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BIOMEDICAL APPLICATIONS

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CHROMBIO. 1659

SIMULTANEOUS DETERMINATION OF 5-HYDROXYTRYPTAMINE, ITS AMINO ACID PRECURSORS AND ACID METABOLITE IN DISCRETE BRAIN REGIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

L-Tryptophan, 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid (5-HIAA) from discrete regions of brain can be resolved by isocratic elution on a C-18 reversed-phase column and quantitated by native fluorescence measurement. This method is rapid and simple and can reproduceably detect 5-hydroxytryptamine, tryptophan and 5-HIAA with picogram sensitivity. The detection of 5-HTP in brain extracts from untreated animals is difficult because of the extremely low endogenous levels of 5-HTP. Quantitation of 5-HTP presents no problem in animals injected with 5-HTP or with an inhibitor of L-aromatic amino acid decarboxylase. Brain tissue requires minimal treatment for assay and the hydroxyindoles are stable enough to allow automated processing.

INTRODUCTION

The neurotransmitter 5-hydroxytryptamine (5-HT) has been implicated as a modulator of a variety of physiological processes, not the least of which is blood pressure and its regulation [1]. The amine itself does not apparently cross the blood brain barrier [2] and, as a result, the amino acid precursors of 5-HT, tryptophan and 5-hydroxytryptophan (5-HTP), are frequently injected systemically to increase the brain concentrations of 5-HT. However, it is becoming apparent that tryptophan and 5-HTP can exert physiological and behavioral effects which are not entirely mediated by 5-HT (synthesized from tryptophan or 5-HTP) but possibly by the amino acids themselves [3—6] or by the nonspecific influence of the amino acids on other neurotransmitter systems [7—9]. For these reasons, it is quite important that the concentrations of tryptophan and 5-HTP, in addition to those of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) be determined in discrete brain regions before and

after 5-HT percursor loading.

A large number of methods are currently available for measuring 5-HT and its precursors and metabolites in biological tissue [10]. However, even recent methods often require derivatization or fluorescence enhancement prior to high-performance liquid chromatography (HPLC) [11] or extraction of the tissue into organic solvents in preparation for HPLC [12], both of which restrict the number of substances in a particular biosynthetic pathway which can be subsequently analyzed. Furthermore, some HPLC methods are applied to whole brain [8, 12—14] which does not completely allow for the discrete analysis of neurotransmitter levels in the dynamic fashion in which they certainly function.

In the present report, we describe a method which allows the resolution and detection of tryptophan, 5-HTP, 5-HT, and 5-HIAA in small brain areas from rats. The method requires no pretreatment of the samples besides deproteination and the use of isocratic elution simplifies the instrumentation. Finally, this procedure takes advantage of the native fluorescence properties of tryptophan, 5-HT, and 5-HIAA for detection and quantitation. Determination of endogenous 5-HTP levels is difficult but estimates can be made by difference.

EXPERIMENTAL

Apparatus

The HPLC apparatus consists of a Dupont Chromatographic Pump Module (Model 870), a Perkin-Elmer Fluorescence Spectrophotometer (Model 650-10LC) and an LKB Potentiometric Recorder (Model 2210). Automated sample injections were performed by a Waters Intelligent Sample Processor (WISP Model 710B).

The chromatographic separation was achieved by reversed-phase using an octadecyl polymer-coated pellicular silica column (Spherisorb ODS, particle size 5 μm; 250 × 4.6 mm I.D. column prepacked from Chromanetics Corp.). A short precolumn (60 × 5 mm I.D.) packed with an octadecyl-coated pellicular support (CO:Pell ODS, Whatman, Clifton, NJ, U.S.A.) was used as a guard column.

The mobile phase consists of 0.01 *M* sodium acetate containing 15% methanol brought to pH 4.85 with acetic acid. This was degassed under vacuum prior to use. Solvent flow-rate was set at 1.4 ml/min and separation was performed at ambient temperature (23°C).

The fluorescence spectrophotometer was set at an emission wavelength of 345 nm while the excitation wavelength was 285 nm. The spectral bandpass was set at 10 nm.

Chemicals and reagents

Tryptophan, 5-HTP, 5-HT (creatinine sulfate), N-acetylserotonin (NAS), and 5-HIAA standards were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol was HPLC grade from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other chemicals and reagents were of the highest quality commercially available. Stock solutions of tryptophan, 5-HTP, 5-HT, 5-HIAA and NAS were made to a concentration of 1.0 mg/ml in distilled, deionized water containing

0.1% ascorbic acid. Stock solutions were frozen and stored at −20°C, protected from light, and were diluted to the appropriate concentration prior to HPLC analysis with the same buffer used to dilute tissue samples (see below).

Tissue preparation

Male Wistar rats were obtained from the NIH animal colony and used when 10—12 weeks of age. Rats were housed in group cages under constant humidity and ambient temperature. The lighting schedule consisted of a 12-h light—dark cycle (7 a.m.—7 p.m.). Food and water were available ad libitum. Animals were sacrificed and the following brain regions were immediately dissected out and frozen on dry ice: anterior hypothalamus, midbrain tegmentum, nucleus tractus solitarius (NTS) and striatum. All samples were stored in liquid nitrogen until assay. Samples were rapidly thawed and prepared for analysis by addition of 100 ng of NAS as internal standard followed by homogenization in 4 volumes (or at least 100 μl) of ice cold 0.16 *N* perchloric acid containing 0.1% EDTA and 0.1% ascorbate. Tissue was homogenized in 1.5-ml polypropylene test tubes with a motor-driven PTFE pestle which had been cut and shaped to fit the test tubes. The homogenate was centrifuged at 40,000 *g* for 20 min at 4°C. The supernatant was then diluted 1:6 with ice cold 0.5 *M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) buffer, pH 6.0 and immediately frozen on dry ice until analysis. We have found that the 5-hydroxyindoles and 5-HIAA in particular are much more stable when diluted into Hepes pH 6.0 than when diluted into $NaPO_4$ buffer, pH 7.9 or Tris·HCl pH 7.4. In some cases rats were injected intraperitoneally with L-tryptophan (methyl ester hydrochloride salt) or 5-HTP dissolved in 0.9% saline. The injection volume was 2 ml/kg and doses are based on the weight of the free amino acid.

RESULTS

A chromatogram demonstrating the resolution of tryptophan, 5-HTP, 5-HT, 5-HIAA, and NAS standards is shown in Fig. 1. It can be seen from Fig. 1 that all substances are sufficiently resolved to allow accurate quantitation. All standards including the internal standard are chromatographed within 18 min. Standards and tissue are stable for at least 7 h when prepared by the protocol described above, allowing automatic sample processing.

These same substances can be detected in brain tissue as shown in Fig. 2A. Identification of the peaks found in the chromatogram of brain tissue was achieved by matching the retention times of peaks from tissue with those of the standard compounds. Further confirmation was achieved by addition of a known quantity of each standard to a duplicate sample of brain extract followed by chromatography of both samples. As can be seen in Fig. 2B the characteristics of the identified peaks remained unchanged, although they were appropriately taller with the added standard, and no new peaks emerged. The first two peaks, eluting at 3 min and 3.7 min, are as yet unidentified, but they are neither tryptamine nor indoleacetic acid. In order to enhance quantitation of the 5-HTP peak in brain tissue from untreated rats it was necessary to add 100 pg of 5-HTP standard to each sample before analysis.

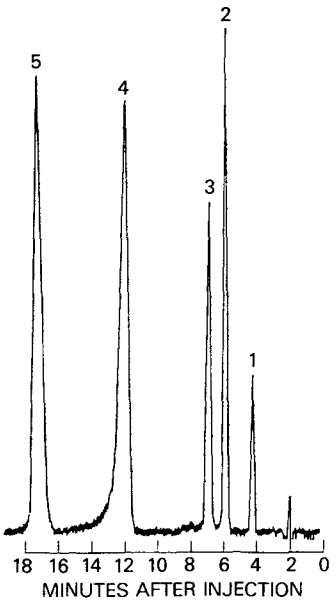

Fig. 1. Chromatogram of standards prepared as described in Methods. Injection volume is 40 μl. Peaks: 1, 5-HTP (200 pg); 2, 5-HIAA (1.20 ng); 3, tryptophan (2 ng); 4, 5-HT (2 ng); and 5, NAS (2 ng).

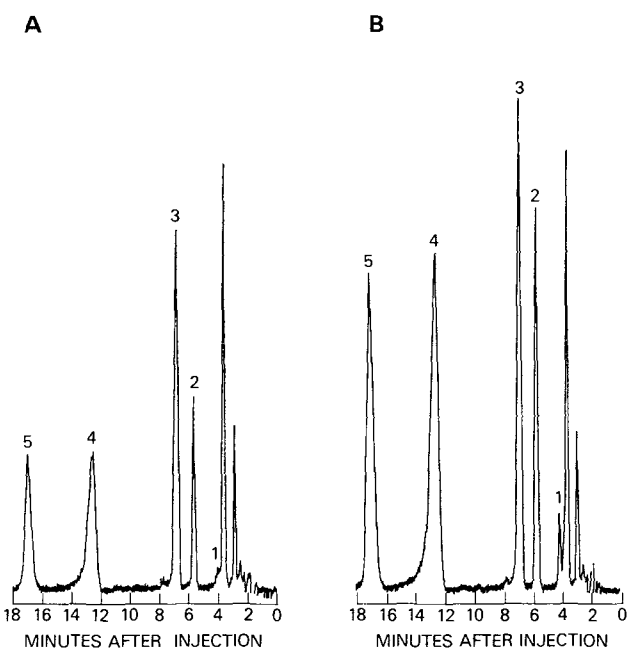

Fig. 2.

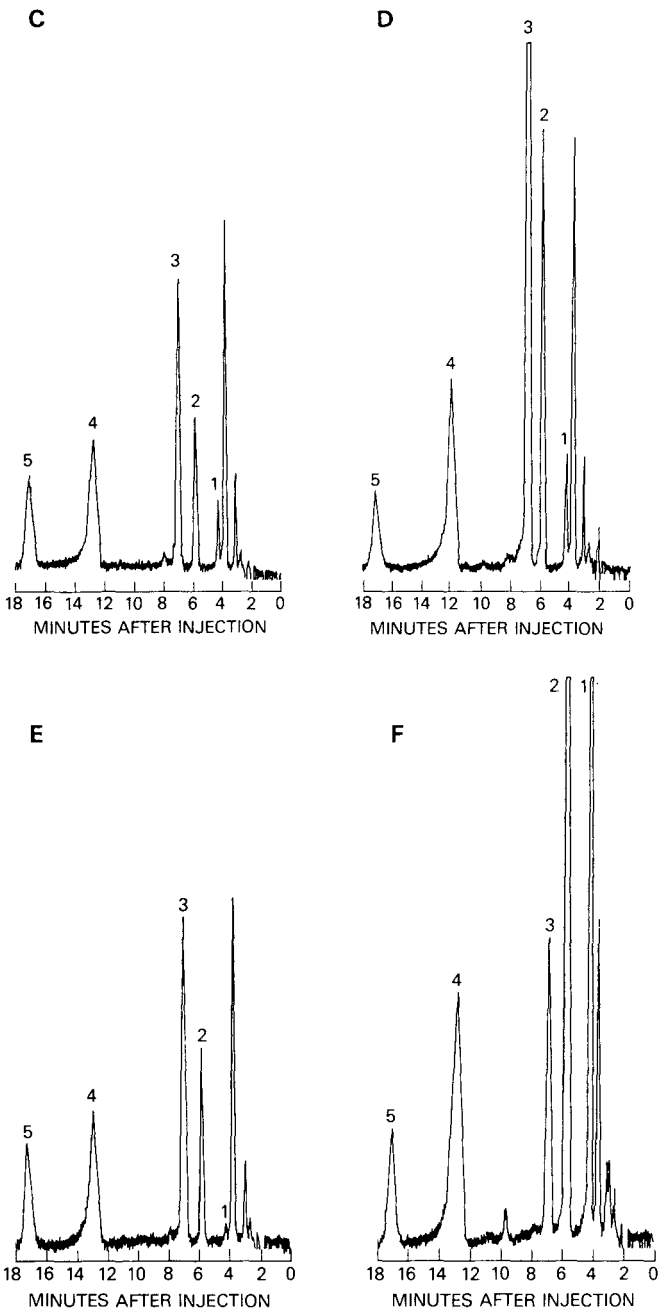

Fig. 2. Chromatogram of brain tissue extracts. (A) Midbrain tegmentum from an untreated rat, (B) same as A but with addition of 100 pg of 5-HTP, 0.60 ng of 5-HIAA, 1 ng of tryptophan, 1 ng of 5-HT, and 1 ng of NAS, (C) extract from midbrain tegmentum with 100 pg of 5-HTP added, (D) midbrain tegmentum from an animal injected with 100 mg/kg of tryptophan 2 h prior to sacrifice, (E) midbrain tegmentum from untreated rat without exogenous 5-HTP, and (F) midbrain tegmentum from an animal injected with 50 mg/kg of 5-HTP 1 h prior to sacrifice. Peaks: 1, 5-HTP; 2, 5-HIAA; 3, tryptophan; 4, 5-HT; and 5, NAS. All injections were made in a volume of 40 μl of which 20 μl was tissue extract.

The endogenous amount of 5-HTP was then calculated as the difference between the 5-HTP standard peak and the 5-HTP peak in the spiked tissue sample. This method of determination should be viewed with some caution, however.

The effects of tryptophan and 5-HTP loading can also be seen in Fig. 2D and F, respectively. An injection of 100 mg/kg of tryptophan 2 h prior to sacrifice substantially increased the brain levels of tryptophan, and moderately increased 5-HT and 5-HIAA while the levels of 5-HTP were not significantly changed. An injection of 50 mg/kg of 5-HTP 1 h prior to sacrifice did not change brain tryptophan levels but increased the levels of 5-HTP, 5-HT, and 5-HIAA. Note the extremely large amounts of tryptophan and 5-HTP remaining in brain after their respective injections (Fig. 2D and F).

The assay is linear over a wide range of standard concentrations as shown in Fig. 3. The chromatographic and detectability data for tryptophan, 5-HTP, 5-HT, 5-HIAA, and NAS are summarized in Table I. Although more sensitive methods are available (see ref. 10), the present method is sensitive enough to allow measurement of the 5-hydroxyindoles from brain tissue. Omission of the tissue dilution step (above) correspondingly increases the peak height of each compound in the brain extracts (data not shown). It should be pointed out too that the present detectability values were determined with a 5:1 signal-to-noise ratio. The coefficient of variation of the absolute fluorescence of 1.0 ng of 5-HT was 5.8% for inter-assay variability and 1.3% for intra-assay variability for all assays over a 2—3 month period. The coefficients of variation for the other indoles were similar to that of 5-HT.

Finally, this procedure has been used to analyze tryptophan, 5-HTP, 5-HT, and 5-HIAA in numerous rat brain areas in untreated animals and in animals injected with tryptophan and the results are summarized in Table II. Recoveries varied between 50—80%. In general, recovery of all compounds was higher with larger homogenization volumes.

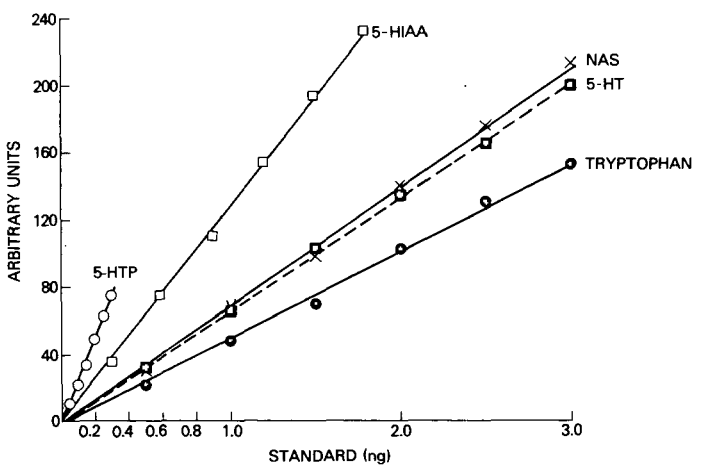

Fig. 3. Linearity of fluorescence response (in arbitrary units) with increasing quantities of 5-HTP, 5-HIAA, tryptophan, 5-HT, and NAS. Each data point represents the average of at least four determinations.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF 5-HTP, 5-HIAA, TRYPTOPHAN, 5-HT, AND NAS

The conditions are described in Methods; λ_{ex} = 285 nm, λ_{em} = 345 nm.

Compound	Retention time (min)	Limit of sensitivity* (pg)
5-HTP	4.2	40
5-HIAA	5.8	75
Tryptophan	6.8	190
5-HT	12.2	145
NAS	17.4	130

*Determined at a signal-to-noise ratio of 5:1.

TABLE II

LEVELS OF 5-HTP, 5-HIAA, TRYPTOPHAN, AND 5-HT IN VARIOUS BRAIN REGIONS FROM UNTREATED ANIMALS OR FROM ANIMALS INJECTED WITH 100 mg/kg TRYPTOPHAN 2 h PRIOR TO SACRIFICE

Values are expressed as ng/mg wet weight, n = 3.

	Tryptophan	5-HTP	5-HT	5-HIAA
Tegmentum	4.38 ± 0.36*	0.04 ± 0.01	1.24 ± 0.01	0.93 ± 0.07
	17.78 ± 4.4**	0.07 ± 0.01	1.92 ± 0.36	2.42 ± 0.29
Anterior	4.92 ± 0.37	0.04 ± 0.01	1.02 ± 0.04	0.52 ± 0.03
hypothalamus	14.03 ± 4.3	0.06 ± 0.01	1.49 ± 0.06	1.04 ± 0.13
NTS	5.18 ± 0.34	0.05 ± 0.01	1.08 ± 0.13	0.51 ± 0.03
	21.20 ± 0.47	0.12 ± 0.01	2.01 ± 0.10	1.61 ± 0.05
Striatum	5.52 ± 0.25	0.04 ± 0.01	0.63 ± 0.02	0.56 ± 0.01
	15.38 ± 4.6	0.04 ± 0.02	1.11 ± 0.15	0.85 ± 0.06

*Top row of numbers for each brain area represents values for untreated rats.
**Bottom row refers to values from tryptophan-treated rats.

DISCUSSION

Chromatography of deproteinized, buffered brain extracts on a reversed-phase column at pH 4.85 allows the resolution of tryptophan, 5-HTP, 5-HT, and 5-HIAA in discrete brain areas. These substances can then be easily quantitated by native fluorescence measurement. Although the present method lacks the absolute sensitivity of electrochemical detection [15—17], it is sensitive enough to allow the measurements of 5-HT, its amino acid precursors, and the major 5-HT metabolite 5-HIAA. The present method is also quite useful for experiments where animals have been treated with tryptophan or

5-HTP in order to increase brain 5-HT concentrations since these 5-HT precursors can be measured simultaneously.

