acid (0.3 *M*) at pH 3.4, which precipitated serum proteins (and reduced the significance of the interferences), and performing the extraction with the ether—isopropanol mixture (8:2). Under these conditions, the percentage recovery for 5′-dFUR quantities between 0.05 and 20 μg/ml was approximately 95%.

Sensitivity, reproducibility and accuracy

Similar, for routine assays, the quantitative limit of sensitivity was about 50 ng/ml plasma. Repeatability was investigated by analysing a plasma pool containing 1 μg of 5′-dFUR and 2 μg of internal standard per ml. The coefficient of variation (s = 0.95) within tests, for 10 successive extractions and assays was 4%.

Application

The proposed technique was used to carry out a pharmacokinetic investigation of 5′-dFUR after intravenous infusion. We studied the decrease with time of 5′-dFUR plasma levels in a pancreatic carcinoma patient with liver metastasis, who had received 1.5 g by short infusion (20 min). The drug disappeared from the plasma rapidly and no measurable residues were present after 2 h (Fig. 2).

A preliminary pharmacokinetic approach showed that this substance appeared to follow a two-compartment open model. For this patient, the pharmacokinetic parameters of elimination half-life and plasma clearance were 10.89 min and 0.879 l/min, respectively.

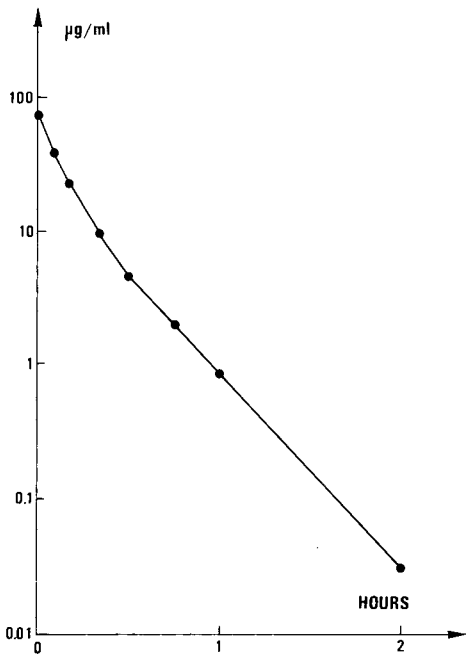

Fig. 2. 5′-dFUR plasma levels in a patient after continuous, 20-min intravenous infusion. Dose administered = 1.5 g.

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Note

Rapid liquid chromatographic determination of probenecid in human cerebrospinal fluid

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Probenecid [*p*-(di-*n*-propylsulfamyl)-benzoic acid] has been used to study the metabolic turnover of dopamine and serotonin in the central nervous system. Previous reports [1—6] have shown that the degree of the probenecid-induced accumulation of the acid metabolites [homovanillic acid (HVA) and 5-hydroxy-indoleacetic acid (5-HIAA)] and the standard dose concentration of probenecid vary considerably in the cerebrospinal fluid (CSF) of individual patients. Thus, the probenecid levels in CSF must be determined before any correlation between the increase of the acid metabolites and the CSF levels of probenecid can be established.

Probenecid concentrations in biological fluids have been measured by both fluorometric and radioenzymatic methods [5, 7]. The general problems associated with these methods were tedious sample preparation and relative nonspecificity. Gas chromatography coupled with electron-capture detection [8, 9] or with mass spectrometry [6] have also been employed to measure probenecid in CSF samples. Although these two techniques have the adequate sensitivity and specificity for the probenecid determination, they required the derivatization procedure prior to analysis. Recently, a technique utilizing high-performance liquid chromatography (HPLC) with UV detection has been described for the determination of probenecid in plasma and urine [10].

In the present paper we have modified this method for the measurement of probenecid in human CSF. Probenecid is isolated from acidified CSF with toluene extraction and then quantitated by liquid chromatography with UV detection at 245 nm. An internal standard is used for correction of the losses during the extraction procedure.

EXPERIMENTAL

Chemicals

Probenecid standard was obtained from Merck Sharp and Dohme (West Point, PA, U.S.A.). Internal standard [*m*-(diisobutylsulfamyl)benzoic acid (DSB)] was synthesized according to the procedure of Mieler [11]. Sodium octylsulfate was purchased from Pfaltz and Bauer (Stamford, CT, U.S.A.). All other reagents were of analytical grade. Water was deionized and then double-distilled in glass.

Apparatus

The liquid chromatograph was constructed from five components: Consta-Metric IIG solvent delivering system (Laboratory Data Control, Riviera Beach, FL, U.S.A.); Model 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.); Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.); a 25 cm × 4.6 mm reversed-phase column of 5-μm octadecyl silica (Bioanalytical Systems, West Lafayette, IN, U.S.A.); and a 3 cm × 4.6 mm guard column of 5-μm RP-18 (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase was prepared by mixing 540 ml of methanol, 260 ml of water, 9 ml of glacial acetic acid, and 750 mg of sodium octylsulfate. The flow-rate was fixed at 1.5 ml/min at ambient temperature. At the end of each day, the system was flushed with methanol—water (1:1, v/v) to prolong the lifetime of the column.

Procedure

Lumbar CSF samples were collected from patients administered probenecid orally (a total dose of 100 mg/kg body weight) at the Bronx Veterans Administration Medical Center, New York. The CSF was immediately frozen and stored at −80°C until analyzed.

To an aliquot of CSF (0.5 ml), 100 μl of 0.15 m*M* DSB and 0.3 ml of 6 *M* hydrochloric acid solution were added. The mixture was extracted twice with 3 ml of toluene and the combined extracts evaporated to dryness under a stream of dry nitrogen at 37°C. The drying procedure was carefully repeated after 1 ml methanol was used to wash the dried residue. The dried residue was dissolved in 200 μl of the mobile phase. Standard curves were prepared by plotting the relative height ratios ($H_{\text{probenecid}}/H_{\text{DSB}}$) in a series of samples containing a known quantity of DSB to which proportionate amounts of probenecid had been added. The free probenecid concentrations were calculated by inverse linear regression analysis. The total probenecid concentrations of five CSF samples were determined as described in the above section except CSF samples were first incubated with an aryl sulphatase + β-glucuronidase enzyme preparation (Type H-1, Sigma, St. Louis, MO, U.S.A.) in 1 *M* sodium acetate buffer (pH 6.2, 200 μl) at 37—38°C for 6 h prior to extraction in order to disassociate probenecid from its conjugate. A small volume (20 μl) of each CSF sample was also injected directly onto the column and the free probenecid level presented in the original sample was calculated from the peak height using a standard curve.

RESULTS AND DISCUSSION

Fig. 1 shows representative chromatograms of human CSF extracts. Identification of the probenecid and DSB peaks observed in CSF samples was based on retention times and cochromatography with the authentic compounds. Under the chromatographic conditions used, the variable-wavelength detector (245 nm) gave better detector responses for probenecid and DSB than the fixed-wavelength detector (254 nm). Therefore, an UV-absorbing wavelength at 245 nm and a flow-rate of 1.5 ml/min were chosen because of the desirable sensitivity and adequate separation time for probenecid and DSB in standard and CSF solution.

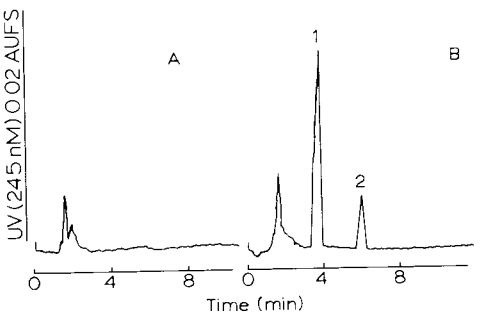

Fig. 1. Chromatograms of human CSF extracts: (A) probenecid-free (blank) CSF without DSB internal standard; (B) CSF containing probenecid (1) and 4.6 μg DSB (2). Conditions were as given in the Experimental section.

No endogenous compounds of CSF interfered with the detection of probenecid and DSB. Linearity between the detector response and the concentration of probenecid injected was observed over the range 2.4—24 μg. The percentage recoveries, estimated by peak height ratios of extracted standard solutions to non-extracted ones, for probenecid and DSB, were 85 ± 5 and 92 ± 3% (n = 4), respectively. The detection limit of probenecid was 250 ng/ml of CSF.

Table I shows free probenecid CSF concentrations determined by both direct injection and toluene extract of CSF samples from fourteen patients administered the same total dose of probenecid (100 mg/kg body weight). The wide range of those CSF probenecid values is in general agreement with previous data using different techniques [5, 6, 8, 9]. Small percentages (2—10%) of probenecid existing in conjugated form [9] were found in the five human CSF samples analyzed for total probenecid.

Although there were small differences among individual CSF probenecid concentrations obtained by direct injection and toluene extraction methods, no statistical discrepancy between the mean value of both methods was noticed (by paired t-test, not significant, Table I). However, we preferred the toluene extraction method since it yielded cleaner chromatograms and gave a longer lifetime of the column. Also, toluene can selectively extract probenecid and DSB from CSF samples and leave the acid metabolites (e.g. DOPAC, HVA, and

TABLE I

COMPARISON OF FREE CSF PROBENECID CONCENTRATIONS BY DIRECT INJECTION AND AFTER TOLUENE EXTRACTION

All patients were administered 100 mg/kg body weight of probenecid in divided oral doses over an 18-h period.

Patient No.	Free CSF probenecid (μg/ml)	
	Direction injection	Toluene extraction
1	17.8	17.0
2	19.0	18.8
3	15.0	15.2
4	14.0	13.0
5	20.9	21.2
6	30.4	29.3
7	22.0	22.3
8	25.4	25.6
9	20.8	21.4
10	18.0	16.2
11	17.8	17.3
12	16.2	15.7
13	13.4	14.8
14	20.2	19.8
$\bar{X}$	19.4	19.1
S.D.	4.3	4.5

5-HIAA) in aqueous solution [12]. Those acids could be reextracted back into an organic phase (e.g. ethyl acetate) and then determined by an appropriate technique.

In conclusion, the procedure described offers a simple and accurate HPLC determination of CSF probenecid concentration and can be done within an hour.

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Note

Quantitative assay of rifampicin and its main metabolite 25-desacetylrifampicin in human plasma by reversed-phase high-performance liquid chromatography

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The microbiological assay of rifampicin does not allow separate determination of the drug and its main metabolite, 25-desacetylrifampicin [1] in biological fluids. Methods able to measure both substances are essential when information about the metabolism of the antibiotic is needed. A thin-layer chromatographic method for the simultaneous assay of rifampicin and some of its transformation products has been developed, but it was applied to urine samples only, in which the amounts to be measured are relatively large [2]. A more sensitive method, which uses high-performance liquid chromatography (HPLC) on silica columns, was recently set up for the analysis of rifampicin and 25-desacetylrifampicin in plasma, down to 0.1 μg/ml [3].

Another recent method based on the same technique for the quantitative assay of rifampicin, 25-desacetylrifampicin, 3-formylrifamycin SV, and 3-formyl-25-desacetylrifamycin SV in human plasma, urine and saliva, down to 0.1 μg/ml, has been reported [4].

Reversed-phase HPLC was recently used for the separation of rifampicin from its degradation products (among them, 25-desacetylrifampicin and 3-formylrifamycin SV) in pharmaceutical formulations [5]. The present method uses the same technique, because reversed-phase columns are less affected by the humidity of the solvents than silica columns, can be more easily washed free of pollutant substances present in the biological fluid extracts and possess a longer life-span. This method allows the separation of rifampicin, 25-desacetylrifampicin, 3-formylrifamycin SV, 3-formyl-25-desacetylrifamycin SV, and N-desmethylrifampicin (see Figs. 1 and 2), but quantitative recovery studies were carried out only for the first two compounds, as they are present in the largest amounts in the plasma of patients treated with the drug. In fact there are negligible quantities in plasma extracts of 3-formylrifamycin SV and 3-formyl-25-desacetylrifamycin SV [6], minute