Apart from quantitation of 5-HT levels in discrete brain regions the present method can be used to study the in vivo rates of 5-HT synthesis and turnover in animals treated with L-aromatic amino acid decarboxylase inhibitors (5-HTP accumulates) and with monoamine oxidase inhibitors (5-HT accumulates) or acid transport inhibitors (e.g., probenecid, 5-HIAA accumulates), respectively. This particular application was demonstrated recently by Reinhard et al. [16] using HPLC with electrochemical detection. Similarly, the in vitro assay of tryptophan hydroxylase, the initial and rate limiting enzyme in the biosynthesis of 5-HT [18], could be improved and extended from the presently employed methods [19—21] by virtue of the increased sensitivity and the ease of measurement of the substrate tryptophan offered by this HPLC method. Meek and co-workers [22, 23] were the first to apply 5-HTP detection by HPLC to the measurement of tryptophan hydroxylase activity. However, the concurrent measurement of tryptophan and 5-HTP levels will allow more careful kinetic analysis of tryptophan hydroxylase, including substrate utilization and reaction stoichiometry. Finally, the present method can be simply adapted to measure L-aromatic amino acid decarboxylase activity in vitro.

One drawback of the current procedure is the detection and quantitation of 5-HTP. Endogenous brain 5-HTP levels are normally quite low and detection and measurement by HPLC with electrochemical detection [15] offers no advantages over the present method. Although the 5-HTP peak is observable (see Fig. 2), it is juxtaposed to an unidentified fluorescent peak in tissue. The endogenous levels of 5-HTP can be estimated by difference without interference from the unidentified peaks, as described above, but quantitation presents no difficulty in rats injected with 5-HTP (Fig. 2F) or with an inhibitor of L-aromatic amino acid decarboxylase (unpublished observations).

In summary, the method described in this report has the advantages of allowing the simultaneous measurement of tryptophan, 5-HTP, 5-HT, and 5-HIAA in discrete brain areas with minimal tissue preparation. Most other HPLC methods for measuring the hydroxyindoles with fluorometric detection require various extraction or ion-exchange steps and these are often coupled with pre- or postcolumn (HPLC) fluorescence enhancement (see ref. 10 for review). Furthermore, fluorescence detection generally involves less technical maintenance than electrochemical detection and provides a more stable baseline. Finally, the present method is more versatile and is far easier to perform than the commonly used radioenzymatic procedures [24, 25] which only measure 5-HT, or the recently described radioimmunoassays for 5-HT and 5-HIAA [26].

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SIMULTANEOUS DETERMINATION OF THE THREE MAJOR MONOAMINE METABOLITES IN CEREBROSPINAL FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple method is described for the simultaneous determination of the three monoamine metabolites, 4-hydroxy-3-methoxyphenylacetic acid, 4-hydroxy-3-methoxyphenylethyleneglycol and 5-hydroxyindole-3-acetic acid, in cerebrospinal fluid by high-performance liquid chromatography with electrochemical detection. Quantitation is accomplished by the standard addition technique. Chromatographic peak heights are corrected for volume effects by comparison with the signal obtained for an added auxiliary reference substance. Sample preparation is kept to a minimum, involving precipitation of proteins by means of perchloric acid and subsequent neutralization. The reproducibility was estimated to be 10%. For one of the metabolites, 4-hydroxy-3-methoxyphenylethyleneglycol, a correlation between the results obtained by this method and a mass fragmentographic method was made, and a satisfactory correlation (r = 0.904, slope = 0.914, intercept 3.26 ng/ml) found. The sensitivity of the method is in the picogram range. The methodology has been applied to measure biogenic amine metabolites in both rabbit and human cerebrospinal fluid. The levels found are in agreement with previously reported values.

INTRODUCTION

The diagnostic significance of the monoamine metabolites, 4-hydroxy-3-methoxyphenylacetic acid (HVA), 4-hydroxy-3-methoxyphenylethyleneglycol (MHPG) and 5-hydroxyindole-3-acetic acid (5-HIAA) is a subject of intensive research in the field of neuropharmacology. The outflow of HVA, MHPG and 5-HIAA in body fluids like cerebrospinal fluid (CSF), plasma and urine is an indication of the turnover of the corresponding biogenic amines, dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT). As

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opposed to blood and urine, CSF is the source that probably best reflects the neuronal activity in the central nervous system.

Many assay methods for one or more of these metabolites in CSF have been described. The highest degree of sensitivity and specificity has been achieved using either gas chromatography with electron-capture or mass fragmentographic detection [1—15], or high-performance liquid chromatography (HPLC) using fluorimetric or electrochemical detection [16—21]. However, each of these techniques has shortcomings in at least one of the following criteria: cost of instrumentation, complex sample preparation involving multi-step derivatization, speed, sensitivity, specificity, flexibility, necessity of using internal standards to correct for and monitor the recovery during sample preparation. To our knowledge, only two studies, one employing gas chromatography with mass spectrometric detection, more specifically mass fragmentography, report the simultaneous determination of the three metabolites MHPG, HVA and 5-HIAA [11], and another, utilizing HPLC with electrochemical detection [21]. Although the technique of mass fragmentography is extremely well suited for the simultaneous determination of various compounds, it remains a very expensive technique which is only available in a few laboratories. Furthermore, the conditions are stringent: a derivatization and extraction procedure which works well for all compounds should be found, and deuterated analogues of the compounds should be available.

In this study, we describe a simple assay method which allows simultaneous determination of the title compounds, by HPLC with electrochemical detection. The method meets to a reasonable degree all the above mentioned criteria, so that it provides a valuable alternative to the more expensive and complex mass spectrometric methods. Compared to the published HPLC method [21], the present method has the advantage that the use of an auxiliary reference standard, i.e. 5-hydroxy-2-indolecarboxylic acid, allows correction for volume variations during sample work-up and injection, ensuring the precision of the determinations at the nanogram level.

MATERIALS AND METHODS

Chemicals

4-Hydroxy-3-methoxyphenylethyleneglycol piperazine salt (HMPG), DL-3-methoxy-4-hydroxymandelic acid (VMA) and DL-3,4-dihydroxymandelic acid (DOMA) were obtained from Calbiochem, Brussels, Belgium; 3,4-dihydroxyphenylacetic acid (DOPAC), DL-3,4-dihydroxyphenylglycol (DOPEG), 5-hydroxy-2-indolecarboxylic acid (5-HICA), homovanillic acid (HVA), 4-hydroxy-3-methoxybenzoic acid and 4-hydroxy-3-methoxybenzyl alcohol were purchased from Aldrich, Beerse, Belgium; β-(3,4-dihydroxyphenyl)-ethanol (DOPET) and 5-hydroxyindole-3-acetic acid (5-HIAA) were obtained from Serva, Heidelberg, G.F.R.; pentafluoropropionic acid anhydride (PFPA) was obtained from Pierce Chemical Co., Rockford, IL, U.S.A.; 1-(4-hydroxy-3-methoxy-phenyl)-1,2-ethane-1,2,2-[$^2H_3$]diol piperazine salt ([$^2H_3$]MHPG) was purchased from Merck, Sharp & Dohme, Pointe Claire-Dorval, Canada; and bovine serum albumin was from Sigma, St. Louis, MO, U.S.A. Inorganic salts and organic solvents were analytical grade from Merck, Darmstadt, G.F.R.; doubly distilled water was used throughout.

High-performance liquid chromatography

Instrumental conditions. The HPLC analyses were performed with a Model 6000 A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), equipped with a Model U6K universal sample injector (Waters Assoc.) and with an electrochemical detector (Model LC4, Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon working electrode. The detector was operated at a potential setting of +0.8 V vs. the reference electrode. The chromatographic trace was registered by a recorder supplied by Kipp & Zonen. A reversed-phase column RSil C18 HL (Alltech, Eke, Belgium, particle size 10 μm, 250 × 4.6 mm I.D.) was used for all experiments. The eluent consisted of a 0.07 *M* NaH_2PO_4 buffer, with 0.1 m*M* disodium EDTA and methanol, in a ratio 9:1. The pH of the eluent was carefully adjusted to 5.4 with sodium hydroxide, since slight variations in eluent pH had a pronounced effect on the retention times, especially those of the acidic metabolites. The solvent flow-rate was 1.5 ml/min, with a corresponding pressure of 10.3 MPa. The eluent was filtered through a Millipore type RA 1.2 μm filter, and degassed before use in an ultrasonic bath (Bransonic 92).

Sample preparation. CSF samples were collected from the cisterna magna of urethane-anesthetized rabbits, by a method previously described [22]. The rabbits were of either sex, weighing between 2.5 and 3.0 kg. Human CSF samples were collected by lumbar puncture from patients suffering from various psychiatric disorders. Samples were immediately frozen until analysed. The further processing of samples before injection is very simple and does not include an extraction step, since this step gives rise to difficulties with the reproducibility of the quantitation (see below). The sample work-up only involves a deproteinization step with perchloric acid, followed by neutralization of the supernatant prior to injection. Of the CSF sample, two 100-μl aliquots are transferred to conical Eppendorf tubes. To one of the aliquots, a mixture of the three metabolites MHPG, HVA and 5-HIAA is added in a 100-μl volume, to the other, 100 μl of doubly distilled water are added. In this way, one part of the sample is spiked with an amount of the three metabolites, approximately equal to the expected amount present in the sample. Typically, additions of 2.5 ng of MHPG and 9.5 ng of HVA and 5-HIAA were used to spike a 100-μl rabbit CSF sample.

For both the spiked and the unspiked aliquot, the rest of the sample work-up is identical: 10 ng of the auxiliary standard 5-hydroxy-2-indolecarboxylic acid (5-HICA) are added in 50 μl, followed by 50 μl of a 2.4 *M* $HClO_4$ solution. Subsequently the samples were kept in the refrigerator or in an ice-bath for 15 min to allow protein precipitation, after which they were centrifuged for 5 min in an Eppendorf 5414 centrifuge. Of the supernatant, 250 μl were then transferred to another Eppendorf tube, which already contained 50 μl of a 5 *M* sodium acetate solution. Of the final neutralized and buffered sample, an aliquot of 100 μl was injected into the chromatographic system.

Quantitative analysis. The ratio of the detector response (peak height) for each compound versus that for the auxiliary standard (5-HICA) was determined, and will be referred to as *R*. In this way, a correction is carried out for volume variations during sample preparation is carried out for volume variations during sample preparation and injection. Calculation of the

metabolite concentration is based on comparison of the spiked and unspiked sample chromatograms, according to the equation

$$X = \frac{R_{\text{X unspiked}}}{R_{\text{X spiked}} - R_{\text{X unspiked}}} \times Y$$

where X = amount of metabolite X in the unspiked sample (ng), Y = amount of added metabolite X in the spiked sample (ng).

Gas chromatography—mass spectrometry

Instrumental conditions. Gas chromatography—mass spectrometry (GC—MS) analysis was carried out on a Finnigan 4000 instrument, connected to an Incos 2000 data system, using the jet separator as GC—MS interface and with the mass spectrometer operated in the electron-impact ionization mode. GC was performed on a 2 m × 2 mm I.D. glass column, packed with 3% SP-2100 coated on Gas-Chrom Q 100—120 mesh (Supelco, Bellefonte, PA, U.S.A.), which was operated isothermally at 155°C. Helium was used as carrier gas at a flow-rate of 25 ml/min. Injector and GC—MS interface temperatures were 250°C and 230°C, respectively. The MS conditions were: electron energy, 70 eV; emission current, 0.3 mA; ion source temperature, 230°C. Using the Incos datasystem software, the mass spectrometer was programmed to monitor ions at *m/z* 311 and 622 for the detection of MHPG and at *m/z* 313 and 625 for the detection of [$^2H_3$]MHPG.

Sample preparation. For the mass fragmentographic analysis of MHPG, the procedure described by Bertilsson [7] was applied with minor modifications. The sample size used was 200 μl for analysis of rabbit CSF and 500 μl for human CSF. After addition of 0.1 volume of a 5% aqueous ascorbic acid solution and of [$^2H_3$]MHPG (16 and 8 ng for rabbit and human CSF, respectively) into 2 volumes of sodium acetate buffer (2.5 *M*, pH 6.2; saturated with NaCl), the samples were allowed to equilibrate for 1 h at room temperature. Subsequently, the samples were extracted twice with 3 ml of ethyl acetate. The combined extracts were evaporated under a nitrogen stream and the residues reacted with 100 μl of a mixture of PFPA and ethyl acetate (1:1, v/v) for 30 min at 60°C. Immediately before GC—MS analysis, the derivatizing reagent was evaporated and the residues redissolved into 15 μl of ethyl acetate. Aliquots of 1.5 μl were used for GC—MS analysis.

Quantitative analysis. Standard curves were obtained with five aqueous standard solutions, each containing known amounts of MHPG covering the concentration range of interest and bovine serum albumin (0.3 mg/ml), and by carrying these standards through the extraction and derivatization steps. Quantitative calculations were based on peak area ratios of the molecular ion record (at *m/z* 622) versus those of its $^2H_3$-labeled analogue (at *m/z* 625). Ratios for the standard mixtures were plotted against the concentration in ng/ml, and an unweighted least-squares linear regression analysis was performed. Using the regression parameters of the calibration curve, the unknown MHPG concentrations and their associated standard errors were estimated. The precision of the assay was found to be 3% (C.V.; n = 5) at the 30 ng/ml level.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic separation of a series of metabolites (DOMA, VMA, DOPEG, DOPAC, MHPG, 5-HIAA, HVA, DOPET) and the auxiliary standard (5-HICA), under the experimental conditions mentioned in the previous section. The position of peaks in the chromatogram can easily be modified by changing the methanol content or the pH of the eluent. Adding methanol will decrease the retention time of all compounds, whereas an increase in pH will decrease selectively the retention times of the acidic compounds (HVA, 5-HIAA, DOMA, VMA, DOPAC, 5-HICA) and will only slightly influence the position of neutral metabolites in the chromatogram. The pH effect is rather drastic (see also ref. 23). Therefore it is absolutely necessary to adjust the pH of the eluent very carefully in order to obtain identical chromatograms with satisfactory resolution between peaks, when proceeding with fresh eluent. We believe that it is possible to obtain a good separation of all these compounds with any commercially available reversed-phase column, simply by optimizing the pH and the methanol content of the eluent.

In order to determine quantitatively the metabolites in CSF, various procedures can be used. Precise and accurate results will only be obtained by

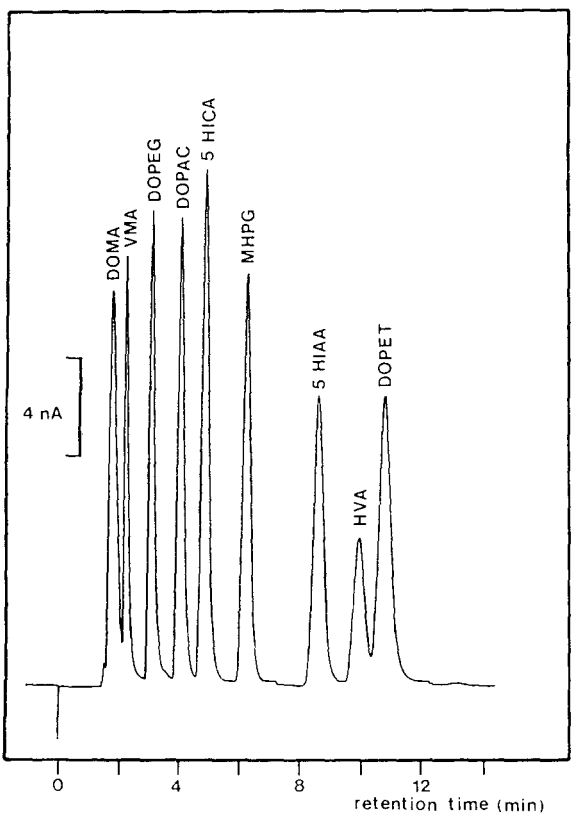

Fig. 1. Chromatogram of a standard mixture containing 25 ng each of DOMA, VMA, DOPEG, DOPAC, 5-HICA, MHPG, 5-HIAA, HVA and DOPET. Experimental conditions as mentioned in Materials and Methods.

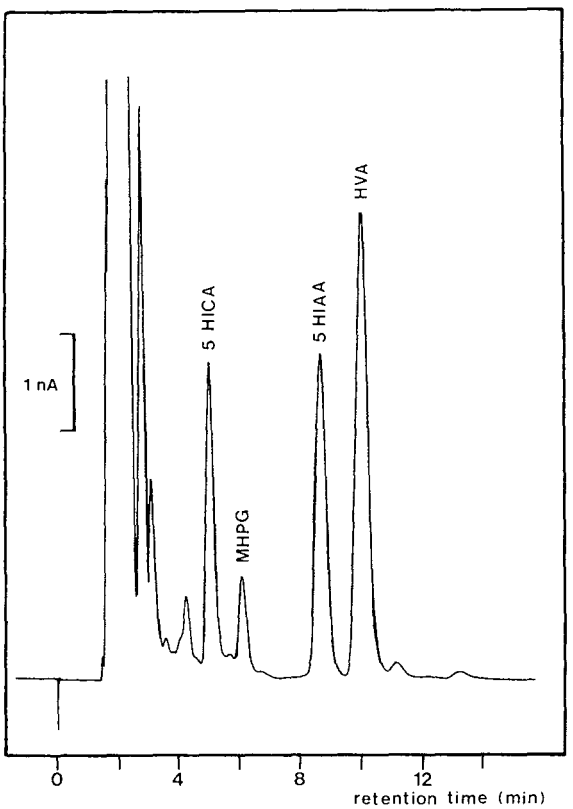

Fig. 2. Chromatogram obtained from an unspiked rabbit cisternal CSF sample. Experimental conditions and sample work-up as mentioned in Materials and Methods.

using an internal standard method. As internal standard one should preferably choose structurally related products, e.g. homologues, of the compounds to be determined, to correct for losses during the extraction procedure or other manipulations in the sample work-up. In preliminary experiments we tried to quantitate the monoamine metabolites with 5-HICA, 4-hydroxy-3-methoxybenzoic acid and 4-hydroxy-3-methoxybenzyl alcohol as internal standards for, respectively, 5-HIAA, HVA and MHPG, and with an ethyl acetate extraction as prepurification step. This procedure gave irreproducible results, even after the internal standard correction, probably because the compounds to be determined and their respective internal standards do not behave similarly during the extraction with ethyl acetate. Therefore we decided to use the standard addition technique, i.e. to use the products themselves as internal standards, and to calculate the metabolite content by comparison of the chromatograms of the spiked and non-spiked samples. Again this time, use of the extraction step gave rise to irreproducible results. Consequently, we decided to eliminate the extraction step by injecting the deproteinized and neutralized CSF supernatant directly into the HPLC system, as described in the Methods section. By proceeding in this manner, we were able to obtain chromatograms for CSF samples that were free from interference, as illustrated in Fig. 2. Satisfactory

resolution is obtained for the three major monoamine metabolites MHPG, 5-HIAA and HVA, and the auxiliary standard 5-HICA. Injections can be repeated every 12 min. Eventually a short precolumn, with backflush possibility, can be incorporated into the chromatographic system, in order to avoid possible contamination of the analytical column. It is our experience, however, that several hundreds of injections directly on the analytical column can be performed without loss of column performance. Peak identification was based on retention behavior and co-chromatography with the standard reference compounds under various chromatographic conditions. The linearity of the method was evaluated by spiking CSF pool samples with increasing amounts of the three metabolites. Plotting the R values (metabolite peak height versus 5-HICA peak height) versus the amount of added metabolite gave linear relationships with satisfactory correlation coefficients (r = 0.9987, 0.9955 and 0.9878 for MHPG, 5-HIAA and HVA, respectively), as is illustrated in Fig. 3. The intercept of the regression lines in Fig. 3 (calculated by linear least-squares analysis), divided by their slopes, gives the amount of metabolite (ng) present in the initial pool sample. For routine measurements, only one spiked sample with one unspiked sample is analysed.