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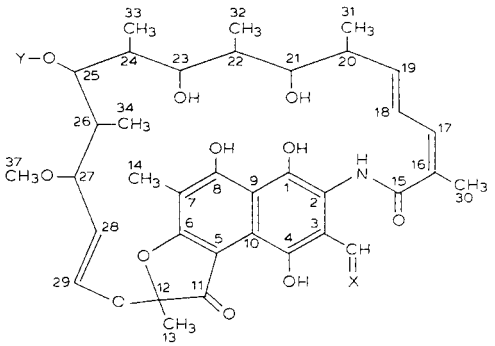


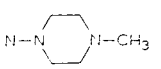
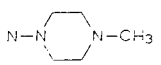
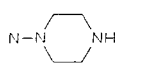
	X	Y
Rifampicin		CH_3CO
25-Desacetylrifampicin		H
3-Formylrifamycin SV	O	CH_3CO
25-Desacetyl-3-Formylrifamycin SV	O	H
N-Desmethylrifampicin		CH_3CO

Fig. 1. Structures of the compounds separated as shown in the chromatogram of Fig. 2. The carbon atom numbering system used here is conventional and does not follow the IUPAC recommendations.

amounts in bile and urine extracts [7], and N-desmethylrifampicin is found only in the urine [2].

EXPERIMENTAL

Materials

Rifampicin, 25-desacetylrifampicin, 3-formylrifamycin SV, 3-formyl-25-desacetylrifamycin SV, and N-desmethylrifampicin were Lepetit working standards of appropriate high purity. Butyl-*p*-hydroxybenzoate from Eastman-Kodak (Rochester, NY, U.S.A.) was used as internal standard. All the solvents and reagents were from Merck (Darmstadt, G.F.R.) or Carlo Erba (Milan, Italy), high-purity grade. The distilled water was filtered through the Millipore Mille-Q system. All glassware, previously washed, was cleaned by heating at 500°C for 6 h. The plasma blanks were obtained from healthy volunteers.

Standard solutions

Rifampicin, 25-desacetylrifampicin and the internal standard, butyl-*p*-hydroxybenzoate, were dissolved in acetonitrile—2-propanol (1:1, v/v) containing 0.5 mg/ml ascorbic acid to prevent oxidation of the compounds to be measured.

Extraction procedure

A 0.5-ml aliquot of heparinized plasma was pipetted into a screw-cap tube in which 10 μl of a solution of the internal standard in acetonitrile—2-propanol (1:1, v/v) had previously been placed. The amounts of the internal standard varied from 0.5 to 5 μg, depending on the expected amounts of the compounds to be determined. The sample was then diluted with 4.5 ml of 1 *M* KH_2PO_4, containing 1 mg/kg sodium ascorbate, and the pH adjusted to 4 with 1 *N* HCl. The diluted sample was extracted with 15 ml of ethyl acetate for 10 min at 300 inversions per minute on a Continental Alter 2864 shaker. After centrifugation at 2500 *g* for 10 min, 14 ml of the organic phase were transferred to a screw-cap tube. The organic phase was then taken to dryness under a stream of nitrogen at 37°C and the residue dissolved in 3.5 ml of 90% aqueous acetonitrile. After extraction with 3 ml of *n*-heptane, the sample was centrifuged and the *n*-heptane phase discarded. Then 3 ml of the acetonitrile phase were transferred to a conical tube and taken to dryness at 37°C under nitrogen. The residue was redissolved in 25—100 μl of acetonitrile—2-propanol (1:1, v/v) according to the expected content of the compounds, and introduced into the microvials of the HPLC automatic sampler.

Instrumentation

Two liquid chromatographs, Hewlett-Packard and Waters, were used during the standardization and application of the analytical method.

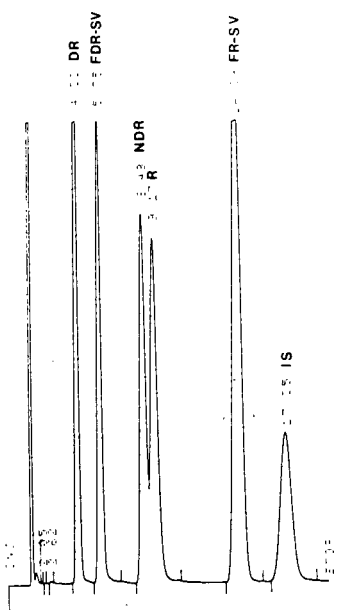


Fig. 2. Chromatogram of a mixture of 1 μg each of 25-desacetylrifampicin (DR), 3-formyl-25-desacetylrifamycin SV (FDR-SV), N-desmethylrifampicin (NDR), rifampicin (R), 3-formylrifamycin SV (FR-SV), and butyl-*p*-hydroxybenzoate (as internal standard, IS) dissolved in acetonitrile—2-propanol (1:1, v/v). The conditions are those described in the section Waters operating conditions.

Hewlett-Packard operating conditions. Instrument: H.P. Model 1084B equipped with a Model 79870A fixed-wavelength (254 nm) UV absorbance detector and a Model 79841A automatic sampler. Injection: 10 µl, containing about 50—250 ng of the compounds. Column: RP-8, 10 µm, 25 cm × 4.6 mm, Brownlee Labs. (Santa Clara, CA, U.S.A.). Elution: isocratic, 38% of B in A, where A = 0.1 *M* KH_2PO_4, pH 3.5 with 0.2 *M* H_3PO_4; B = acetonitrile. Flow-rate: 2 ml/min. Temperature: 30°C.

Waters operating conditions. Instrument: Waters Associates equipped with Model 660 solvent programmer, a Model 440 fixed-wavelength (254 nm) UV absorbance detector and a Model 710A sample programmer W.I.S.P. Injection, column, and flow-rate: see previous section. Elution: isocratic, 40% of B in A, where A = 0.1 *M* KH_2PO_4, pH 3.5 with 0.2 *M* H_3PO_4; B = acetonitrile containing 10% water. Temperature: ambient. Quantification: Hewlett-Packard Model 3380A computing reporting integrator.