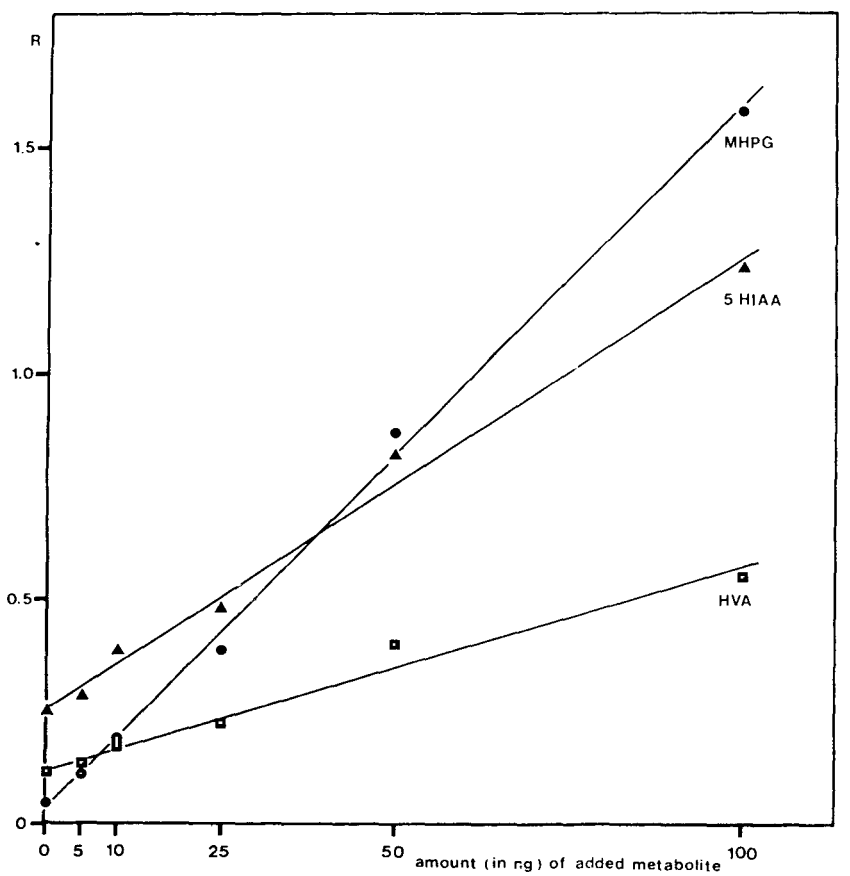

Fig. 3. Plot of R values (metabolite peak height versus 5-HICA peak height) versus the amount of metabolite added (in ng) to a rabbit cisternal CSF sample. Experimental conditions and sample work-up as described in Materials and Methods.

TABLE I

METABOLITE LEVELS IN HUMAN AND RABBIT CSF, DETERMINED BY THE PRESENT METHOD

Results are expressed as mean ± standard deviation. The number of samples is indicated in parentheses.

Sample origin	Concentration (ng/ml)		
	MHPG	5-HIAA	HVA
Human lumbar CSF	5.3 ± 2.4 (7)	19.8 ± 5.1 (11)	40.7 ± 20 (11)
Rabbit cisternal CSF	28 ± 9 (11)	111 ± 17 (12)	136 ± 31 (12)
Rabbit CSF pool*	45 ± 4 (9)	170 ± 15 (9)	298 ± 38 (9)

*CSF pool originating from non-treated and drug-treated rabbits.

In Table I are summarized the results of the present assay method, as applied to both human and rabbit CSF samples. The data for human lumbar CSF samples are in agreement with previously reported values [11]. It should be mentioned that the value obtained for human CSF only represents a mean of values obtained from patients with various psychiatric disorders, different age, sex, etc. The figures for rabbit cisternal CSF are consistently higher than for human CSF and are also in agreement with literature values [24--26]. The standard deviation is smaller here, probably because all animals were healthy, of the same weight and were treated in the same manner (anesthetized with urethane). To test the reproducibility of the present method, a CSF pool, originating from non-treated and drug-treated animals, was divided into 100-μl aliquots and analysed. As shown in Table I, the reproducibility (expressed as standard deviation) is about 9% for MHPG and 5-HIAA, and 13% for HVA. In order to obtain reproducible results it is essential to use precise volumetric material (i.e. glass micropipettes) for the transfer of CSF samples and for the addition of standards. The volumetric errors associated with the further processing of the sample are corrected for by comparison with the auxiliary reference substance (5-HICA).

For a number of CSF samples (both human and rabbit) we determined MHPG both by the present HPLC method and by the mass fragmentographic method described by Bertilsson [7] (see also Methods section). Since mass fragmentography is by far the most sensitive and specific method for quantitative analysis, a comparison of the results of the two methods will give an idea of the validity of the alternative HPLC method. A correlation of $r = 0.904$ was found, which is acceptable. The regression line equation is $Y = 0.914X + 3.26$ (ng/ml) (Fig. 4).

From the results of this correlation, and the reproducibility data given in Table I, we can conclude that for the determination of MHPG, and probably also for the other metabolites 5-HIAA and HVA which occur at higher levels in CSF, the present method offers an alternative which can easily be applied to routine analysis, and which does not require costly instrumentation and extensive samples preparation. For most of the samples we analysed, the

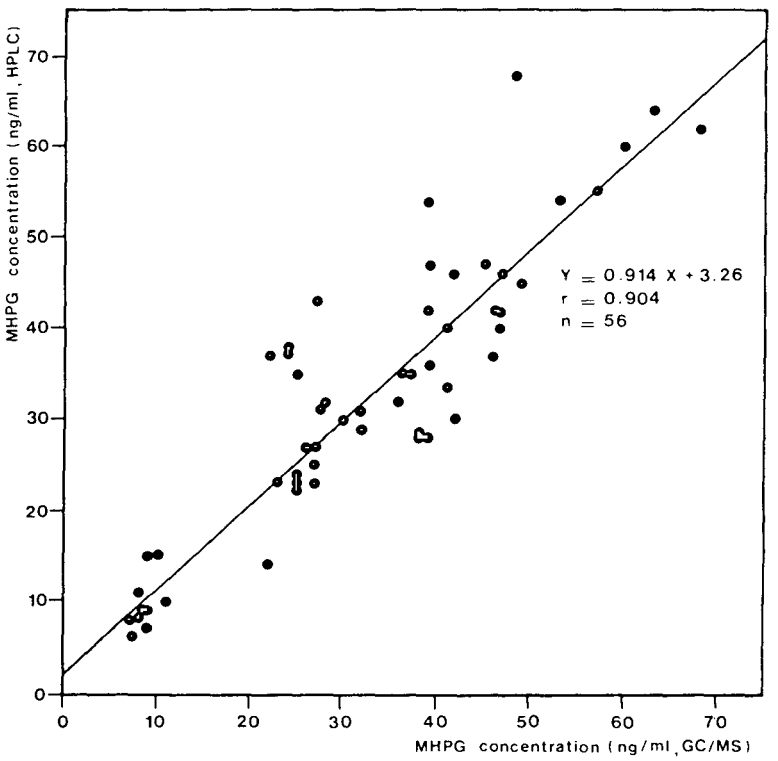

Fig. 4. Correlation between data sets obtained by mass fragmentography and the HPLC method, for MHPG levels in rabbit cisternal and human lumbar CSF. Experimental conditions and sample work-up for both methods are described in Materials and Methods.

metabolite levels were high enough to allow accurate detection, starting from a 100-μl CSF sample. The detector response remains appropriate for most metabolite levels encountered, which correspond typically with injections on column between 500 pg (for MHPG) and 15 ng (for HVA). Only when high sensitivity is required, e.g. for the determination of MHPG in human lumbar CSF, where the reproducibility of the HPLC method remains rather poor, does mass fragmentography remain the technique of choice.

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SEPARATION OF THE INDOLEALKYLAMINES AND THEIR ANALOGOUS TETRAHYDRO-β-CARBOLINES BY LIQUID CHROMATOGRAPHY

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SUMMARY

Liquid chromatographic procedures for the separation of the N-methylated and analogous tetrahydro-β-carboline derivatives of 5-hydroxytryptamine, 5-methoxytryptamine, and tryptamine are described. The methods involve the use of normal-phase chromatography and chromatography on both ODS and strong cation-exchange columns. The judicious use of these procedures allows the unique chromatographic characterisation of each of the compounds studied. When combined with fluorescence monitoring of the column eluents sub-nanogram quantities of both the indolealkylamines and tetrahydro-β-carbolines can be detected.

INTRODUCTION

Both the psychotomimetic indolealkylamines [1—4] and their structurally analogous tetrahydro-β-carbolines [5—9] have now been detected in mammalian body fluids and tissues including those of the human. Enzymatic mechanisms for the in vivo and in vitro synthesis of these compounds have also been described [10—13].

While liquid chromatographic procedures for the separation of the indole-

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amines and their β-carboline derivatives, prior to analysis, have previously been described [6, 14, 15] on-line detection has largely been confined to UV monitoring.

In a recent study [10] we presented a rapid liquid chromatographic procedure for the separation and detection of the products of rabbit lung indoleamine N-methyltransferase activity. In addition a preliminary study of the separation of tryptamine, its N-methylated derivatives and their β-carboline analogues was presented. We now report a detailed and systematic study of the separation and detection of the N-methylated derivatives and analogous β-carbolines of tryptamine, 5-hydroxytryptamine and 5-methoxytryptamine. Several chromatographic systems involving the use of strong cation-exchange, silica and ODS reversed-phase columns are described. The judicious use of these procedures may permit the unique chromatographic characterisation of each of these compounds. When combined with fluorometric detection subnanogram quantities of both the indolealkylamines and β-carbolines can be detected.

EXPERIMENTAL

Materials

L-Cysteine hydrochloride, N,N-dimethyltryptamine (DMT), N-methyltryptamine (NMT), 5-hydroxytryptamine (serotonin) creatinine sulphate (5HT), 5-hydroxy-N,N-dimethyltryptamine (bufotenin) oxalate (5OHDMT), 5-methoxytryptamine hydrochloride and 5-methoxy-N,N-dimethyltryptamine (5MeODMT) were purchased from Sigma (St. Louis, MO, U.S.A.); tryptamine hydrochloride from Calbiochem (Los Angeles, CA, U.S.A.); and N-methyl-5-hydroxytryptamine oxalate (NMS) from Aldrich (Milwaukee, WI, U.S.A.). 1,2,3,4-Tetrahydro-β-carboline (THBC), 2-methyl-1,2,3,4-tetrahydro-β-carboline (MTHBC), 6-hydroxy-1,2,3,4-tetrahydro-β-carboline (6OHTHBC), 6-methoxy-1,2,3,4-tetrahydro-β-carboline hydrochloride (6MeOTHBC), and 5-methoxy-N-methyltryptamine (5MeONMT) were kindly donated by Dr. S.A. Barker, University of Alabama.

All solvents used for chromatography were of analytical purity and glass distilled prior to use. The mobile phase for the silica column (anhydrous methanol containing 0.4% 5 *M* aqueous ammonia and 1 m*M* cysteine hydrochloride) was stored at room temperature for at least 24 h and any precipitate formed was removed by filtration prior to use of the mobile phase.

Instrumentation

Separation of the indoleamines and their analogous β-carbolines was achieved on a Perkin-Elmer Series 3B liquid chromatograph using a silica column (DuPont Zorbax, 25 cm × 4.6 mm I.D., 6 μm). The mobile phase was anhydrous methanol containing 0.4% aqueous ammonia and 1 m*M* cysteine hydrochloride. The flow-rate was 1.5 ml/min.

Separations were also achieved using a strong cation-exchange column (Whatman Partisil 10 SCX, 25 cm × 4.6 mm I.D., 10 μm). The mobile phase was methanol—0.053 *M* phosphoric acid/ammonia buffer, pH 4.0 (30:70) with a flow-rate of 1.5 ml/min. A reversed-phase chromatographic procedure

involving an ODS column (DuPont Zorbax 25 cm × 4.6 mm I.D., 6 μm) with a mobile phase consisting of acetonitrile—0.053 *M* phosphoric acid/ammonia buffer, pH 7.0 (70:30) at a flow-rate of 2 ml/min was also used.

The spectroscopic detectors used included a Perkin-Elmer 650-10S fluorescence spectrometer, a Perkin-Elmer 3000 fluorescence spectrometer and a Perkin-Elmer LC-75 variable-wavelength UV detector with an auto control scanning accessory.

Spectroscopic studies

The optimum wavelengths for detection by both fluorescence spectroscopy and UV absorption spectroscopy were determined for each species by halting the flow of the mobile phase as each component entered the appropriate detector flow cell. Excitation and emission spectra were recorded and the wavelengths corresponding to the maximum fluorescence of each indoleamine species were determined. The wavelengths of maximum UV absorption were obtained by scanning the UV spectra using the LC-75 auto control unit.

RESULTS

Spectroscopic detection

During the development of procedures for the separation of the indoleamines and their analogous β-carbolines on a silica column a number of param-

TABLE I

SPECTRAL CHARACTERISTICS OF INDOLEAMINES AND β-CARBOLINES CHROMATOGRAPHED ON SILICA, PARTISIL 10 SCX AND ZORBAX ODS COLUMNS

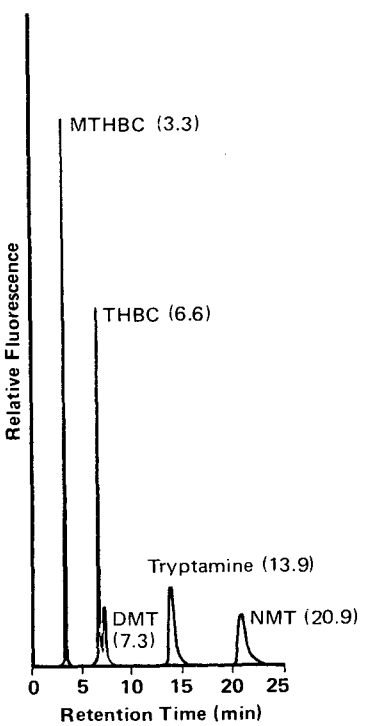
Indoleamines and β-carbolines (10 nmol) were chromatographed on a silica, SCX* or ODS** column and the UV absorption and fluorescence spectra recorded as described under Methods. Minimum detectable limits (MDL) (fluorescence) were estimated by analysis of peak heights obtained after chromatography of 0.5—5.0 pmol of each derivative. Each value represents the mean of three determinations. The coefficients of variation in peak heights were < 5%.

Compound	λ_{max} UV (nm)	λ_{ex} fluorescence (nm)	λ_{em} fluorescence (nm)	MDL fluorescence (pmol)
Tryptamine	220; 220**	278; 276**	348; 346**	0.16; 0.07**
NMT	220; 219**	279; 275**	348; 343**	1.2; 0.3**
DMT	221; 219**	278; 276**	347; 343**	0.4; 0.62**
5HT	222	276	338	0.11
NMS	220	276	337	0.82
5OHDMT	222	276	337	0.06
5-Methoxy-tryptamine	221; 220*; 220**	273; 270*; 274**	334; 335*; 335**	0.12; 0.09*; 0.03**
5MeONMT	220; 220*; 220**	276; 270*; 272**	335; 333*; 335**	0.36, 0.12*; 0.08**
5MeODMT	222; 220*; 220**	277; 270*; 271**	335; 336*; 334**	0.09; 0.24*; 0.08**
THBC	225; 220*; 220**	278; 265*; 269**	350; 345*; 345**	0.18; 0.1*; 0.12**
MTHBC	223; 220*; 219**	278; 265*; 271**	349; 343*; 346**	0.06; 0.3*; 0.19**
6OHTHBC	222; 220*	276; 269*	333; 334*	0.22; 0.07*
6MeOTHBC	225; 218*; 220**	277; 270*; 269**	333; 332*; 332**	0.23; 0.08*; 0.08**

eters for their detection and estimation both by fluorescence spectroscopy and UV absorption spectroscopy were examined.

In Table I the excitation and emission wavelengths for maximum fluorescence of the indoleamines and β-carbolines are presented together with the minimum detectable limits (defined as signal-to-noise ratio > 2) for fluorescence detection. The values of λ_{max} for the absorption spectra are also given in Table I.

In addition to silica, separations of the various indoleamines and β-carbolines were also achieved on Partisil 10 SCX and Zorbax ODS columns. The optimum wavelengths for detection by both fluorescence and UV absorption together with the minimum detectable limits for fluorescence detection for 5-methoxytryptamine, its N-methylated derivatives, and a number of β-carboline analogues of the N-methylated derivatives of 5HT, tryptamine and 5-methoxytryptamine, chromatographed on Partisil 10 SCX, are presented



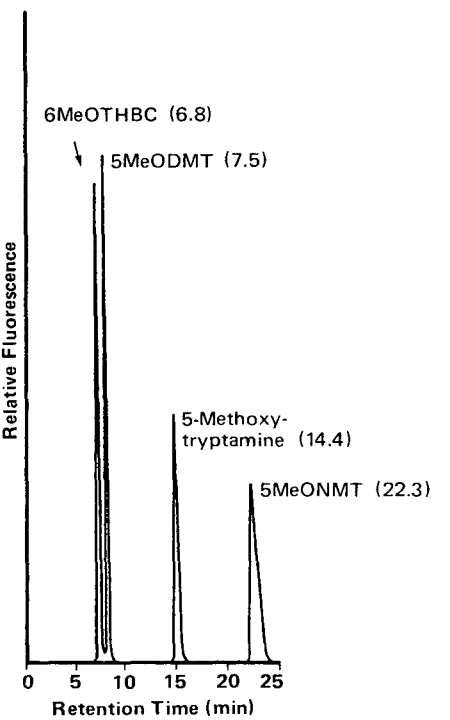


Fig. 1. Chromatogram of tryptamine, its N-methylated derivatives and their analogous β-carbolines. A mixture composed of 2 nmol of each derivative in 10 μl of methanol was applied to the DuPont silica column. The compounds were detected by fluorescence spectroscopy (λ_{ex} 278 nm, λ_{em} 347 nm). Retention times are indicated in parentheses.

Fig. 2. Chromatogram of 5-methoxytryptamine, its N-methylated derivatives and 6MeOTHBC. A mixture composed of 2 nmol of each derivative in 10 μl of methanol was applied to the silica column. The compounds were detected by fluorescence spectroscopy (λ_{ex} 277 nm, λ_{em} 335 nm).

in Table I. Similar data for tryptamine, 5-methoxytryptamine and their N-methylated derivatives and analogous β-carbolines separated on Zorbax ODS are also presented.

Chromatography

To achieve complete separation of tryptamine, 5HT, 5-methoxytryptamine and their various N-methylated derivatives and analogous β-carbolines a number of chromatographic procedures employing a silica column, a strong cation-exchange column and an ODS reversed-phase column were utilised.

A chromatogram of a mixture containing tryptamine, NMT, DMT, THBC and MTHBC separated on a DuPont silica column is presented in Fig. 1. A similar chromatogram illustrating the complete separation of 5-methoxytryptamine, 5MeONMT, 5MeODMT and 6MeOTHBC is also presented (Fig. 2). During the chromatography of 5HT, NMS, 5OHDMT and 6OHTHBC the resolution of 5OHDMT and 6OHTHBC remained incomplete (Fig. 3). Our studies have indicated that complete separation of these two compounds can be achieved by reducing the concentration of aqueous ammonia in the mobile phase to 0.2% although this is accompanied by marked increases in the retention times of 5HT and NMS. While the silica column permits the complete separation of tryptamine, 5HT and 5-methoxytryptamine from

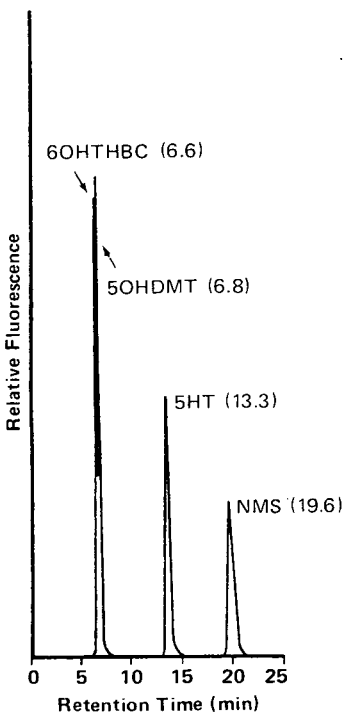


Fig. 3. Chromatogram of 5HT, its N-methylated derivatives and 6OHTHBC. A mixture composed of 2 nmol of each derivative in 10 μl of methanol was applied to the silica column. The compounds were detected by fluorescence spectroscopy (λ_{ex} 276 nm, λ_{em} 337 nm).

their respective N-methylated derivatives and β-carbolines, structurally analogous members of these three indoleamine series were found to display very similar chromatographic characteristics. To permit the separation and unique characterisation of these various derivatives, additional chromatographic procedures involving both a strong cation-exchange column and an ODS reversed-phase column were developed. Complete resolution of the mono- and N,N-dimethylated derivatives of 5HT and tryptamine (Fig. 4) and also those of 5-methoxytryptamine (Fig. 5) was achieved using a Partisil 10 SCX column. While the related β-carbolines 6OHTHBC, THBC, and MTHBC could also be completely separated from one another on the SCX column, in contrast to their behaviour on silica, they remain unresolved from their corresponding indolealkylamine analogues. While the Partisil 10 SCX column is invaluable in permitting the separation of the 5-hydroxylated indolealkylamines and β-carbolines from either the tryptamine or 5-methoxytryptamine series of analogues, members of the tryptamine and 5-methoxytryptamine series of derivatives could not be resolved from one another by this chromatographic system.

To achieve the desired separation of the mono- and N,N-methylated derivatives of tryptamine and 5-methoxytryptamine, chromatography on a Zorbax ODS column was employed. Under these conditions complete separation of

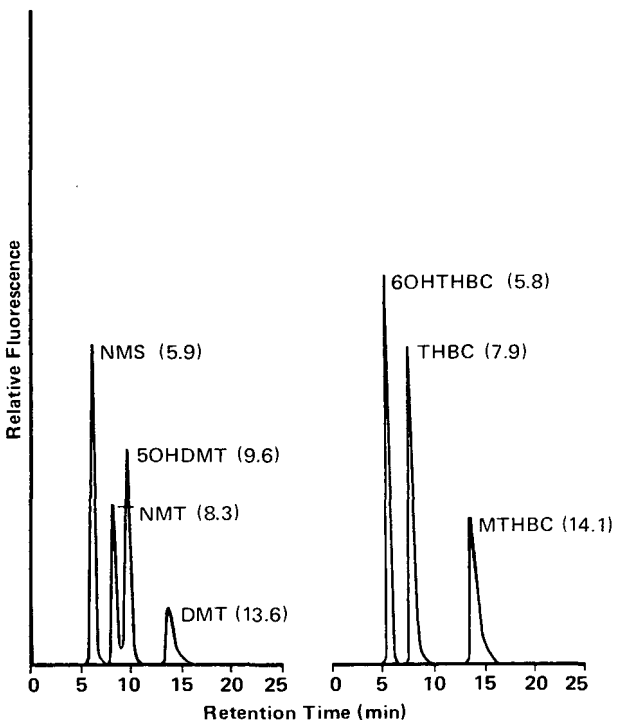


Fig. 4. Chromatograms of N-methylated derivatives of tryptamine and 5HT (left) and their analogous β-carbolines (right). A mixture composed of 2 nmol of each derivative in 10 μl of methanol was applied to a Partisil 10 SCX column. The compounds were detected by fluorescence spectroscopy (λ_{ex} 280 nm, λ_{em} 357 nm).

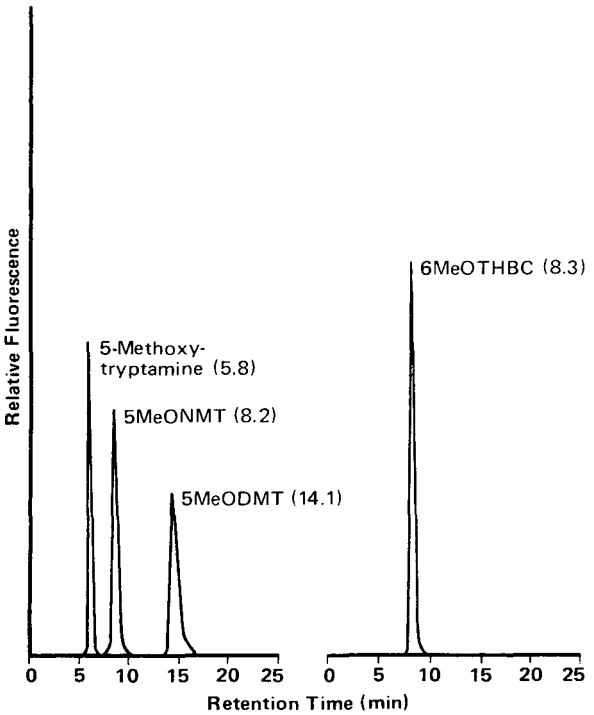


Fig. 5. Chromatograms of 5-methoxytryptamine its N-methylated derivatives (left) and 6MeOTHBC (right). A mixture composed of 2 nmol of each derivative in 10 μl of methanol was applied to a Partisil 10 SCX column. The compounds were detected by fluorescence spectroscopy (λ_{ex} 270 nm, λ_{em} 336 nm).

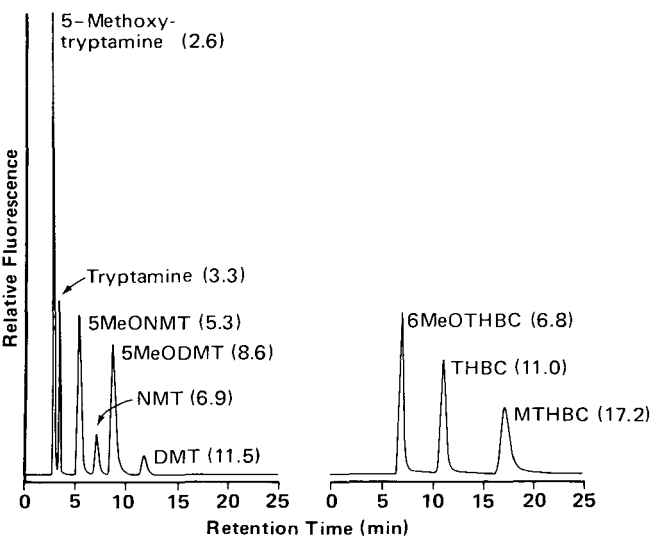


Fig. 6. Chromatograms of tryptamine, 5-methoxytryptamine their N-methylated derivatives (left) and analogous β-carbolines (right). A mixture composed of 1 nmol of each derivative in 10 μl of methanol was applied to a Zorbax ODS column. The compounds were detected by fluorescence spectroscopy (λ_{ex} 276 nm, λ_{em} 343 nm).

the methylated derivatives of both tryptamine and 5-methoxytryptamine could be achieved (Fig. 6). The β-carbolines 6MeOTHBC, THBC and MTHBC were also completely resolved from one another.

DISCUSSION

In a preliminary study [10] we reported that the mono- and N,N-dimethylated derivatives of tryptamine and their tetrahydro-β-carboline analogues can be separated by normal-phase liquid chromatography on a silica column. In this initial study, however, detection was limited to UV monitoring at 220 nm and indoleamines other than those of the tryptamine series were not examined.

Our current studies have shown that in addition to the separation of the tryptamine derivatives, the N-methylated and tetrahydro-β-carboline analogues of 5HT and 5-methoxytryptamine can also be resolved on silica. The chromatographic characteristics of analogous members of these three series of indoleamines were found to be very similar indicating that the substituent at the 5-position of the indole moiety (6-position of the β-carboline moiety) has only a minor influence on the chromatography of these compounds on silica. Although samples of 6-methoxy-2-methyl-tetrahydro-β-carboline and 6-hydroxy-2 methyl-tetrahydro-β-carboline were not available, by analogy with the behaviour of other derivatives in this series, their chromatographic characteristics are likely to be similar to those of MTHBC.

In contrast to normal-phase chromatography, during ion-exchange chromatography on Partisil 10 SCX the retention time characteristics of the indolealkylamines remain very similar to those of their respective analogous tetrahydro-β-carbolines. While chromatography on Zorbax ODS has the potential to resolve NMT, DMT and 5MeONMT from their individual tetrahydro-β-carboline analogues simultaneous separation of this series of compounds could not be achieved. These chromatographic systems however are invaluable for the separation of analogous members of the three indoleamine and tetrahydro-β-carboline series because of the differential influence of the hydroxy, methoxy and hydrogen substituents at the 5-position of the indole moiety and the 6-position of the β-carboline moiety on the chromatography of these compounds.

On-line detection of the indolealkylamines and tetrahydro-β-carbolines can be achieved by either UV absorption spectroscopy or by fluorescence spectroscopy. We have previously compared the use of both these techniques for the detection of 5HT, tryptamine and their mono- and N,N-dimethylated derivatives chromatographed on Partisil 10 SCX [10]. In addition to affording greater specificity the use of fluorescence spectroscopy greatly enhanced the detectability of these compounds. The minimum detectable amounts for fluorescence detection of the indolealkylamines and tetrahydro-β-carbolines chromatographed using the systems described above are within the sub-nanogram range.

The ability of normal-phase chromatography to group the various indoleamines and tetrahydro-β-carbolines according to their degree of N-methylation affords a unique method for the purification of specific classes of these

compounds. We have recently described the use of such procedures for the purification of 5OHDMT and DMT isolated from human urine [16]. When combined with analysis by reversed-phase or cation-exchange chromatography particularly in combination with fluorescence detection, such techniques may provide highly sensitive and specific methods for the detection and quantitation of indolealkylamines and tetrahydro-β-carbolines isolated from body fluids [16].

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PURIFICATION OF SALT-SOLUBLE CROSS-LINKED ELASTIN BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

The hydrophobic protein elastin, which is a major constituent of vascular and lung tissue is fragmented in several pathological conditions. The nature of the soluble fragments is not well understood. Such fragments bind to alkyl chains linked to agarose. Elution, which is effected by dimethylformamide and sodium dodecyl sulphate, is optimal from the decyl-agarose column. Dialysis of the eluates against buffered sodium chloride precipitates elastin, thus further purifying the salt-soluble cross-linked elastin.

INTRODUCTION

Fibrous elastin, which is an important constituent of several tissues, including blood vessels, lung and skin, is one of the most stable mammalian proteins [1—3]. In disease states such as atherosclerosis, emphysema and pseudoxanthoma elasticum the degradation rate of elastin is increased [4, 5]. Also, insoluble elastin is strongly hydrophobic [4, 6]. Therefore it is likely that soluble polypeptide fragments of elastin extracted from tissue might exhibit hydrophobic properties different from the other proteins in the extract. This suggests that hydrophobic interaction chromatography might be useful for isolating soluble elastin from tissue extracts. Additionally, it has been shown that both α-elastin and soluble degraded elastin bind to decyl-agarose [7]. This report presents an analysis of the binding and eluting properties of elastin fragments when such fragments are applied to agarose columns substituted with variable length aliphatic carbon chain ligands.

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MATERIALS AND METHODS

Animal surgery and tissue extraction

Arteriovenous fistulae were fashioned between the right external jugular vein and the right common carotid artery in sheep as described by Stehbens [8]. At various times post-operatively (between 352 and 580 days), the vascular tissue was removed and the loose, perivascular fascia and fat were discarded. The venous tissue in the vicinity of the anastomoses was removed, weighed and stored at −70° C until required.

The cutting of the tissue and the extraction with 0.2 *M* sodium chloride in 0.02 *M* sodium phosphate, pH 7.4 (extraction buffer) were performed as previously described [9]. In brief, this involved cutting the tissue finely, shaking for 16 h at 4° C, followed by centrifugation at 28,000 *g* for 40 min at 4° C. The supernatant was used for the chromatography of soluble elastin.

Hydrophobic interaction chromatography

Tissue extracts (0.5 mg protein) were applied at room temperature to 1-ml columns of alkyl-substituted agarose (Miles-Yeda, Rehovot, Israel) which had been previously equilibrated with extraction buffer. The agarose was substituted with alkyl chains containing either 0(C_0), 2(C_2), 4(C_4), 6(C_6), 8(C_8), or 10(C_{10}) carbons. The columns were developed firstly with 2 ml of extraction buffer followed by 2 ml of 50% dimethylformamide (DMF), 2 ml of 1% sodium dodecyl sulphate (SDS) and finally a further 2 ml of extraction buffer.

Electrophoresis

SDS—slab gel electrophoresis using a 10% polyacrylamide running gel and a 4% polyacrylamide stacking gel was performed according to the method of Laemmli [10] with the following modification. The Tris—HCl separating gel buffer was at a pH of 9.5.

The total volume of each eluate was dialysed against water, lyophilized and redissolved in 150 μl of a solution of 1.5 *M* Tris—HCl, pH 8.8, 3.0% SDS, 0.1% bromophenol blue and 15% glycerol. Where indicated, β-mercaptoethanol at a final concentration of 1% was added. These samples were placed in boiling water for 3 min before being electrophoresed on polyacrylamide gels. After electrophoresis gels were stained with 0.1% Coomassie Blue dissolved in 50% methanol—10% acetic acid and then destained with a 10% methanol—7% acetic acid mixture.

Precipitate formation

The formation of an insoluble fraction from the different eluates occurred spontaneously on dialysis against 2 × 1 l of extraction buffer at room temperature for 24 h. Centrifugation at room temperature at 2000 *g* for 20 min sedimented the insoluble material. The supernatant was discarded and the residue was washed twice with extraction buffer.

Hydrolysis and amino acid analysis

Protein hydrolysis was performed in vacuo in constant boiling hydrochloric

acid for 65 h at 110°C. Amino acid analysis was conducted on a Beckman 119CL amino acid analyzer.

Protein determination

Two methods of determining protein levels were used. The concentration in the tissue extract was estimated by the method of Lowry et al. [11] using bovine serum albumin as the standard. The protein concentration in the column fractions was determined from the amino acid composition of aliquots that had been hydrolyzed and analyzed.

RESULTS

Aliquots of the buffered sodium chloride extracts from the venous extracts were applied to each of the six columns of agarose containing different alkyl ligands. The columns were then eluted sequentially with extraction buffer, DMF, SDS and extraction buffer again as described in Materials and Methods.

SDS—polyacrylamide gel electrophoresis

The distribution of protein eluted by each of the solvents when passed through each of the columns is illustrated in the SDS—polyacrylamide gel patterns in Fig. 1. These gel patterns indicate that all of the column eluates are heterogeneous. All display a number of bands over a wide range of molecular sizes. The gel of extraction buffer eluates confirms that the longer carbon chain ligands retain more protein (Fig. 1A). However this trend does show some variability with different tissue extracts. With each of the extracts examined, DMF consistently elutes the greatest amount of protein from the C_8 and C_{10} columns (Fig. 1B). The gel electrophoresis of the SDS and the second

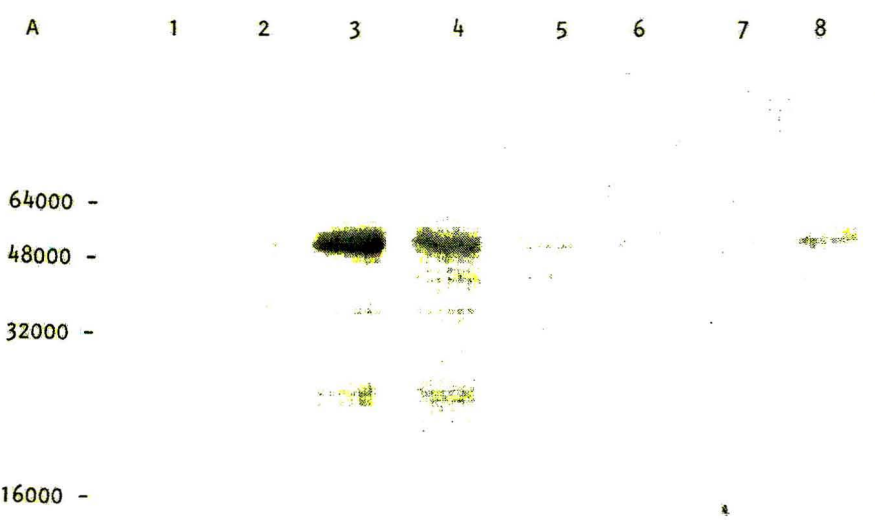

Fig. 1.