RESULTS AND DISCUSSION

Known amounts of rifamycin and 25-desacetylrifamycin using standard solutions prepared as described in Experimental, were added to human plasma samples in order to have the same concentrations of each compound. Three concentration levels were established, based on the amounts of rifamycin and 25-desacetylrifamycin expected to be in the plasma: 0.5, 2, and

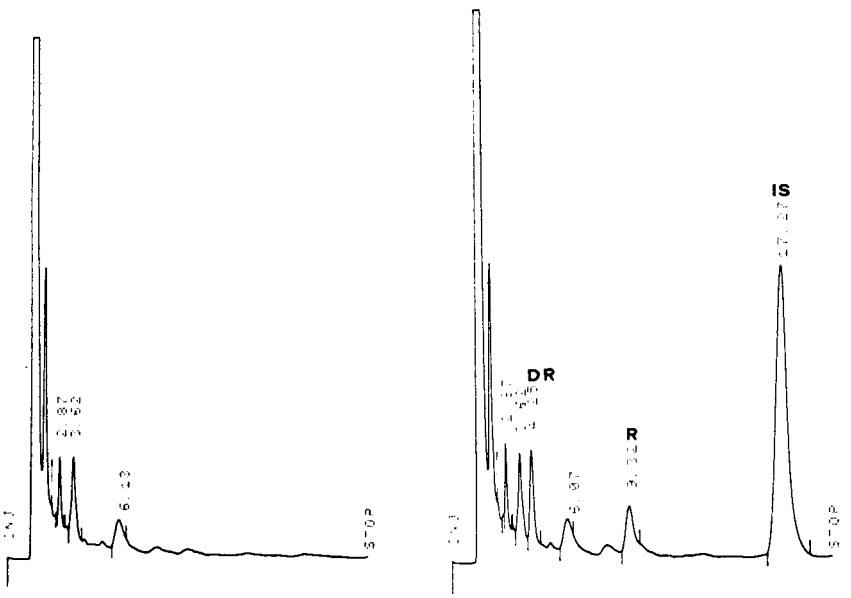


Fig. 3. Chromatogram of an extract of blank human plasma. The amount of extract injected is equivalent to 0.2 ml of plasma. (See Hewlett-Packard operating conditions and Results section.)

Fig. 4. Chromatogram of a sample prepared as described under Experimental. Amount of extract injected is equivalent to 0.2 ml of plasma with 25-desacetylrifampicin (DR, 0.5 µg/ml), rifampicin (R, 0.5 µg/ml), and internal standard (IS, 2 µg/ml) added. The conditions are described in the section Hewlett-Packard operating conditions.

TABLE I

RESULTS OF RECOVERY TRIALS

Different amounts of rifampicin (R) and 25-desacetylrifampicin (DR) were added to 0.5 ml of human plasma.

Added (μg)	Found (μg)				Average found (μg)	Standard deviation (S.D.)	Relative S.D.	Variance	Standard error	Average recovery (%)
R 0.236	0.191	0.186	0.212	0.192	0.195	0.011	5.875	$1.31 \cdot 10^{-4}$	0.006	82.6
DR 0.229	0.202	0.198	0.195	0.202	0.199	0.003	1.708	$1.15 \cdot 10^{-5}$	0.002	87.3
R 0.976	0.957	1.016	1.030	1.038	1.010	0.037	3.627	0.001	0.018	103.5
DR 0.910	0.851	0.894	0.905	0.895	0.886	0.024	2.710	0.001	0.012	100.0
R 4.95	5.00	4.84	4.87	4.90	4.903	0.069	1.417	0.005	0.035	99.0
DR 4.84	4.38	4.15	4.32	4.32	4.293	0.099	2.309	0.010	0.050	88.6

10 μg/ml. Four samples were prepared for each concentration level, extracted and analyzed as previously indicated under Experimental.

The chromatograms for two human plasmas are shown in Figs. 3 and 4.

The results of the recovery trials are listed in Table I. The precision of the measurements is good, even at the lowest concentrations, for both the compounds. In fact, the relative standard deviation for 25-desacetylrifamycin was always less than 3, while that of rifamycin was 5.87 at 0.47 μg/ml, and was less than 4 at the higher concentrations. The average recovery values were in the range of 80—90% for samples of about 0.5 μg/ml and in the range of 88—103% for the other samples. The linearity of the recovery was very good over the range of concentration 0.5—10 μg/ml, as demonstrated by the equations $y = -0.00196 + 0.99219x$ ($r^2 = 0.99936$) for rifamycin and $y = 0.03782 + 0.88051x$ ($r^2 = 0.99878$) for 25-desacetylrifamycin. The limit of detection was 0.2 μg/ml.

The method was used in the assay of rifamycin and 25-desacetylrifamycin in the plasma of healthy volunteers orally given a single dose of 600 mg per day. Fig. 5 shows the plasma concentrations of rifamycin and 25-desacetylrifamycin found in one subject during the first 12 h of treatment.

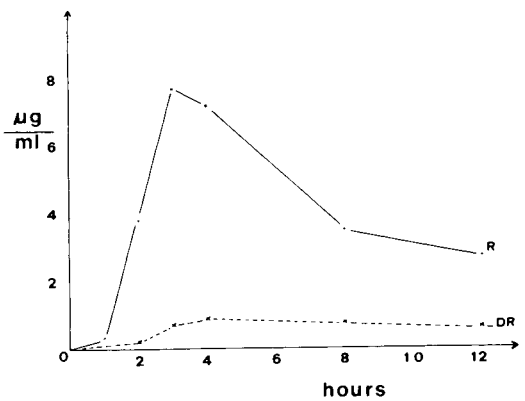


Fig. 5. Concentrations (μg/ml) of rifampicin (R) and 25-desacetylrifampicin (DR) in the plasma of one healthy volunteer after oral administration of 600 mg of rifampicin, during the first 12 h of treatment.

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Note

High-performance liquid chromatographic determination of cephalexin in human plasma, urine and saliva

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Cephalexin, 7-(D-α-amino-α-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid (Fig. 1A) is one of the most commonly used oral cephalosporin antibiotics. Following oral administration, cephalexin is well absorbed and is largely eliminated by the kidney as unchanged drug [1—3]. Its elimination half-life of about an hour in normal subjects may be increased to 20—30 h in patients with severe renal dysfunction [4,5].

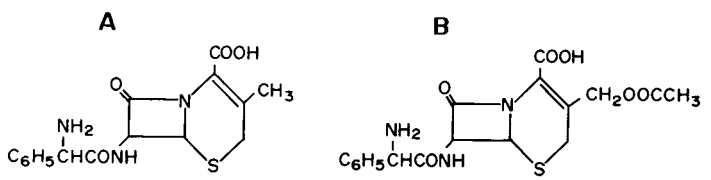

Fig. 1. Chemical structures of cephalexin (A) and cephaloglycin (B).

In severe systemic infections cephalexin dosage of 3—4 g daily for adults and 100 mg/kg/day for children have been recommended [1]. Recently, it was suggested that some pediatric patients with osteomyelitis may require as much as 200 mg/kg/day for a period of 6—16 months [6,7]. A good correlation has been observed between cephalexin serum concentration and concentration in synovial fluid of patients with suppurative arthritis [8]. Monitoring of cephalexin serum concentration is important to assure compliance and gastrointestinal absorption [9,10].

Several methods have been reported for the analysis of cephalexin using microbiological [3,11,12], spectrophotometric [13,14], fluorimetric [15—17] and polarographic [18] techniques but high-performance liquid chromatography (HPLC) is rapid, simple, sensitive and specific for cephalexin.

One HPLC method is available for determination of cephalexin in plasma [19] and two for urine [19,20]. However, no internal standard was employed placing too much reliance on accurate volume transfers and on comparing the peak of cephalexin to the standard for quantification. The procedure is unsuitable for pediatric use because a large sample (500 μl) of plasma was required. Furthermore, these methods are not preferred for routine monitoring of plasma or urine cephalexin concentration due to long retention time for plasma (16 min) and urine (8—16 min).

Since pK_a values of cephalexin are 5.2 and 7.3 [21], and plasma protein binding is about 15% [22], cephalexin may be present in saliva. Monitoring of salivary concentration has been shown to be of value for a number of drugs [23], especially in pediatric patients because saliva can be collected by non-invasive techniques. No methods have been reported for measurement of cephalexin in saliva.

This report describes a rapid, sensitive, simple, reproducible and specific HPLC technique for the determination of cephalexin in small volume samples of plasma, urine and saliva, using cephaloglycine (Fig. 1B) as an internal standard. To demonstrate its clinical utility plasma, urinary and salivary concentrations of cephalexin in a normal subject are presented.

MATERIALS AND METHODS

Chemicals and reagents

Cephalexin, cephaloglycin and tobramycin were obtained from Eli Lilly (Indianapolis, IN, U.S.A.), carbenicillin from Roerig (New York, NY, U.S.A.), gentamicin from Sigma (St. Louis, MO, U.S.A.), and chloramphenicol and its sodium succinate ester from Parke Davis (Ann Arbor, MI, U.S.A.). Methanol (glass distilled) HPLC grade, was purchased from Mallinckrodt (Paris, KY, U.S.A.).

Chromatographic equipment and conditions

The reversed-phase HPLC system consisted of Consta Metric pump II G (Laboratory Data Control, Riviera Beach, FL, U.S.A.), μBondapak C_{18} column, 3.9 mm × 30 cm, 10 μm (Waters Assoc., Milford, MA, U.S.A.), analytical fixed-wavelength UV detector, Model 153 (Beckman Instruments, Fullerton, CA, U.S.A.) and a recorder, Series 5000 (Fisher Recordall, Houston Instruments, Houston, TX, U.S.A.).

Acetic acid (0.5%) was added to methanol—water (20:80) to prepare the mobile phase. It was pumped at 2 ml/min for analysis of plasma and saliva samples and at 1.7 ml/min for urine samples. Chart speed of the recorder was set at 0.2 in./min.

Standards

Cephalexin (10 mg) and cephaloglycin (10 mg) were each dissolved in distilled water (10 ml) to give a concentration of 1 mg/ml. Appropriate amounts of these standard solutions were added to the plasma, urine and saliva samples to yield concentrations of 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40 and 50 μg/ml of cephalexin. Two standard curves were constructed for cephalexin in each body

fluid: one from 0.2—10 μg/ml using 0.5 μg of cephaloglycin and another from 10—50 μg/ml with 5 μg of cephaloglycin. All samples were stored at —20°C.

Assay procedure

Biological fluids (100 μl) containing known amounts of cephalexin were placed in polypropylene microcentrifuge tubes. Methanol (200 μl) containing internal standard was then added to the mixture. The resulting mixture was vortexed for 5 sec and then centrifuged at 9360 *g* for 5 min. The supernatant was transferred to another set of polypropylene test tubes and evaporated at 40°C under a gentle stream of nitrogen. The residue was dissolved in 75 μl of the mobile phase, vortex mixed for 30 sec, and 50 μl of this were injected onto the HPLC column. The detector was set at 0.005—0.08 a.u.f.s. (wavelength 254 nm).

Calculations

The concentrations of cephalexin in the unknown plasma, urine and saliva samples were calculated by comparing its cephalexin:cephaloglycin peak height ratios with those obtained from cephalexin standard curves for plasma, urine and saliva.

Recovery and precision

Cephalexin was added to drug-free plasma, urine and saliva and then analyzed by the procedure described above but without any added internal standard. Fifty microliters of the supernatant were injected and peak heights corresponding to cephalexin measured. Absolute recoveries were calculated by comparing these peak heights with peak heights obtained by direct injection of pure standards.

Precision of the method was evaluated by analysis of plasma, urine and saliva standards containing cephalexin and cephaloglycin concentrations of 50, 25, 10 and 0.5 μg/ml. These samples were analyzed five times by two individuals.

Specificity

Commonly used antibiotics such as gentamicin (10 μg/ml), tobramycin (10 μg/ml), carbenicillin (15 μg/ml), chloramphenicol sodium succinate (20 μg/ml) and chloramphenicol (20 μg/ml) were tested using this procedure for potential interference with cephalexin and cephaloglycin.

Clinical application

A normal adult volunteer (age 29) received 1 g of cephalexin as an oral suspension (Keflex®, Eli Lilly). Blood and saliva (stimulated by paraffin wax) samples were obtained simultaneously at 0, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0, 6.0 h after drug administration. Urine samples were collected hourly for the first 6 h and at normal voiding hours thereafter for a total of 24 h. The specimens were stored at —20°C and analyzed within a week.