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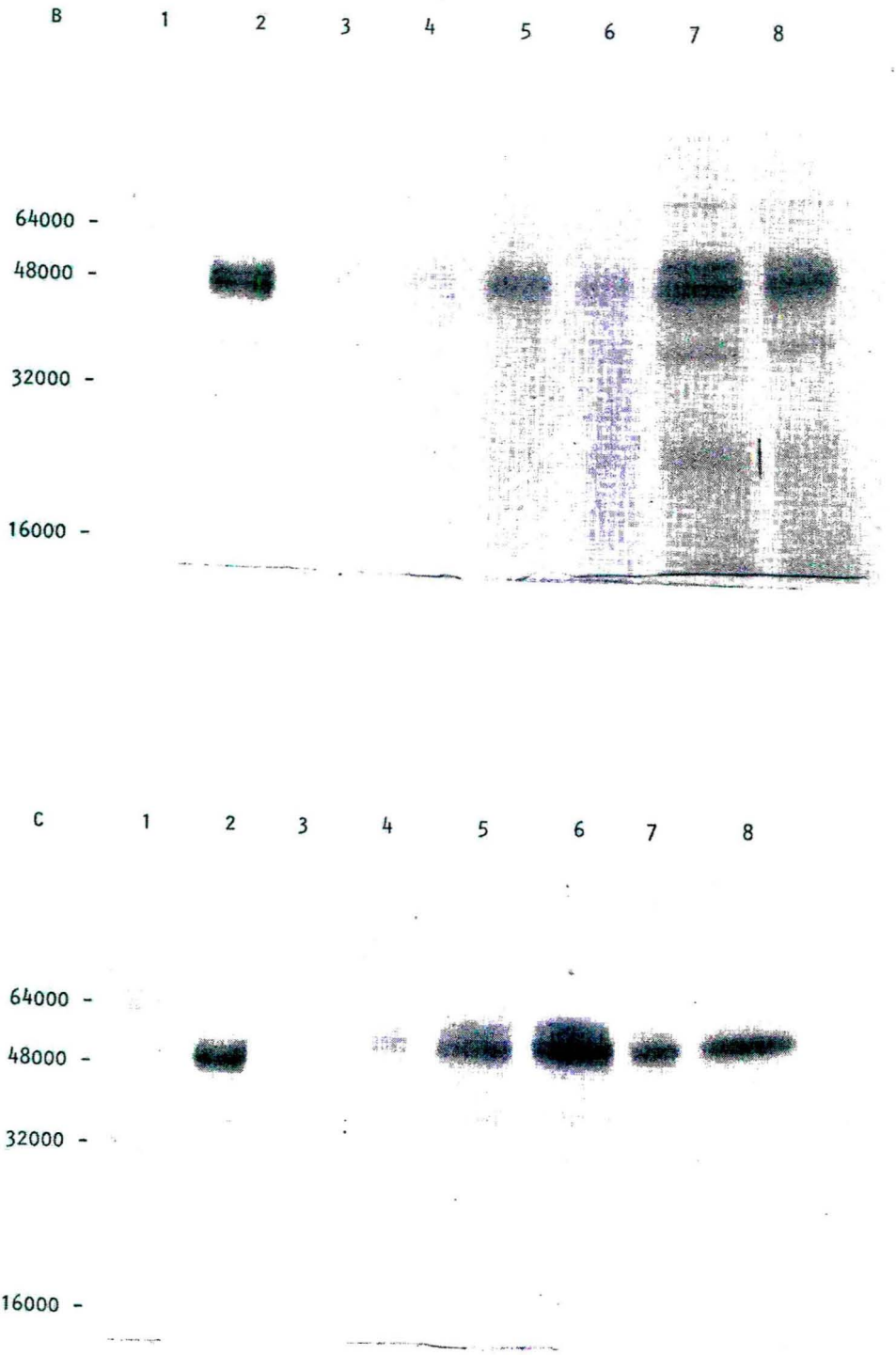
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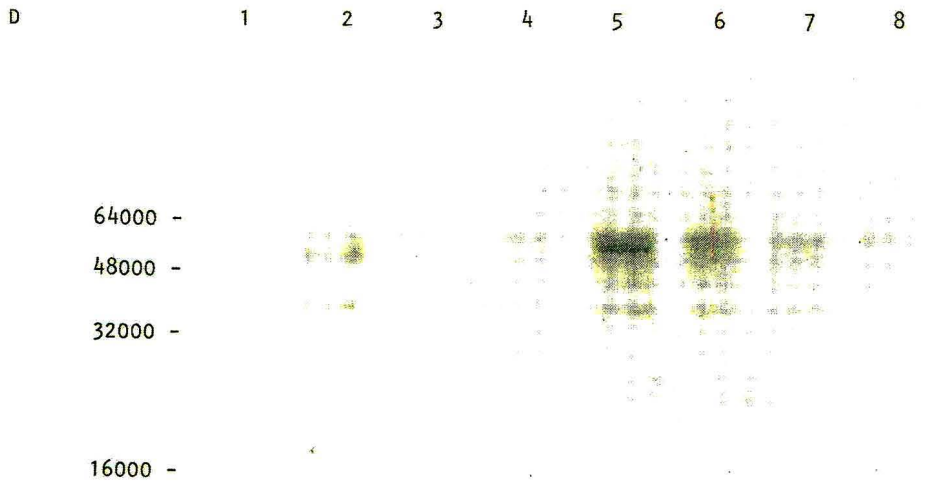

Fig. 1. SDS—polyacrylamide gel electrophoresis of eluates from agarose column substituted with alkyl chains. The total eluate volume was concentrated before preparation for electrophoresis. (A) Extraction buffer eluates; (B) DMF eluates; (C) SDS eluates; (D) second extraction buffer eluates. See Materials and Methods for conditions of electrophoresis. Lane 1: cross-linked haemoglobin; Lane 2: tissue extract; Lane 3: C_0 eluate; Lane 4: C_2 eluate; Lane 5: C_4 eluate; Lane 6: C_6 eluate; Lane 7: C_8 eluate; Lane 8: C_{10} eluate.

extraction buffer eluates suggest that these solvents are eluting, non-specifically, the remaining bound protein (Fig. 1C and D).

When electrophoresis is performed on samples that have been treated with β-mercaptoethanol, much of the slowly migrating material appears to diminish quantitatively. Concomitantly there is an increase in faster migrating species. This is illustrated for the eluates from the C_{10} column (Fig. 2).

Amino acid analyses and protein elution profiles

The protein hydrolyzates were analyzed so as to determine the protein concentration of each eluate. These analyses also provided a comparison of the amino acid composition of each fraction. Fig. 3 indicates that, of the extraction buffer eluates, the highest protein concentration is in that from the C_0 column. DMF is most effective at removing bound proteins from the C_8 column while of the SDS eluates, that from the C_{10} had the highest protein concentration. For the second extraction buffer elution, the highest protein concentrations were from the agaroses with the shorter chain ligands.

Elastin elution

As a measure of the binding and eluting behaviour of soluble cross-linked elastin which is known to be present in the extracts [7], the desmosine plus isodesmosine concentrations of the eluates (as determined by amino acid analyses) are shown in Fig. 4. There was no cross-link detectable in the extraction buffer eluate. The highest concentration amongst the DMF eluates is found in the C_{10} eluate whilst amongst the SDS eluates only the C_2 and C_{10}

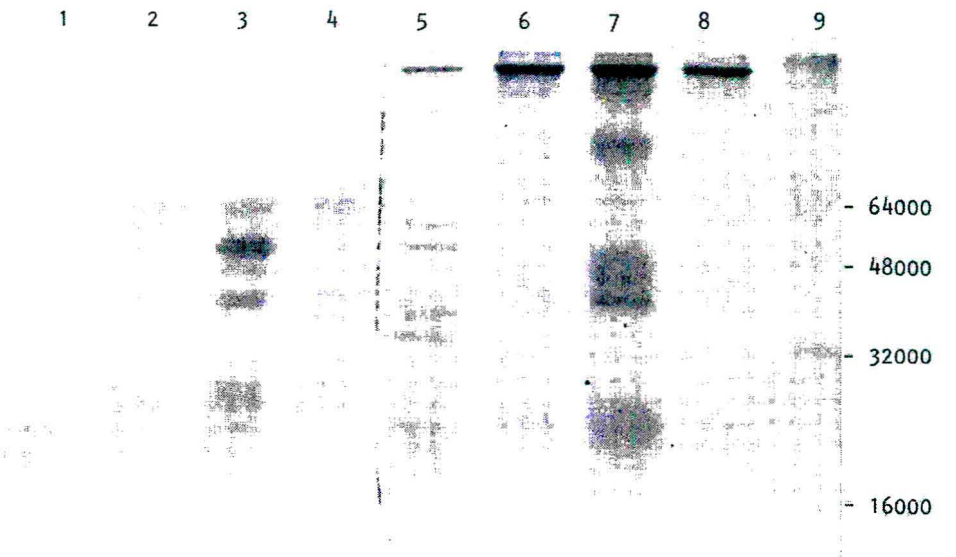

Fig. 2. Polyacrylamide gel electrophoresis of eluates from C_{10} column. Lanes 1 and 5: extraction buffer eluates; Lanes 2 and 6: DMF eluates; Lanes 3 and 7: SDS eluates; Lanes 4 and 8: second extraction buffer eluates; Lane 9: cross-linked haemoglobin; Lanes 1—4: in presence of β-mercaptoethanol; Lanes 5—9: in absence of 1% β-mercaptoethanol.

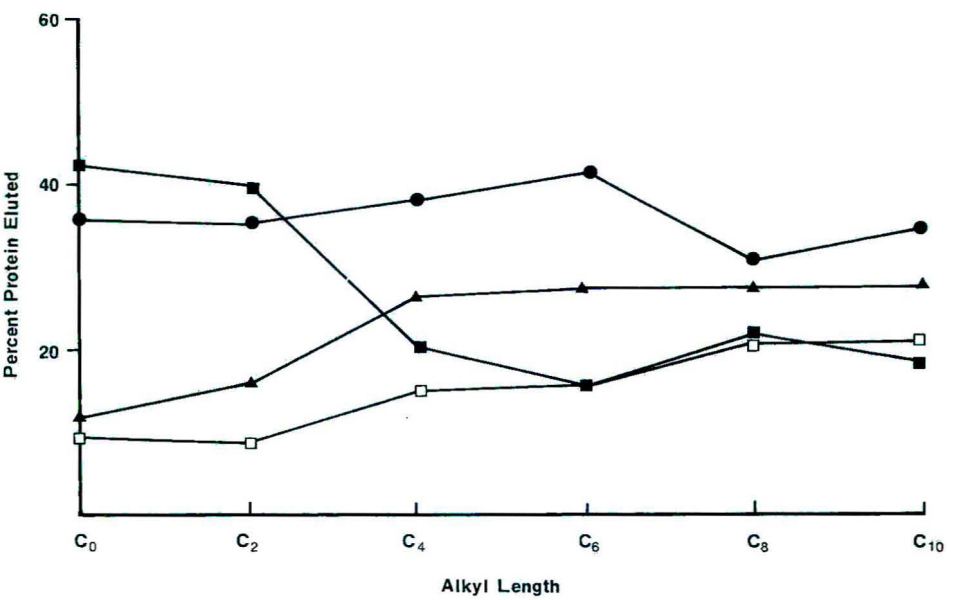

Fig. 3. Distribution of protein in eluates from alkyl-substituted agarose columns. (▪—▪) Extraction buffer eluate; (□—□) DMF eluate; (▲—▲) SDS eluate; (●—●) second extraction buffer eluate.

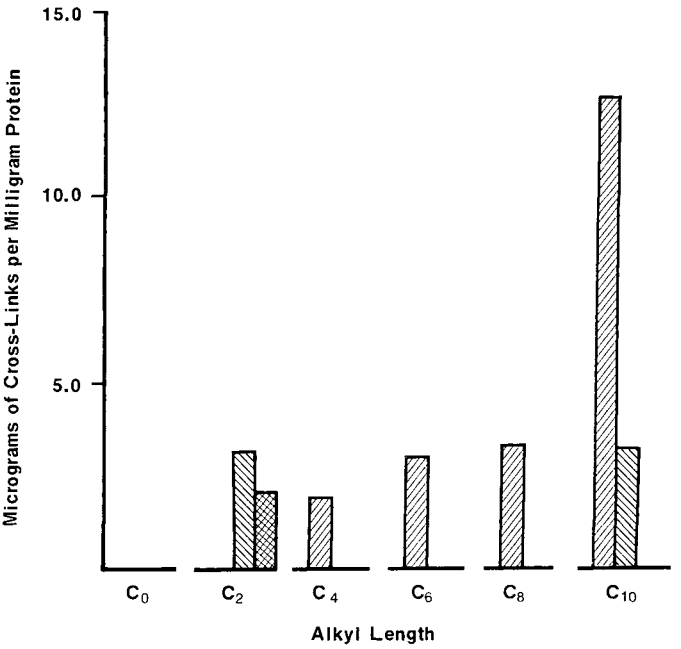

Fig. 4. Distribution of elastin cross-links (isodesmosine and desmosine) in eluates from alkyl-subtituted agarose columns. (▨) DMF eluate; (▧) SDS eluate; (▩) second extraction buffer eluate.

contained significant concentrations of these cross-links. There was cross-link also present in the second extraction buffer eluate from the C_2 column.

Precipitate formation

After dialysis of the eluents against extraction buffer at room temperature, some formed precipitates. Varying amounts of precipitates formed from the DMF and SDS eluates and the extent of these seemed to parallel the distribution patterns of the cross-links (data not shown). When these precipitates were washed with extraction buffer, dried, hydrolyzed and analyzed, there was a noticeable compositional change. As an example, the composition of the DMF eluate from the C_{10} column is compared with the precipitate obtained from the same eluate (Table I). As determined by their difference indices [12], precipitate resulting from dialysis of the DMF eluate has greater similarity than the DMF eluate itself to α-elastin [7] and to fibrous elastin [9].

DISCUSSION

The recent detection of polypeptides containing elastin cross-links in the salt extracts from vascular tissue has important implications with regard to the turnover of fibrous elastin [7]. Analyses of these polypeptides indicated the presence of both desmosine and isodesmosine. Thus it would appear that the protein(s) containing these is a fragment of the insoluble fibrous molecule and that it is not essential to cleave to pyridinium cross-links per se so as to

TABLE I

AMINO ACID COMPOSITION OF SOLUBLE ELASTIN FRACTIONS COMPARED WITH FIBROUS ELASTIN

Cross-links expressed as leucine equivalents.

	Residues per 1000 residues			
	DMF eluate from C_{10} column	Precipitate from DMF eluate from C_{10} column	α-Elastin [7]	Fibrous elastin [9]
Lysine	82.0	22.3	30.7	7.9
Histidine	33.9	11.2	13.6	3.0
Arginine	30.9	18.7	6.8	11.1
Hydroxypyroline	—	—	—	22.4
Aspartic acid	102.1	34.9	1.8	15.2
Threonine	—	19.0	9.2	16.6
Serine	14.8	33.2	9.0	17.8
Glutamic acid	146.3	67.2	20.4	25.8
Proline	62.0	85.8	105.3	103.0
Glycine	162.9	265.2	241.1	304.5
Alanine	104.8	215.2	288.7	214.7
Valine	86.0	80.9	111.7	118.3
Methionine	—	—	—	1.7
Isoleucine	16.3	26.2	17.5	23.2
Leucine	95.4	58.4	57.5	58.9
Tyrosine	7.5	11.8	13.4	13.9
Phenylalanine	38.4	34.1	34.5	29.7
Isodesmosine	—	6.3	29.3	4.4
Desmosine	16.7	9.9	7.2	6.2
Lysinonorleucine	—	—	2.2	1.3
Difference index [12] (comparison with α-elastin)	36.6	16.7		
Difference index [12] (comparison with fibrous elastin)	38.4	12.3		

solubilize the fibre. The ability to isolate such soluble elastin would assist the characterization of it, which in turn, may aid the elucidation of the mechanism of cleavage. By taking advantage of the strong hydrophobic nature of elastin [4, 6], it has been possible, by chromatography on decyl-agarose, to purify partially the soluble cross-linked elastin [7]. This hydrophobic characteristic has been used previously for the purification of soluble elastin by means of relatively higher concentrations of alcohols [13]. This report provides further data on the behaviour of cross-linked elastin when chromatographed on agaroses substituted with alkyl chains.

The gel electrophoretic patterns suggest that the lowest protein binding for vascular tissue extracts is to the C_0- and C_2-substituted matrices (Fig. 1A). This is supported by the quantitative protein determinations (Fig. 3). As a protein eluent, DMF is most effective for the material bound to the longer

alkyl chain columns (C_8 and C_{10}) (Figs. 1B and 3) while SDS appears to be most suitable for the C_6 (Fig. 1C) and C_{10} matrix (Fig. 3). The second extraction buffer elution was most effective for the shorter chain ligands.

DMF and SDS eluates, when electrophoresed in the absence of β-mercaptoethanol, indicate a predominance of high molecular weight species. As this material is reducible by the addition of β-mercaptoethanol and as elastin contains neither free thiols nor disulphide linkages, it would appear that there are disulphide-containing proteins associated with the soluble elastin. Although the nature of these is unknown, one possibility is that they might be microfibrillar proteins which contain disulphides and which are known to associate with fibrous elastin [14]. The predominant bands in the reduced samples comigrate with proteins of approximately 40,000 to 50,000 daltons. The protein species eluted by each solvent appear to be rather similar for each of the six columns — it is the quantities that vary.

Difficulties were experienced in determining the protein concentration of the eluted fractions by colorimetric techniques such as the method of Lowry et al. [11], the biuret method [15] and the method according to Bradford [16]. Components in the eluents interfered with colour development in each case. Hydrolysis and amino acid analysis of an aliquot of each fraction not only yielded the composition but also provided a very accurate determination of the protein concentration [17]. Such data provide quantitative confirmation that the greatest binding capacity for vascular proteins is provided by the C_6, C_8 and C_{10} columns (Fig. 3). DMF is most effective at removing protein from the C_8 and C_{10} columns (about 20% of the bound protein) while SDS removed between 24% and 27% of that bound to the C_4—C_{10} columns. There is further elution of protein when a second extraction buffer elution is performed. As indicated in Fig. 3, the amount eluted by this solvent is rather constant (between 34% and 42%) for all columns except for the C_8 where the level is only about 28% of the applied protein.

The yield of cross-links (desmosine plus isodesmosine) as determined by amino acid analysis can be used as an index of the elastin fragments present in an extract [18]. The absence of cross-links in any of the first extraction buffer eluates indicates that elastin binds to these substituted agaroses. DMF elutes cross-link containing material from the C_4—C_{10} columns. By far the highest level of cross-link per mg of protein is in the DMF eluate from the C_{10} column. This indicates that the best preparation of soluble elastin from hydrophobic interaction chromatography is in this eluate. There is some elution of cross-link by SDS from both the C_2 and C_{10} columns. There is no cross-link apparent in any of the eluates from the unsubstituted agarose (C_0) column. A possible explanation is that there are reduced quantities of protein (including elastin) bound to this column as indicated by the relatively high concentration in the extraction buffer eluate (Fig. 2). The low level of cross-links in such a heterogeneous protein mixture would make detection difficult.

When the DMF and the SDS eluates from these substituted agarose columns are dialyzed against extraction buffer, varying amounts of precipitates form. The weights of precipitate as determined gravimetrically closely parallel the elastin content of the fractions. As shown in Table I, these precipitates have a composition more akin to elastin than do the eluates from which they arise.

This suggests that precipitation further purifies the elastin fragments in the extracts. The nature of the precipitate which appears to be fibrous and the mechanism of its formation are currently under investigation.

Hydrophobic interaction chromatography can be used for the isolation of salt-soluble cross-linked elastin from extracts of vascular tissue. Alkyl chain ligands have been used with the optimal appearing to be 10 carbons long. Little difference is detected between the use of alkyl chains and alkyl chains with ω-amino groups (data not shown).

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CHROMBIO. 1646

AN AUTOMATIC METHOD FOR DETERMINATION OF GLYCOSYLATED HEMOGLOBINS USING LOW-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

Several studies have revealed a correlation between blood levels of glucose and hemoglobin A_{1c} (HbA_{1c}), a minor form of hemoglobin (Hb) present at elevated concentrations in patients with diabetes mellitus. To facilitate a clinical study of the level of circulating HbA_{1c} we have developed an automatic chromatographic system. An efficient separation of HbA_{1c} from HbA_0 and other rapid hemoglobins (HbA_{1a}, HbA_{1b}) was achieved on Bio-Rex-70 columns using three buffers. This system allows the daily analysis of 40 samples. The mean level of HbA_{1c} in normal subjects was 5.4 ± 0.4%. The method also detects the presence of elevated levels of HbF and the most frequent forms of abnormal hemoglobin (HbS, HbC).