RESULTS AND DISCUSSION

Typical chromatograms of cephalexin and cephaloglycin in plasma, urine and saliva are shown in Fig. 2. The peaks are sharp and symmetrical allowing use of peak heights rather than peak areas to quantitate detector response. Detector response (peak height) was linear (correlation coefficients > 0.99) over 0.2—50 μg/ml concentration range for cephalexin, with all curves passing through origin. Peak height ratios of cephalexin:cephaloglycin were also linear over the same concentration range.

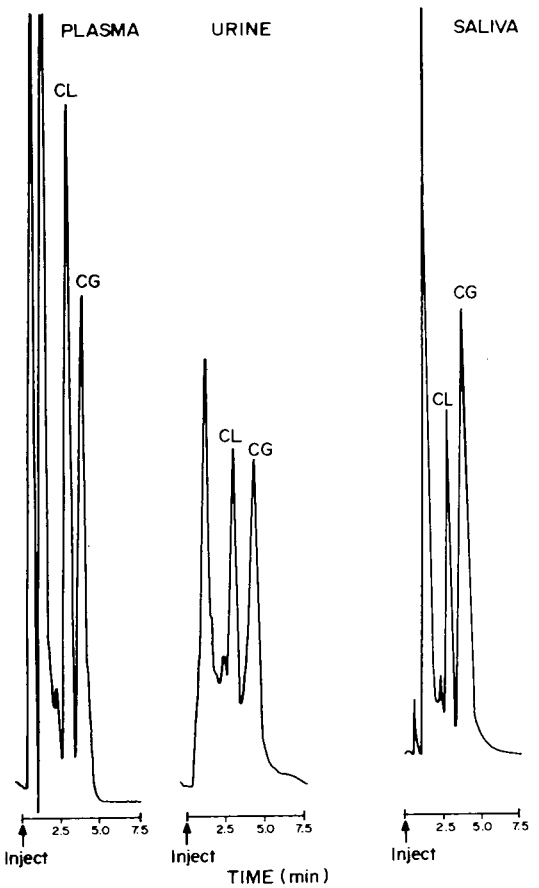

Fig. 2. Chromatograms of cephalexin (CL) and cephaloglycin (CG) in plasma (CL, 40 μg/ml; CG, 50 μg/ml), urine (CL, 30 μg/ml; CG, 27 μg/ml) and saliva (CL, 20 μg/ml; CG, 50 μg/ml). a.u.f.s.: 0.04.

The retention times for cephalexin and cephaloglycin were about 3.0 and 4.0 min respectively. This is a definite advantage over the currently available HPLC procedures [19,20] because this method uses an internal standard and allows rapid analysis of cephalexin in plasma, urine and saliva.

The limit of detection was 0.2 μg/ml for cephalexin in the three body fluids. Recoveries of cephalexin and cephaloglycin ranged from 96—102% (Table I)

TABLE I

RECOVERIES ON EXTRACTION OF CEPHALEXIN AND CEPHALOGLYCIN FROM PLASMA, URINE AND SALIVA

Each value is the mean of five determinations.

Compound	Concentration (μg/ml)	Recovery (% ± S.D.)		
		Plasma	Urine	Saliva
Cephalexin	50	101.9 ± 2.2	97.3 ± 3.3	98.1 ± 1.4
	25	97.6 ± 4.8	96.7 ± 2.7	96.5 ± 2.4
	10	96.8 ± 3.2	97.7 ± 3.8	102.0 ± 2.8
	0.5	97.1 ± 3.1	96.0 ± 4.9	97.7 ± 2.8
Cephaloglycin	50	98.1 ± 3.4	98.8 ± 2.3	98.7 ± 2.9
	25	102.0 ± 4.3	98.9 ± 3.2	100.3 ± 4.1
	10	97.8 ± 2.7	96.1 ± 4.8	97.7 ± 3.8
	0.5	96.5 ± 5.1	97.9 ± 3.7	98.1 ± 2.7

TABLE II

REPRODUCIBILITY OF CEPHALEXIN DETERMINATION IN PLASMA, URINE AND SALIVA

In all cases $n = 5$.

	Concentration (μg/ml)	Coefficient of variation (%)		
		Plasma	Urine	Saliva
Within-day	50	2.7	3.2	2.9
	25	4.2	4.5	3.4
	10	3.0	3.9	2.3
	0.5	2.4	6.0	4.8
Day-to-day	50	2.9	4.1	4.4
	25	3.2	3.4	3.7
	10	3.0	4.9	5.0
	0.5	5.0	3.8	4.2

while precision varied from 2.3—6% (Table II). Daily variations in cephalexin concentrations were < 5%. This assay was specific for cephalexin in that commonly used antibiotics (gentamicin, tobramycin, carbenicillin, chloramphenicol and its sodium succinate ester) did not interfere with its measurement. In these experiments cephalexin did not lose its potency during the storage for ⩽ 7 days prior to analysis.

Plasma concentrations of cephalexin are shown in Fig. 3. The peak plasma concentration of 32 μg/ml at 1 h after oral administration and an elimination half-life of about 1 h are consistent with reported findings [1, 24, 25]. Cephalexin concentration in saliva et 0.5 h and 1 h were 2.8 and 3.3 μg/ml, respectively. No cephalexin was detectable beyond 1 h after drug administration. Various factors including salivary flow-rate, pH fluctuation and low partition coefficient or lipid solubility of cephalexin may be responsible for relatively low concentrations of cephalexin in saliva [23,26].

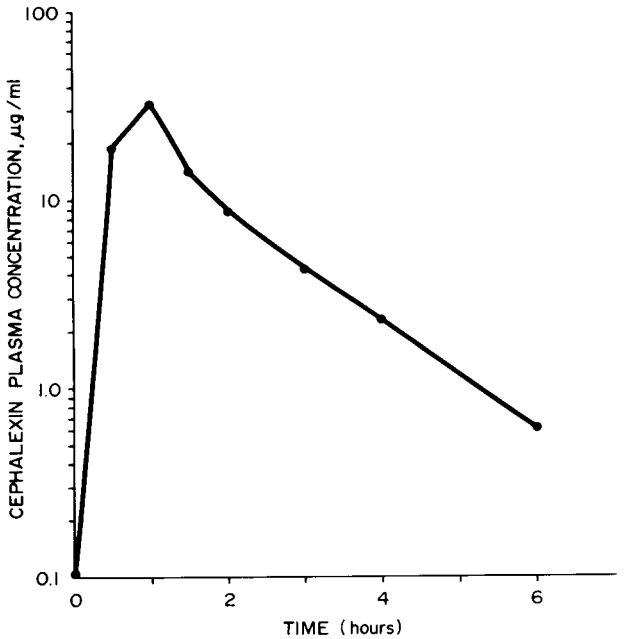

Fig. 3. Plasma concentrations of cephalexin following an 1-g oral dose.