INTRODUCTION

The determination of the level of glycosylated hemoglobins (Hbs) provides a follow-up of diabetes mellitus [1]. The original chromatographic method by Trivelli et al. [2] is too time consuming to be widely used in clinical analysis. Several attempts have been made to set up a rapid, low-cost and reliable procedure to assay glycosylated Hbs. A method for the radioimmunoassay of HbA_{1c} proposed by David et al. [3] has not been developed on a routine basis. Recently, an affinity technique using the capacity of boronate to form complexes with 1,2-*cis*-diol has been described by Mallia et al. [4]. This technique measures the total glycosylated fractions, including those of HbA_0. Its routine use implies a precise knowledge of assayed fractions. Several groups have reported HbA_{1c} assay by gel scanning after isoelectric focusing (IEF) [5—9] with an improved separation of HbA_{1c} from HbA_0; nevertheless, the method has not yet been used for routine experiments on a large scale. Rapid chromatographic methods, especially those using microcolumns, have been developed [10—16]. Some of them propose the measurement of the total glycosylated Hbs

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designated as HbA_1. Unfortunately, the preparation in the laboratory of microcolumns needed for HbA_1 determination is excessively time consuming. Commercially available microcolumns are highly temperature dependent and require careful attention [17, 18]. Therefore several groups have been prompted to develop other types of fast and automatic chromatographic methods for estimation of glycosylated Hbs using liquid chromatography under high [19, 20] or medium range [21] pressure.

This article presents the results obtained with the previously described [22] automatic peptide analyser adapted to the determination of glycosylated Hbs. A three-buffer procedure allowed complete and rapid peak separation of HbA_{1a+1b}, HbA_{1c} and HbA_0, although the pressure remained lower than 4.5 bars. Up to 200 samples could be analysed in one week with, in addition, the detection of abnormal levels of HbF and of several abnormal Hbs.

MATERIALS AND METHODS

Chemicals

The following analytical grade chemicals, $NaH_2PO_4(H_2O)$, $Na_2HPO_4(2H_2O)$, KCN, H_3PO_4, NaOH, chloroform and NaCl were obtained from Merck (Darmstadt, G.F.R.). Bio-Rex-70 resin (200—400 mesh) was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.).

Preparation of buffer solutions

Solutions	Na_2HPO_4	NaH_2PO_4	KCN	NaCl
1	0.02 *M*	—	0.008 *M*	—
2	—	0.04 *M*	0.008 *M*	—
3	0.02 *M*	—	0.008 *M*	0.1 *M*
4	—	0.04 *M*	0.008 *M*	0.1 *M*

Buffer A was obtained by mixing solutions 1 and 2 to give a final pH of 6.76 ± 0.01. Buffer B was obtained by mixing solutions 3 and 4 to give a final pH of 6.56 ± 0.01. Buffer C was obtained by adding NaCl up to 0.3 *M* to buffer A; no adjustment of the pH is required.

Preparation of the column resin

The resin (50 g of Bio-Rex-70, 200—400 mesh) was washed three times with 1 l of distilled water during which fine particles were discarded, then washed with 500 ml of 0.5 *N* NaOH and rinsed with water. The pH of the slurry was adjusted to pH 2.2 with H_3PO_4 (85%). After removal of the supernatant the resin was suspended in solution 1. Then NaOH (10 *N*) was added to bring the final pH to 6.76.

The resin suspension was poured in the column (6.25 mm × 15 cm) to give a 5—6 cm bed height, packing the Bio-Rex-70 resin (200—400 mesh) by pumping buffer C at a flow-rate of 17 ml/h at room temperature. The column should be conditioned by operating it during ten buffer cycles prior to any sample analysis. The back-pressure should not exceed 4.5 bars.

Equipment

This consisted of two Accu-Flow pumps (Beckman, Palo Alto, CA, U.S.A.), several pneumatic valves (CAV 3031, PA 875, SR, R 6031 V6), adjustable columns (6.25 mm × 15 cm) and connectors from Chromatronix (Berkeley, CA, U.S.A.), a Mikrorapid Centrifuge from Hettich (Tüttlingen, G.F.R.), centrifuge tubes from Eppendorf (Hamburg, G.F.R.) and an Ismatec peristaltic pump (Zürich, Switzerland). A sample distributing rack TD 15 T3 was supplied by Gilson (Villiers le Bel, France) and colorimeters (fitted with 418-nm and 570-nm filters) by Technicon (Dublin, Ireland). All connections were made with PTFE tubing (0.8 × 1.50 mm and 0.5 × 1.50 mm) (Habia, Montmirail, France). A Dynamaster recorder with two channels and point-by-point recording was obtained from Elliot Automation (Vichy, France). The integrator (ICAP 10) was made by Delsi (Suresnes, France). The programmer was constructed from Crouzet components (Valence, France) by Touzart & Matignon (Paris, France).

Samples

Normal blood samples were obtained from normal blood donors free of any medication who were tested for normoglycemia, normal mean reticulocytes and normal hematocrit, absence of any hemoglobinopathy and no persistence of fetal hemoglobin using IEF separation [23] and by dosage of HbF according to the method of Betke et al. [24].

Chromatographic system

A sample injector was added to the chromatographic system already described by Blouquit et al. [22]. Samples were injected on the column from the specimens in the sampler rack by means of a 20-μl loop. The filling of this loop was controlled by the peristaltic pump. Sample injection on the column was done by commutation of V_1 and V_2, the two three-way pneumatic valves shown in Fig. 1. This system was equipped with two columns, C_1 and C_2, one being equilibrated with buffer A while the other was running. Column selection was done through V_3 and V_4, the two four-way pneumatic valves (Fig. 1). The flow-rate of the pumps was 17 ml/h. Buffer selection was done through R, a six-way rotating valve (Fig. 1) under the following program. (1) Sample injection. (2) Buffer A: elution of the fast migrating fractions, i.e. HbA_{1a1}, HbA_{1a2}, HbA_{1b}, during 4 min. (3) Buffer B: elution of HbA_{1c} during 12 min. (4) Buffer C: elution of the remaining HbA_0 and HbA_2 fractions during 16 min. (5) Buffer A: rinsing the loop sampler for 2 min. The complete cycle lasts 36 min. The chromatography was performed at ambient temperature between 20°C and 25°C; no noticeable variations were observed.

Calculation

The absorbance of the eluate was measured at two wavelengths, 418 nm for HbA_{1c} and 570 nm for HbA_0. An integrator interfaced between the colorimeter (570 nm) and the recorder allowed a precise estimation of the percentage of HbA_{1c}. Moreover, it was possible to check the integration count

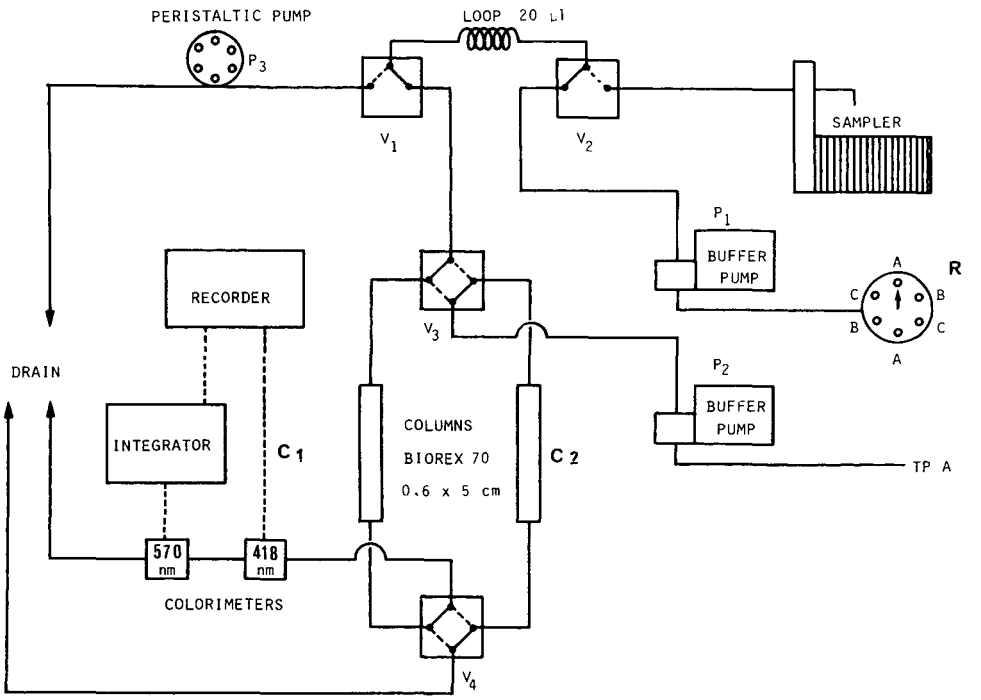

Fig. 1. Flow diagram of the chromatographic system for the determination of glycosylated hemoglobins. The sampler rack, two three-way valves (V_1, V_2) a six-way valve (R), two four-way valves (V_3, V_4), a peristaltic pump (P_3) and the buffer pumps (P_1, P_2) are controlled by a punched-card programmer.

by a manual estimation of the fraction with the formula

$$\frac{\text{Area HbA}_{1c}\ (418\ \text{nm})}{\text{Area HbA}_{1c}\ (418\ \text{nm}) + \text{Area HbA}_0\ (570\ \text{nm}) \times 10^{\star}}$$

Preparation of the hemolysates

Blood was withdrawn into an anticoagulant solution and stored at 4°C until use. A 1-ml aliquot of blood was washed twice with isotonic salt solution. During these washings care was taken, not to remove any red blood cells, since those present in the upper layer of the pellet were principally constituted of reticulocytes, low in HbA_{1c}. Their loss might alter the assay resulting in an artificial increase in old red cells rich in HbA_{1c}. The red blood cell pellet was hemolyzed by the addition of 600 µl of distilled water and 300 µl of chloroform. This mixture was shaken for 30 sec and then centrifuged at 4°C during 30 min at 8000 *g*. The supernatant was then diluted five times with buffer A and stored at 4°C for no more than 48 h. The final concentration in Hb was 10—20 g/l.

*The ratio $\epsilon_{418}/\epsilon_{570}$ (where ϵ = molar absorptivity) of the Hb measured with the filters used has been found to be very close to 10.

RESULTS

Storage effect

The level of glycosylated Hbs was correlated with the age of the red blood cells (RBCs) [25], the time during which these RBCs were in contact with glucose [26] and perhaps with still unknown factors. For all these reasons special attention was given to the choice of the anticoagulant medium and to the conditions of storage of the samples. Blood from a normal individual was withdrawn into three different anticoagulant media, citric acid—dextrose (ACD), EDTA and heparin. No significant difference in the HbA_{1c} level was observed amongst these three samples. Since the oldest RBCs, the richest in HbA_{1c}, are the most susceptible to hemolysis, ACD was selected as anticoagulant. This medium was the most satisfactory in preventing hemolysis in stored samples. RBCs of normal volunteers and of diabetic patients were stored at 4°C for up to eight days. The levels of HbA_{1c}, as well as that of HbA_1 (A_{1a} + A_{1b} + A_{1c}) were not significantly modified during the eight days storage on ACD either in normal or diabetic individuals. These results were in accordance with already reported HbA_1 data using Bio-Rad microcolumns [17]. The HbA_{1c} level was also determined on fresh hemolysates diluted with five volumes of buffer A and stored at room temperature in the sample rack of the analyser. Stable levels of HbA_{1c} were observed over a period of 36 h.

Chromatographic patterns

Examples of chromatographic patterns obtained with hemolysates from a normal control and from a patient with diabetes mellitus are shown in Fig. 2. The separation pattern of the different peaks is comparable to that obtained with the original method of Trivelli et al. [2] and with that obtained with the high-performance liquid chromatographic (HPLC) techniques [19, 20]. In contrast to Cole et al. [20], we did not observe detectable deflection of the baseline after the introduction of the second buffer, as has been noted when performing the chromatography without sample introduction.

A comparison between the results obtained by manual planimetry of the curves and by a computing integrator was made. An excellent regression curve (r = 0.97) was obtained over the range of HbA_{1c} concentration of clinical interest (4—20%).

The reproducibility of our chromatographic method was tested by comparing fifteen runs of two blood samples, one from a normal volunteer and the other from a diabetic patient. The mean HbA_{1c} level in the normal hemolysate was 5.8 ± 0.19% with a coefficient of variation (C.V.) of 3.2%. The mean HbA_{1c} level in the patient's hemolysate was 14.5 ± 0.23%, C.V. 1.5%.

Values from normal and diabetic subjects

Seventy-four normal control individuals ranging in age from 22 to 51 years were studied. HbA_{1c} levels ranged from 4.9% to 6.2%. The mean HbA_{1c} level was 5.4 ± 0.4%, C.V. 7.4%. The present method was used routinely in our laboratory to determine the HbA_{1c} level of a large number of patients, most of whom were suffering from diabetes. During this study approximately

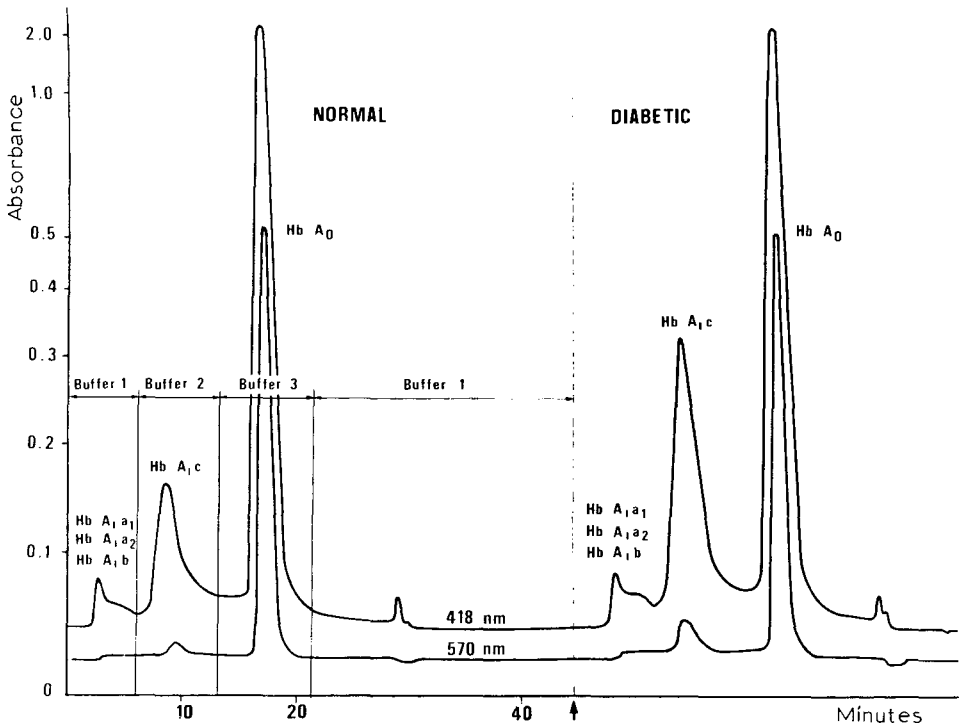

Fig. 2. Typical chromatographic separations of glycosylated hemoglobins using the buffer system described under Materials and methods. Chromatographic profile on the left side is typical of a normal hemolysate, and on the right side of a diabetic patient. The injection of the diabetic blood sample is indicated by the arrow. HbA_{1c} detection was effected at 418 nm, and of HbA_0 at 570 nm. The path-length of each colorimeter cuvette was 15 mm.

10,000 samples were studied. The level of HbA_{1c} ranged from 4% to 18%.

Diabetes mellitus and abnormal hemoglobins

In several instances, the chromatographic patterns were modified due to the presence of an abnormal hemoglobin in the hemolysate. Fig. 3 (right side) shows the elution profile given by the hemolysate of a heterozygous HbA/HbS sicklemic patient. In this profile, HbS_0 eluted just after HbA_0. Fig. 3 (left side) shows the elution profile obtained from the hemolysate of a homozygous sicklemic patient. The major peak represents HbS_0, while the peak within the elution volume of HbA_{1c} was mainly constituted by HbF as judged by IEF. Estimation of glycosylated HbS could not be demonstrated in this chromatographic system. Fig. 4 shows the elution profile of the hemolysate from a HbA/HbC heterozygous patient. HbC_0 was eluted after the peaks of HbA_0. In all cases, quantitative evaluation of the level of the glycosylated hemoglobins contained in the hemolysate was questionable [27, 28]. Fig. 5 shows the elution profile produced by an hemolysate containing 5% of HbF, as determined by the method of Betke et al. [24]. HbF was eluted with the peak of HbA_{1c}, but careful inspection of the chromatographic pattern shows the presence of an unusual shoulder on the descending

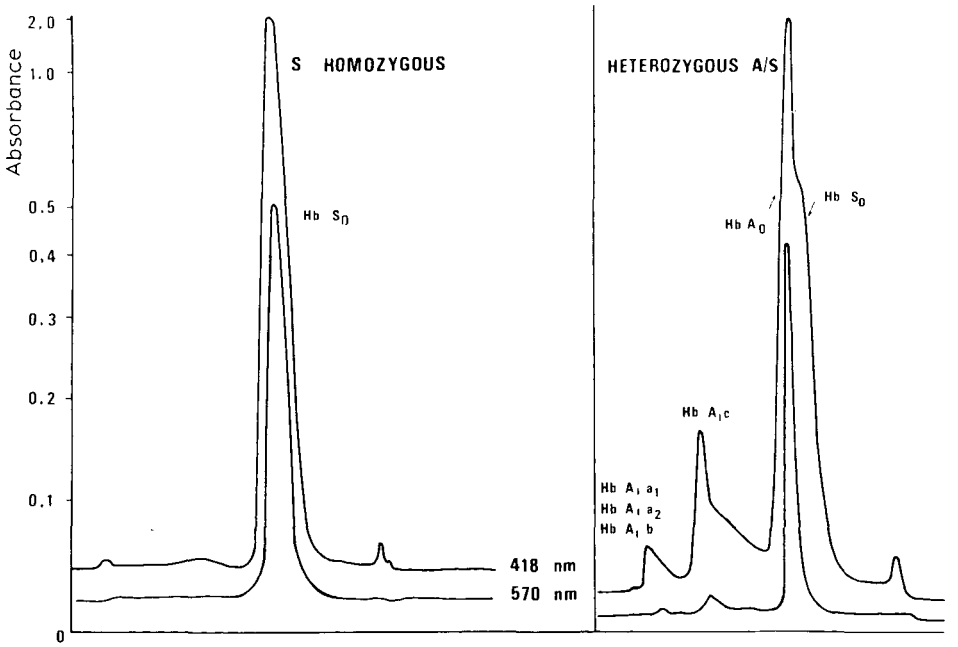

Fig. 3. Right side: a typical elution pattern obtained from the hemolysate of a heterozygous HbA/HbS sicklemic subject. Left side: a typical elution pattern obtained from the hemolysate of an homozygous sicklemic patient.

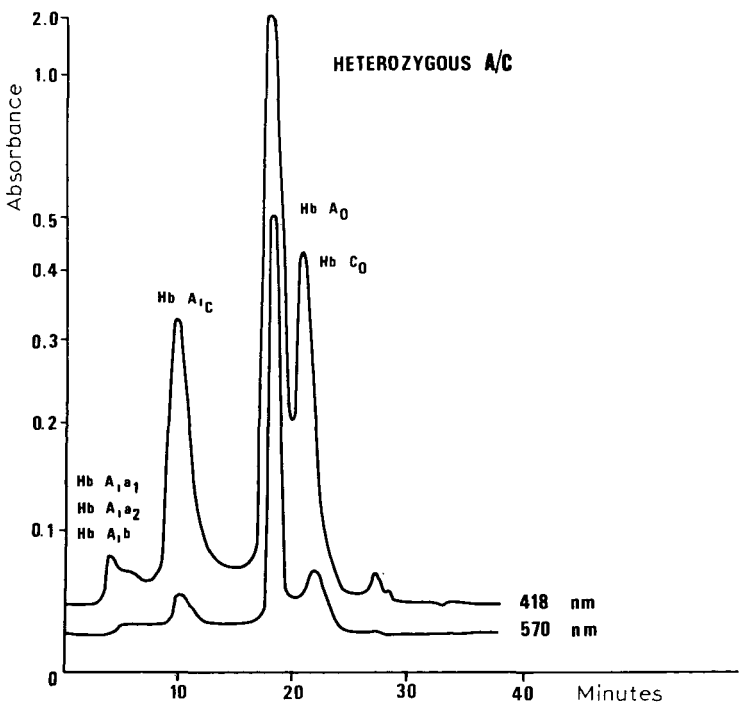

Fig. 4. A typical chromatographic profile obtained from the hemolysate of a HbA/HbC heterozygous subject.

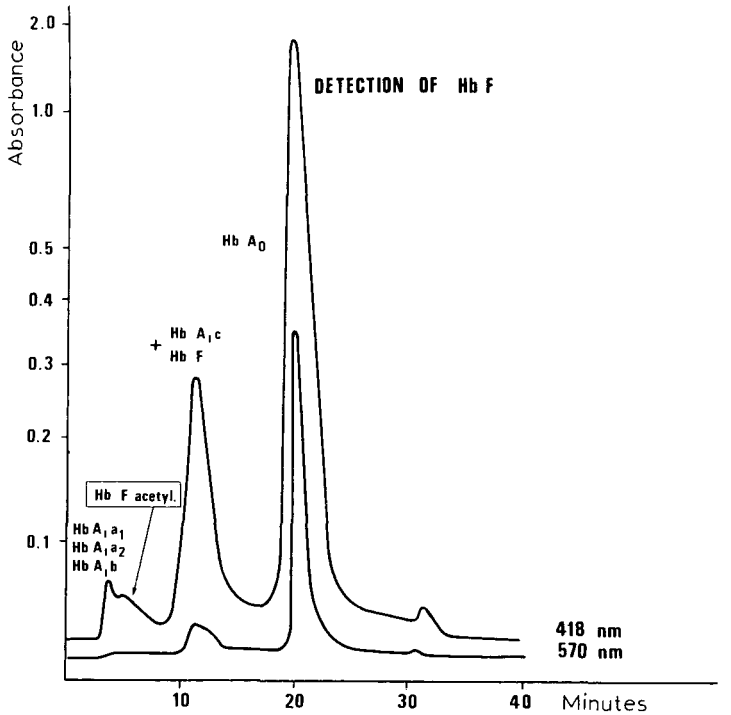

Fig. 5. Chromatographic profile typically seen in hemolysates of patients with HbF persistence. HbF was estimated by the technique of Betke et al. [24] and corresponded to 5% of the total.

side of the HbA_{1a+b} peak which represented the acetylated HbF fraction, the nature of which was definitively demonstrated after IEF analysis of this peak.

DISCUSSION

The method described in this article derives from the original technique for estimation of HbA_{1c} as described by Trivelli et al. [2]. Our method is characterized by its ability to be automated, permitting more than 40 daily tests. The three-step buffer system was used to achieve a complete separation of $HbsA_{1a+1b}$, HbA_{1c} and HbA_0. Its reproducibility was comparable to that obtained with HPLC. Evaluation of the amount of HbA_{1c} can be performed either by manual planimetry or by use of a computing integrator.

The levels of HbA_{1c} in hemolysates stored at room temperature remained stable for at least 36 h. Refrigeration of the sampler did not appear necessary. In contrast to most other authors, we have used ACD in order to prevent to the utmost degree possible hemolysis which might artificially lower the HbA_{1c} level by a selective lysis of the oldest cells rich in HbA_{1c}. We have observed that samples taken in ACD could be stored at 4°C without a significative modification of their HbA_{1c} level for a long period of time. Conversely, one should be careful not to eliminate the youngest RCBs during washing which could modify the overall HbA_{1c} level.

The performance of our system compares favourably to that obtained with either the HPLC method of Cole et al. [20] and of Davis et al. [19], or the method of Wajcman et al. [21]. Nevertheless, it must be emphasized that HPLC resulted in a slightly inferior separation to that obtained with our technique, while it requires expensive equipment and an electronic adjustment of the baseline to minimize graph artefact due to buffer change. The method described by Wajcman et al. gives considerable overlapping of the peaks of HbA_{1a+b} and HbA_{1c} and needs a rigorous monitoring of the pH of the first buffer. The low pressure, which was less than 7 bars, permitted the use of the same column for more than 200 times without repacking.

We found that the introduction of buffer B for the elution of HbA_{1c} greatly improves the resolution of the system by promoting a rapid elution of HbA_{1c} and HbF like in other systems but without eluting HbA_0. However, acetylated HbF was eluted in the descending side of the HbA_{1a+b} peak which permitted the detection of HbF level over 3%. The presence of several abnormal hemoglobins induced modification of the chromatographic profile. HbS was very easy to detect while it produced a shoulder on the descending side of the HbA_0 peak. The presence of HbC in hemolysate was revealed by an extra peak after the HbA_0. Hb Hope [29], which is not rare in the black population, was eluted in our system as a constituent of the HbA_{1c} peak hampering any evaluation of the HbA_{1c} level. These observations imply that a correct evaluation of the amount of HbA_{1c} cannot be done when most of the abnormal hemoglobins among the most frequent are present.

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CHROMBIO. 1645

DETERMINATION OF PORPHYRINS IN URINE BY DIRECT INJECTION ON A LIQUID CHROMATOGRAPHIC COLUMN COATED WITH TRIBUTYLPHOSPHATE

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SUMMARY

To separate and quantify urinary porphyrins, acidified and centrifuged urine was injected on a reversed-phase high-performance liquid chromatographic column, LiChrosorb RP-18, coated with tributylphosphate. A linear pH gradient from pH 4.40 to pH 6.50 was applied in the eluent using phosphate buffers containing methanol (9:1). The method permits detection of uro- and coproporphyrin down to concentrations normally present in urine and also selective determination of the hepta-, hexa- and pentacarboxylic porphyrins present in urine from patients suffering from porphyria cutanea tarda.

INTRODUCTION

Porphyrias are diseases caused by defects of enzymes in the biosynthesis of haem. Different porphyrins are accumulated depending on the break in the metabolic pathway, and for correct diagnosis it is important to have simple and selective analytical methods to measure the porphyrin content in urine, faeces and erythrocytes. The use of liquid chromatography for the separation and quantitation of porphyrins in biological specimens has been reviewed [1]. Most high-performance liquid chromatographic (HPLC) methods presented used some extraction, concentration and/or esterification step before injection of the sample on the chromatographic column and the porphyrins were detected spectrophotometrically.

The analysis of urinary porphyrin carboxylic acids by direct injection of urine or acidified urine on the liquid chromatographic column [2—6] can easily be done in reversed-phase liquid chromatography in combination with fluoro-

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metric detection. The only specimen pretreatment that is necessary is filtration or centrifugation of the urine to get rid of solid particles.

The separation of porphyrins on columns with hydrophobized silica as the solid phase can be performed in different ways. Englert et al. [2] used a gradient of acetonitrile in phosphate buffer pH 7.5 in the eluent, while Ford et al. [3] used a gradient of methanol in phosphate buffer pH 3.5. Rather high concentrations of acetonitrile and methanol were necessary to elute the porphyrins from the column. The urinary porphyrins have also been separated by reversed-phase ion-pair chromatography with tetrabutylammonium as counter-ion added to the eluent [4—6]. The separation was performed at pH 7.5 in the isocratic mode [4] or by applying a multilinear gradient of methanol in the eluent [5, 6]. A separation on an ion-exchange HPLC column has also been demonstrated using a methanol—acetic acid gradient in the eluent [7].

In the present work the hydrophobic support was coated with tributylphosphate (TBP), to separate the urinary free porphyrin carboxylic acids. TBP is a liquid with hydrophobic properties due to the three butyl groups. It also has strong hydrogen-binding properties due to the phosphate group, and TBP-coated columns thus give high retention of hydrogen-donating compounds like carboxylic acids. TBP has previously been used in extraction chromatography for the separation of metal ions [8], but also in HPLC in the separation of organic substances such as carboxylic acids [9—11], amino acids [11] and catecholamines [12]. The analysis of 5-hydroxyindoleacetic acid by direct injection of urine on a column coated with TBP in combination with fluorometric detection [10] has been running for some time as a routine method in our laboratory. With TBP present, high selectivity for carboxylic acids is obtained with respect to other endogenous compounds in urine. It was our intention to use the same chromatographic system to analyse the urinary porphyrin carboxylic acids. Our method involves direct injection of acidified urine on the column, pH-gradient elution of the porphyrins and fluorometric detection. With this method it is possible to separate and quantify octacarboxylic (uro)porphyrin, hepta-, hexa- and pentacarboxylic porphyrin and tetracarboxylic (copro)porphyrin.

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of two pumps, Constametric III and Constametric II, combined with a solvent programmer, Gradient Master Model 1601 (Milton Roy Company, Riviera Beach, FL, U.S.A.), a Rheodyne injector with 20-μl loop and a Schoeffel FS 970 fluorescence detector. The excitation wavelength was set at 405 nm and an emission cut-off filter below 550 nm was used. The isocratic experiments were performed with a Milton Roy Minipump with pulse dampener (LDC, Model 711-26). A Spectro Monitor III (LDC) UV detector with variable wavelength was also used in some of the experiments.

The separation column was of precision bore 316 stainless steel, 100 mm $\times$ 3.9 mm I.D., packed with LiChrosorb RP-18, 5 μm (Merck, Darmstadt,

G.F.R.). A water-bath (Heto Lab Equipment A/S, Denmark) was used for thermostating.

Chemicals

Tributylphosphate (TBP) and methanol as well as all the other chemicals were of analytical grade and obtained from Merck.

The buffers were prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate with an ionic strength of 0.1 using distilled water.

Coproporphyrin dihydrochloride type III isomer, uroporphyrin octamethyl ester type III isomer and a synthetic mixture of tetra-, penta-, hexa-, hepta- and octacarboxylic porphyrins type I isomers including mesoporphyrin IX as a dicarboxylic porphyrin were obtained from Porphyrin Products (Logan, UT, U.S.A.). The uroporphyrin III octamethyl ester was hydrolysed overnight in 6 mol/l hydrochloric acid (dark, 4°C) to obtain the uroporphyrin III carboxylic acid.

Chromatographic conditions

The separation column was packed at 35 MPa with the support suspended in methanol [13].

The eluents were prepared by first mixing buffer and methanol (9:1) and the solution was then equilibrated with TBP. During the development of the method the eluents were saturated to 90% with TBP and the column and the reservoirs were thermostated at 25.0°C by circulating water [11]. In the final method, however, we found it possible to use eluents saturated to 100% with TBP and ambient temperature. The pH of the eluent was always measured before the addition of methanol. In the gradient studies buffer to eluent A was prepared by dissolving 13.8 g of $NaH_2PO_4 \cdot H_2O$ in 1000 ml of distilled water and buffer to eluent B by dissolving 5.2 g of $NaH_2PO_4 \cdot H_2O$ and 3.6 g of $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml of distilled water.

The columns were coated with TBP by injection of 350 μl of TBP on the column, followed by recycling of the eluent overnight [11].

The hold-up volume of the column, V_m, was determined by injection of coproporphyrin III in an eluent of pH 8, where coproporphyrin III was unretained. The retention volume of the samples, V_R, was measured and the capacity ratio, k', was calculated by $(V_R - V_m)/V_m$.

In the isocratic experiments the porphyrins were dissolved in 0.1 mol/l hydrochloric acid and diluted with the eluent.

Analytical method

Standards were prepared by dissolving the synthetic mixture of octa-, hepta-, hexa-, penta-, tetra-, tri- and dicarboxylic porphyrins, containing 10 μmol of each porphyrin, in 4 ml of 1.0 mol/l hydrochloric acid. The standard was further diluted to 0.045—0.28 μmol/l with 0.1 mol/l hydrochloric acid and injected on the chromatographic column. The peak heights of the standards were measured and standard curves were prepared by plotting the peak height of each porphyrin against the concentration. The standards were protected from light and stored in darkness at 4°C.

Collection of 24-h urine was made in dark plastic bottles with the addition of sodium carbonate (2 g/l of urine). Prior to analysis 5.0 ml of the 24-h urine

were adjusted to about pH 2 by the addition of six droplets of 6 mol/l hydrochloric acid. The acidified urine was centrifuged before injection. When higher concentrations of porphyrins were found, a fresh aliquot of the 24-h urine was diluted 1:25 with 0.1 mol/l hydrochloric acid and analysed again.

The porphyrin carboxylic acids were eluted from the column by applying a pH gradient in the eluent from pH 4.40 (eluent A) to pH 6.50 (eluent B). The gradient was started immediately after injection of the sample. The Gradient Master was adjusted to give a linear gradient in 10 min, 15-min delay at eluent B and a return to eluent A in 1 min. After 15 min at eluent A a new injection was made. The flow-rate was 0.8 ml/min.

The peaks obtained in urine were identified by comparing the observed retention times with those of the standards, and the concentrations were calculated from the peak heights given by the sample and the standard. For coproporphyrin the concentrations were calculated from the peak area measured by multiplying the peak height by the peak width at half the peak height.

RESULTS AND DISCUSSION

Retention model

The chromatographic support, LiChrosorb RP-18, was modified in the study by adsorption of tributylphosphate (TBP). TBP is a strong hydrogen acceptor and is able to form hydrogen bonds with acidic groups.

The capacity ratio of a sample distributed to the adsorbed TBP layer is given by

$$k' = (V_s/V_m) \times D \tag{1}$$

where $C_{HA,org}$ and $C_{HA,aq}$ are the total concentrations of the acid in the stationary and the mobile phase, respectively. The distribution constant of the acid, K_D is given by

$$D = \frac{C_{HA,org}}{C_{HA,aq}} = \frac{[HA]_{org}}{[HA]_{aq} + [A^+]_{aq}} \tag{2}$$

where $C_{HA,org}$ and $C_{HA,aq}$ are the total concentrations of the acid in the stationary and the mobile phase, respectively. The distribution constant of the acid, K_D is given by

$$K_D = \frac{[HA]_{org}}{[HA]_{aq}} \tag{3}$$

When the acid is retained on the column in uncharged form and proteolysis occurs in the mobile phase, the equation for the capacity ratio can be transformed to

$$k' = (V_s/V_m) \times \frac{K_D}{1 + (K_a^x/a_{H^+})} \tag{4}$$

$$\log k' = \log [(V_s/V_m) \times K_D] - \log (1 + K_a^x/10^{-pH}) \tag{5}$$

where K_a^x is the apparent dissociation constant including the medium factor

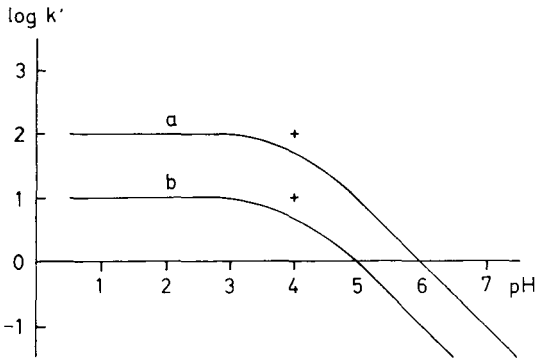

Fig. 1. Relation between pH of the eluent and the retention of acids. (a) Acid with K_D = 170 and pK_a^x = 4. (b) Acid with K_D = 17 and pK_a^x = 4. + = intersection point (pH = pK_a^x) for the two asymptotes according to eqn. 5. (V_s/V_m) is set at 0.6.

(cf. ref. 14) and a_{H^+} is the hydrogen activity in the mobile phase.

The relation between the capacity ratios of two acids containing one carboxylic group each and pH of the eluent according to eqn. 5 is illustrated in Fig. 1. The graphs in Fig. 1 have two asymptotes, one with a slope of 0 and one with a slope of −1. They intersect at a pH equal to pK_a^x for the acids [11,15].

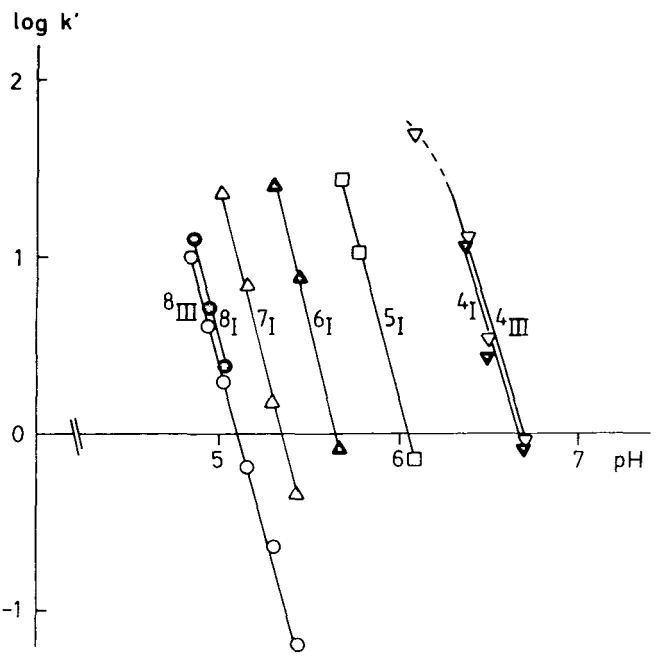

Fig. 2. Change in retention of porphyrin carboxylic acids with pH (isocratic mode). 8_{III} (○) = octacarboxylic (uro)porphyrin III, 8_I (●) = octacarboxylic (uro)porphyrin I, 7_I (△) = heptacarboxylic porphyrin I, 6_I (▲) = hexacarboxylic porphyrin I, 5_I (□) = pentacarboxylic porphyrin I, 4_I (▼) = tetracarboxylic (copro)porphyrin I, 4_{III} (▽) = tetracarboxylic (copro)porphyrin III. Solid phase: LiChrosorb RP-18, 5 μm, coated with TBP. Eluent: buffer—methanol (9:1) with 90% relative saturation of TBP.

Retention of porphyrin carboxylic acids

The porphyrins studied contain between two and eight carboxylic acid groups. The pK_a values of the acidic groups are not known but the degree of dissociation in the eluent could be related to pH. The capacity ratios of the porphyrins are decreased by increasing pH in the eluent as shown in Fig. 2. The slopes of the lines for the octa-, hepta-, hexa- and pentacarboxylic porphyrins were 3.7—4.1, which indicates that the net charge of the porphyrins in the pH range studied for each porphyrin should be about 4. The slopes of the lines for tetracarboxylic porphyrins were close to 3.

Mesoporphyrin IX could not be eluted from the column even at pH 8. A higher pH should be needed but further increase in pH is limited by the stability of the silica support.