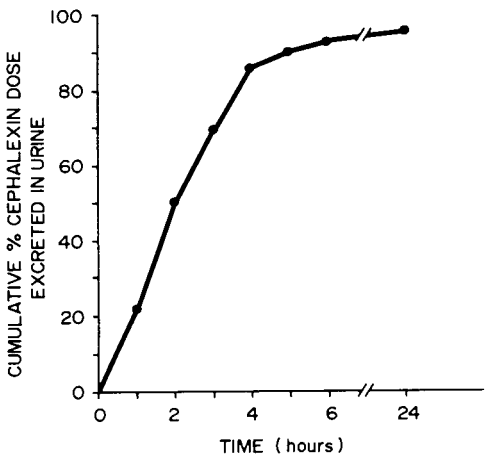

Fig. 4. Cumulative urinary excretion of cephalexin following oral administration of 1 g cephalexin.

Fig. 4 describes the cumulative urinary excretion of cephalexin during a 24-h period following drug administration. About 92% of the dose was excreted within 6 h while 95% was recovered within 24 h after cephalexin administration. These data are in agreement with other reports [1,19,20,24].

This assay system has proven simple, rapid, reproducible, sensitive and specific for determination of cephalexin in plasma, urine and saliva. The sample size required in this procedure makes it suitable for individualizing

cephalexin therapy in pediatric patients or for performing pharmacokinetic studies which requires multiple samples of biological fluids.

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Book Review

Biomedical and biological applications of isotachophoresis (Proc. 1st Symp., Baconfoy, May 4—5, 1979) (Analytical Chemistry Symposia Series, Vol. 5), edited by A. Adam and C. Schots, Elsevier, Amsterdam, Oxford, New York, 1980, VII + 278 pp., price Dfl. 120.00, US$ 58.50, ISBN 0-444-41891-1.

Biochemistry and the biological sciences including medicine require increasingly demanding and more numerous tasks in the analysis of ionogenic components of biological samples. High accuracy and speed in quantitative determinations are especially required, with a simultaneous high degree of qualitative resolution of trace amounts of various compounds. In this respect isotachophoresis has been used increasingly in recent years in addition to classical electromigration methods. The specific advantage of isotachophoresis of the carrierless type consists in its simple quantitative interpretation together with the possibility of automation of the microanalytical separation process. Isotachophoresis, especially in its capillary form, is used in inorganic and organic analysis, including, microanalysis of ionogenic biomacromolecules. In order to extend the application of isotachophoresis it is necessary to become acquainted not only with the principle of the technique, but also with the possibilities of using various electrolyte systems and the way of selecting them.

Therefore the publication of Biochemical and Biological Applications of Isotachophoresis should be welcomed. This book surveys and collects for the first time data on the present-day use of isotachophoresis in biochemistry, biology, pharmacology and toxicology. The 278 pages of text comprise 24 review articles of which the majority (21 articles) are devoted to practical applications of capillary isotachophoresis. In the introductory part a survey of indispensable theoretical information is presented in an accessible form; this can be recommended especially to those readers who are meeting the method for the first time and/or intend to make active use of it. The description of the newly conceived equipment and the adaptations of commercial capillary apparatus are presented in two articles. This part is suitable for those who are interested in the processing of samples with a high content of salts. The technique of preparative and analytical isotachophoresis in gel is discussed in two articles. A large part of the text (10 articles) is devoted to the analysis of the proteins or their interaction with drugs. For doctors the analyses of the celebrospinal fluid will surely be of great interest. Five articles are devoted to the analysis of urine

components; the rest of the text includes kinetic studies of enzymatic processes in ribosomal liver fractions, the analysis of nucleic acid components, low-molecular-weight carboxylic acids, etc.

The electrolyte systems suitable for the analysis of the above-mentioned groups of substances given in this book are of especial value. The book describes a relatively large number of analytical application possibilities with the accent on practical microanalytical use of the available commercial instruments for capillary isotachophoresis. It is a pity that the organizers of the symposium have not provided the book with a subject index to ease the reader's rapid orientation in the problems of this relatively young methodological approach.

The book can provide a good source of information both for scientists and for those working in biochemistry, biology, pharmacy and medicine who intend to introduce isotachophoresis into their laboratories as a routine microanalytical method.

Prague (Czechoslovakia) Z. PRUSÍK

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JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

8th INTERNATIONAL SYMPOSIUM ON BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY

The 8th International Symposium on Biomedical Applications of Chromatography will be held at the Charles University Faculty of Pharmacy in Hradec Králové, Czechoslovakia, on September 7–11, 1982. Lectures (invited or selected), posters and discussions will deal with methods and interpretations of all types of chromatography as applied in clinical and experimental medicine and relevant border disciplines. Special emphasis will be placed on detectors; other technical problems important in biomedical work (*e.g.*, sample preparation and the question of internal standard); peptides (especially hormonal) and proteins (especially enzymes); metabolic deviations (including prenatal diagnosis and heterozygote screening; and drug monitoring.

The Symposium is organized by the Chromatography groups of the Czechoslovak Chemical Society (Chairman: K. Macek, Prague) and of the Society for Clinical Medicine and Laboratory Diagnostics of the German Democratic Republic (J. Wagner, Leipzig). For further information contact Dr. K. Macek, Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia.

EIGHTH EUROPEAN WORKSHOP ON DRUG METABOLISM

The 8th European Workshop on Drug Metabolism will be held at the University of Liège, Belgium, from September 5th to the 9th, 1982. As with the previous workshops, the aims of the meeting will be to provide the participants with a global view of the concepts of drug and xenobiotic metabolism, as well as with opportunities for discussing recent trends and developments in drug metabolism research. Workshops, including live demonstrations, will also be organized throughout the duration of the meeting.

For further information, please contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium, telephone (32-41)-56.24.80/81.

CALENDAR OF FORTHCOMING EVENTS

Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2)

Nov. 16–17, 1981 Washington, DC, U.S.A.	**The International Symposium on HPLC of Proteins and Peptides** Contact: Shirley E. Schlessinger, Symposium Manager, International Symposium on HPLC of Proteins and Peptides, 400 East Randolph, Chicago, IL 60601, U.S.A. (Further details published in Vol. 208, No. 2)
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. J. Albaigés, General Secretary, Plaza de Espana, Barcelona–4, Spain. Tel: 223–31 01.
Nov. 26th, 1981 Cardiff, Wales, Great Britain	**Trace Organic Analysis of Biological Type Samples** Contact: The Royal Society of Chemistry, Analytical Division (Western Region), Burlington House, London W1V 0BN, Great Britain.
Dec. 1–5, 1981 São Paulo, Brazil	**XI Pan American Congress of Pharmacy and Biochemistry** Contact: A.Ph.A. Division of Communications, 2215 Constitution Ave., N.W., Washington, DC 20037, U.S.A.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.
Dec. 10th, 1981 London, Great Britain	**Biological Methods Group Annual General Meeting and Discussion on "Biotechnology"** Contact: The Royal Society of Chemistry, Analytical Division (Biological Methods Group), Burlington House, London W1V 0BN, Great Britain.
Jan. 19–20, 1982 Amsterdam, The Netherlands	**Symposium on "Detection in High-Performance Liquid Chromatography"** Contact: Mrs. Peschier, Hewlett-Packard Nederland B.V., Analytical Department, van Heuven Goedhartlaan 121, 1181 KK Amstelveen, The Netherlands (Tel.: 020-47 20 21). (Further details published in Vol. 212, No. 2)
March 8–12, 1982 Atlantic City, NJ, U.S.A.	**1982 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Linda Briggs, Program Secretary, Pittsburgh Conference, Department J-057, 437 Donald Road, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 212, No. 2)
March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**183rd American Chemical Society National Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 5–8, 1982 Las Vegas, NV, U.S.A.	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. (Further details published in Vol. 212, No. 3)
April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)
April 15–17, 1982 Tokyo, Japan	**International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.

April 19–22, 1982 Barcelona, Spain	**International Congress on Automation in Clinical Laboratory** Contact: Dr. R. Galimany, Sección de Automatización, Laboratorio de Analisis Clinicos, C.S. "Principes de Espana", Hospitalet de Llobregat, Barcelona, Spain.
April 20th, 1982 Loughborough, Great Britain	**Electrochemical Analysis in the Pharmaceutical Industry** Contact: The Royal Society of Chemistry, Analytical Division (Electroanalytical Group), Burlington House, London W1V 0BN, Great Britain.
April 20th, 1982 Belfast, Northern Ireland, U.K.	**Derivative Spectroscopy and its Applications in Bioanalytical and Environmental Chemistry** Contact: The Royal Society of Chemistry, Analytical Division (Northern Ireland Region), Burlington House, London W1V 0BN, Great Britain.
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umweltforschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.
April 27–30, 1982 Munich, G.F.R.	**Biochemische Analytik Conference** Contact: Prof. I. Trautschold, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, G.F.R. (Further details published in Vol. 224, No. 3)
May 11–14, 1982 Ghent, Belgium	**4th International Symposium on Quantitative Mass Spectrometry in Life Sciences** Contact: Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium.
May 20th, 1982 Bath, Great Britain	**Development of Chromatographic Techniques in Cancer Therapy** Contact: The Royal Society of Chemistry, Analytical Division (Western Region), Burlington House, London W1V 0BN, Great Britain.
June 6–11, 1982 Kansas City, MO, U.S.A.	**International Symposium on the Synthesis and Application of Isotopically Labeled Compounds** Contact: Dr. Alexander Susán, Scientific Secretary of the Symposium, c/o Midwest Research Institute, 425 Volker Boulevard, Kansas City, MO 64110, U.S.A. Tel: (816) 753-7600, extension 268. (Further details published in Vol. 225, No. 1.)
June 7–11, 1982 Philadelphia, PA, U.S.A.	**VI International Symposium on Column Liquid Chromatography** Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published in Vol. 211, No. 3).
June 17 + 18, 1982 St. Andrews, Scotland	**Advances in Immunoassay: Techniques and Applications** Contact: The Royal Society of Chemistry, Analytical Division (Scottish Region), Burlington House, London W1V 0BN, Great Britain.
June 21–23, 1982 Bordighera (near San Remo), Italy	**International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences** Contact: Dr. Alberto Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20517 Milan, Italy. Tel.: 35.54.546; Telex: 331268 NEGRI I. (Further details published in Vol. 225, No. 1.)

July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
Aug. 15–21, 1982 Perth, Australia	**The 12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia
Aug. 30–Sept. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A-1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
Sept. 5–9, 1982 Liège, Belgium	**Eighth European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81.
Sept. 6–9, 1982 Bath, Great Britain	**4th European Symposium on Chemical Structure – Biological Activity: Quantitative Approaches** Contact: Dr. J.C. Dearden, School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.
Sept. 7–11, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia.
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)
Aug. 28–Sep. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459

PUBLICATION SCHEDULE FOR 1981

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

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

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ELECTROPHORESIS

A SURVEY OF TECHNIQUES AND APPLICATIONS

Part A: Techniques

Z. DEYL, Czechoslovak Academy of Sciences, Prague (editor)
F. M. EVERAERTS, Z. PRUSÍK, and P. J. SVENDSEN (co-editors)

JOURNAL OF CHROMATOGRAPHY LIBRARY 18

This first volume in a two part set, deals with the principles, theory and instrumentation of modern electromigration methods. The second volume will be concerned with details of applications of electromigration methods to diverse categories of compounds, although a few applications are already discussed in Part A.

Some electromigration methods have become standard procedures because of their extensive use in analytical and preparative separations. These are discussed together with newer developments in the field. Hints are included to help the reader to overcome difficulties frequently arising from the lack of suitable equipment. Adequate theoretical background of the individual techniques is included. A theoretical approach to the deteriorative processes is presented in order to facilitate further development of a particular technique and its application to a special problem.

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