The separation factor between the type I and type III isomers ($\alpha = k'_I/k'_{III}$) was 1.2—1.3 for uroporphyrin and 0.77—0.91 for coproporphyrin. Note the difference in retention order. No complete separation was obtained between the isomers due to rather broad peaks under isocratic conditions.

The porphyrins were eluted from the column by a linear increase of pH in the eluent, from pH 4.40 to pH 6.50. The samples were injected at pH 4.40, where all porphyrins are strongly retained on the column, and the gradient was started. A linear gradient in 10 min at a flow-rate of 0.8 ml/min was found to be suitable to elute the porphyrins one after the other from the column. After 15 min at pH 6.50 a return to pH 4.40 was made in 1 min.

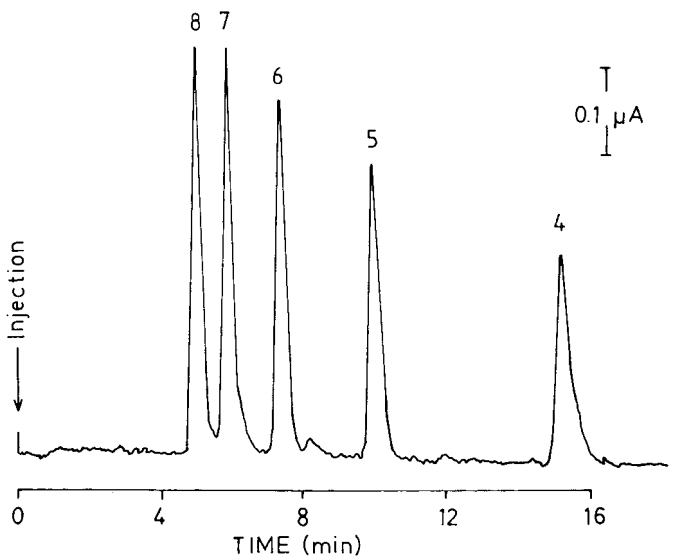

Fig. 3. Separation of a standard mixture of porphyrin carboxylic acids. 8 = octacarboxylic (uro)porphyrin, 7 = heptacarboxylic porphyrin, 6 = hexacarboxylic porphyrin, 5 = pentacarboxylic porphyrin, 4 = tetracarboxylic (copro)porphyrin. Solid phase: LiChrosorb RP-18, 5 μm coated with TBP. Eluents: A = buffer pH 4.40—methanol (9:1) with 90% saturation of TBP; B = buffer pH 6.50—methanol (9:1) with 90% saturation of TBP; linear gradient from 0 to 100% B in 10 min with 15 min delay. Flow-rate, 0.8 ml/min; pressure, 16.3 MPa; fluorescence detection, excitation at 405 nm, emission cut-off at 550 nm; time constant 6 sec. Sample: 20 μl porphyrin standard 0.18 μmol/l (3.6 pmol of each porphyrin).

The concentrations of the buffer ions $H_2PO_4^-$ and HPO_4^{2-} are changed in the eluent during the gradient. The phosphate ions have no fluorescence and only a minor drift in the baseline was observed during the gradient elution. The ions have, however, different weak UV-absorption properties at 405 nm and it was possible to follow the reequilibration of the column to pH 4.40 by connecting

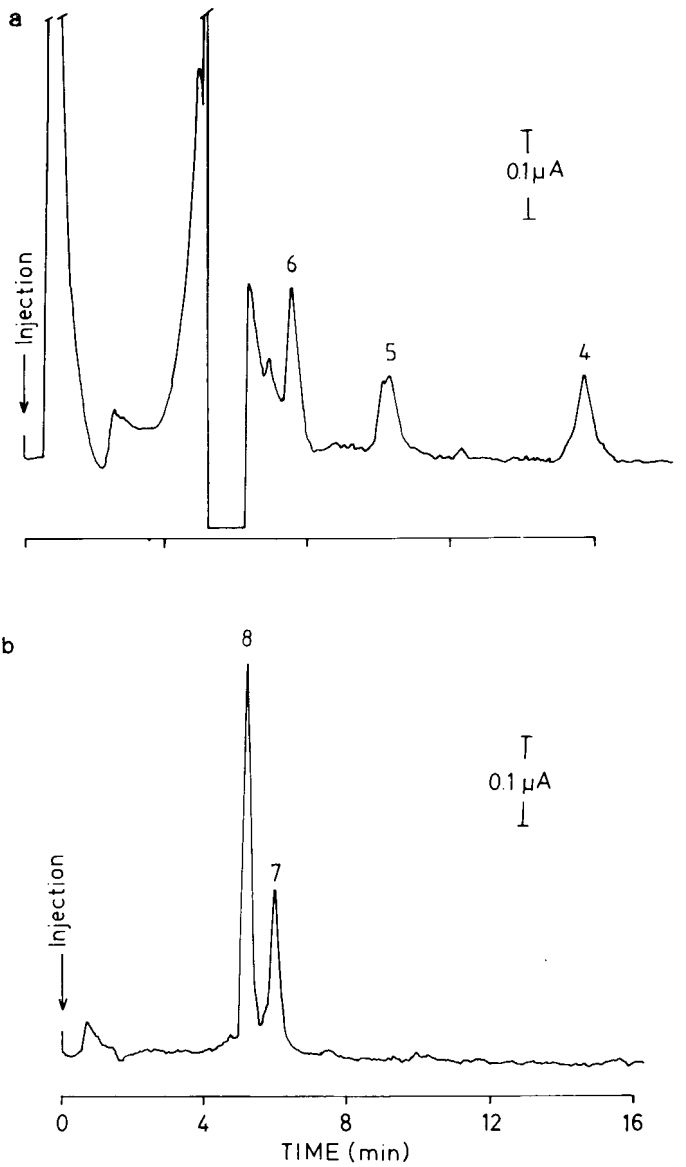

Fig. 4. Chromatogram obtained from a patient suffering from porphyria cutanea tarda (E.A./810924). (a) Sample: 20 μl of undiluted urine injected. When the uroporphyrin peak appeared there was a break in power due to overloading of the photomultiplier in the detector. Urine concentration of coproporphyrin = 0.09 μmol/l. (b) Sample: 20 μl of urine diluted 1:25 with 0.1 mol/l HCl injected. Urine concentration of uroporphyrin = 4.3 μmol/l and of heptacarboxylporphyrin = 1.8 μmol/l. Conditions and peaks as in Fig. 3.

TABLE I

URINARY PORPHYRIN EXCRETION IN TWO PATIENTS (H.H. AND E.A.) SUFFERING FROM PORPHYRIA CUTANEA TARDA

Excretion was estimated on three occasions each and compared to the mean excretion in four healthy individuals. Excretion is expressed in μmol per 24 h.

Initials/date	Uroporphyrin	Heptacarboxylic porphyrin	Hexacarboxylic porphyrin	Pentacarboxylic porphyrin	Coproporphyrin
H.H./810811	4.9	1.8	0.10	0.20	0.25
H.H./811009	0.73	0.92	–*	–	0.19
H.H./820315	0.59	0.49	0.03	–	0.04
E.A./810924	4.5	1.9	0.07	0.05	0.10
E.A./811014	5.6	2.6	**	**	**
E.A./820311	0.78	0.41	–	0.04	0.06
Normals (mean ± S.D., n = 4)	0.006 ± 0.004	–	–	–	0.05 ± 0.02

*– = below detection limit.
** = not quantified.

a UV detector in series with the fluorescence detector. The column was re-equilibrated to pH 4.40 in 12—13 min and then a new injection could be made. A chromatogram of the porphyrin standard containing 0.18 μmol/l of each porphyrin is shown in Fig. 3.

Analysis of porphyrins

Urine samples have been analysed from patients. Results from two patients suffering from porphyria cutanea tarda are shown in Table I. The chromatograms obtained with undiluted urine and with urine diluted 25 times with 0.1 mol/l HCl are shown in Fig. 4a and b. Coproporphyrin type III isomer, present in human urine to 95% of the total coproporphyrin [6], had a slightly higher retention than coproporphyrin type I isomer used as standard. This was compensated for by calculating the concentration from the peak area instead of the peak height. A chromatogram obtained from a healthy individual is shown in Fig. 5.

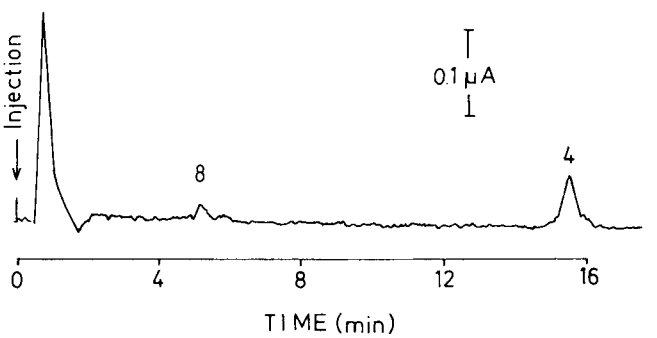

Fig. 5. Chromatogram obtained from a healthy individual. Sample: 20 μl of urine injected. Urine concentration of uroporphyrin 0.006 μmol/l and of coproporphyrin 0.04 μmol/l. Conditions and peaks as in Fig. 3.

TABLE II

IMPRECISION OF THE METHOD AT TWO CONCENTRATIONS

Estimations were made from repeated injections of porphyrin standard (n=8) and expressed as coefficient of variation (C.V. %).

Amount	Uroporphyrin	Heptacarboxylic porphyrin	Hexacarboxylic porphyrin	Pentacarboxylic porphyrin	Coproporphyrin
3.6 pmol (0.18 μmol/l)	5.9	6.8	6.3	6.9	5.4
0.8 pmol (0.04 μmol/l)	5.5	4.7	5.2	6.1	9.4

The imprecision of the method calculated from eight determinations of 3.6 pmol and 0.8 pmol porphyrin standard is given in Table II.

The detection limit defined as the amount that gave a signal two times the noise, was 0.08 pmol (0.004 μmol/l) of uroporphyrin and 0.28 pmol (0.014 μmol/l) of coproporphyrin.

The method presented has now been used as a routine method for more than one year in our laboratory without any deterioration of the column. The

simple specimen handling, the high sensitivity and specificity make it a valuable tool to obtain correct diagnosis and to follow the course of porphyria cutanea tarda.

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SEPARATION AND QUANTITATIVE DETERMINATION OF RADIOLABELED PROSTAGLANDINS, THROMBOXANES, 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ AND OTHER ARACHIDONIC ACID METABOLITES PRODUCED IN BIOLOGICAL MATERIAL

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SUMMARY

Evaluation of several thin-layer chromatographic procedures for the separation of various labeled arachidonic acid metabolites (including 6-keto-prostaglandin $F_{1\alpha}$) produced in the biological system is described. Manual scanning and autoradiography of the plates developed by two-dimensional thin-layer chromatography was also done for locating the radioactivities due to arachidonic acid metabolites other than thromboxane B_2 and the classical prostaglandins ($PGF_{2\alpha}$, PGE_2, and PGD_2).

INTRODUCTION

Arachidonic acid (AA) is rapidly metabolized in the biological system by two main enzymatic pathways initiated separately by cyclooxygenase and lipoxygenase [1]. Cyclooxygenase converts arachidonic acid into the pivotal prostaglandin endoperoxides, which are then transformed into thromboxane A_2 (TxA_2), the classical prostaglandins, 12-hydroxy-heptadecatrienoic acid (HHT), malondialdehyde (MDA), and prostacyclin (PGI_2). Thromboxane A_2 is the main product in blood platelets while PGI_2 is chiefly produced by blood vessels. Thromboxane A_2 synthesis in platelets is usually accompanied by a simultaneous production of thromboxane B_2 (TxB_2), HHT and MDA. Prostacyclin is very unstable in the biological system and is readily converted non-enzymatically into 6-keto-$PGF_{1\alpha}$ whose determination gives a quantitative idea of the level of PGI_2. In platelets, lipoxygenase produces 12(*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE) which is subsequently converted into the corresponding hydroxy compound (HETE). In blood platelets, the lipoxygenase pathway has recently been shown to produce three more hydroxy

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fatty acids, viz. 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid [2], 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid (THETA) [2], and 10-hydroxy-11,12-epoxyeicosa-5,8,14-trienoic acid (EPHETA) [3].

In leukocytes a new family of conjugated lipoxygenation products are formed from AA, which serves as a substrate for 5-lipoxygenase. These are collectively called leukotrienes, viz. 5,12-diHETE or leukotriene B_4 and the leukotrienes C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4) [4]. The latter three compounds make up what is known as SRS-A, a slow-reacting substance of anaphylaxis, an important mediator of hypersensitivity responses that causes slow contraction of smooth muscle during allergic reaction.

The relative formation of prostaglandins, thromboxanes, hydroxy fatty acid (HPETE) and prostacyclin in the cardiovascular system is of great interest as these compounds exhibit opposite biological effects, i.e. whereas TxA_2 stimulates [5], PGE_1 [6], PGD_2 [7], HPETE [8] and PGI_2 [9] inhibit platelet aggregation/release reaction. There are several reports found in the literature on the chromatographic separation of prostaglandins [10—17]. In these reports information is lacking on the separation of prostaglandins from thromboxanes probably because thromboxanes were discovered either at the same time or later after the reports were published. Later some reports were published in which separation of TxB_2 from other prostaglandins and their quantitation have been described [18—20]. In the latter reports, TxB_2 was separated from the prostaglandins by unidimensional thin-layer chromatography (TLC).

There is one report which deals with the differential separation of thromboxanes from prostaglandins by two-dimensional TLC and the radiolabeled products were determined semiquantitatively with the help of a radioscanner [21]. Later, with the discovery of prostacyclin, its separation as a stable metabolite (6-keto-$PGF_{1\alpha}$) from other AA metabolites which are formed concomitantly in the biological system, and determination were desirable. Thus, as several closely related biologically active compounds are formed from AA, it is necessary to evaluate the various methods of resolution of such compounds and finally develop a convenient schedule for the separation and quantitation of these compounds useful for routine work. In the present report, therefore, methods are described for the separation and quantitation of labeled AA metabolites produced in human blood platelets and in the aorta and lung of rat.

EXPERIMENTAL

Authentic PGE_2, $PGF_{2\alpha}$, PGD_2, TxB_2 and 6-keto-$PGF_{1\alpha}$ were obtained from ONO Pharmaceutical Company, Osaka, Japan; HHT and HETE were from Dr. D.H. Nugteren, Unilever Research, Vlaardingen, The Netherlands, as generous gifts. Unlabeled AA was purchased from Nu Chek Prep., Elysian, MN, U.S.A.; and [1-^{14}C] AA (spec. act. 58.4 mCi/mmol) was from The Radiochemical Centre, Amersham, Great Britain. All organic solvents and chemicals were of analytical grade and were purchased from Merck, Darmstadt, G.F.R. Silica gel G plates (0.25 mm, 20×20 cm) were prepared in our laboratory and were activated (1 h) before use. In some separations, prepared plates (DC-Alufolien, Kieselgel 60 F_{254}, 20×20 cm, Merck) were used. Authentic PGs, TxB_2, HHT, and HETE were used as methanolic solutions. 6-Keto-$PGF_{1\alpha}$ was dissolved in

methanol—ethyl acetate (3:1, v/v). AA was dissolved in toluene. The solutions were stored at −20°C.

Blood platelets

Preparation of platelet suspension, incubation and extraction

Platelet suspension was prepared as described earlier [22]. Platelet suspension (300 μl, 10^8 platelets) was incubated with labeled arachidonate (final concentration 20 μ*M* in total incubation volume of 315 μl) at 37°C for 10 min. Extraction of the incubation mixture was done in ethyl acetate and the solvent evaporated. The residue was dissolved in 200 μl of ethanol and used for TLC separation.

Chromatographic separation of AA metabolites

AA metabolites were separated by TLC. Reference standards (3—5 μg) were first applied on the plate followed by incubation extract containing the radiolabeled AA metabolites as one single spot (diameter ca. 7 mm) with the help of a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). Plates were developed using the ascending technique in tightly closed rectangular tanks (Desaga, Heidelberg, G.F.R.) at room temperature (21°C). Chromatography tanks were not equilibrated with the solvent mixture as this has been reported to result in less efficient separation [23]. During TLC developments, a solvent run of ca. 17 cm from the application point to the solvent front was maintained.

Separation of ^{14}C-labeled HHT, HETE, and AA. This was conveniently done by unidimensional TLC resolution of 25 μl of the ethanolic extract by solvent system I (chloroform—methanol, 90:3, v/v). Spots were visualized in iodine, marked, scraped off and their radioactivity counted.

Unidimensional TLC separation of ^{14}C-labeled AA metabolites. This was achieved by resolving 25 μl of the ethanolic extract in the three solvent systems II (chloroform—methanol—acetic acid, 90:8:6, v/v), III (benzene—dioxane—acetic acid, 40:20:2, v/v), and IV (diethyl ether—methanol—acetic acid, 90:1:2, v/v). Classical PGs and TxB_2 were used as standard reference compounds. Their spots were visualized in iodine, marked, scraped off, and their radioactivity counted. The remaining portion of the developed part of the plate was divided into several areas corresponding to 1.2 cm along the direction of the development × 2.5 cm (breadth), scraped off and counted.

Separation of ^{14}C-labeled $PGF_{2\alpha}$, PGE_2, PGD_2 and TxB_2 by two-dimensional TLC. On the left-hand bottom corner of a plate were spotted authentic samples of the above reference standards. On the same spot were placed 25 μl of ethanolic solution of incubation extract. The plate was developed in two sets of solvent systems, II and III or II and IV. Spots were visualized in iodine, marked, scraped off and their radioactivity counted.

Separation of ^{14}C-labeled HHT, HETE, AA, $PGF_{2\alpha}$, PGE_2, PGD_2 and TxB_2 on a single TLC plate by a three-solvent development. TLC plates spotted as described above were subjected to three successive developments in solvents I, II, III or I, II, IV. The details of this method have been reported earlier [24].

Autoradiography of TLC plates after two-dimensional development. On the

left-hand bottom corner of the TLC plates (DC Alufolien, Kieselgel 60 F_{254}, 20 × 20 cm) were placed reference standards (PGs and TxB_2) followed by 25 μl of the ethanolic extract. The plates were developed in solvents II and III or II and IV as described for ^{14}C-labeled AA metabolites, the spots were visualized in iodine, and marked. The plates were then exposed to X-ray film for 7 days. Areas containing strong radioactivity produced marks on the X-ray plates.

Manual scanning of TLC plate after two-dimensional development. Two TLC plates were used. For two-dimensional development, the reference compounds and the extracted material were spotted as described above. One of the plates was developed in solvents II and III, and the other plate in solvents II and IV. The spots were visualized in iodine, marked, scraped off and counted. The remaining portion of the plate was divided into 1.3 cm × 1.3 cm areas which were carefully scraped off and their radioactivity counted.

Aorta and lung (rat)

Preparation of homogenate, incubation and extraction

Incubation medium in the case of lung consisted of 500 μl of lung homogenate, that for aorta consisted of a piece of aorta in 250 μl of phosphate buffer (0.06 *M*, pH 7.4, 2 m*M* EDTA). Lung homogenate was prepared in phosphate buffer (1:4, w/v). The two preparations were incubated with labeled AA (final concentration for aorta 25 μ*M*, for lung 12.5 μ*M*) for 15 min at 37°C. The incubation mixture was extracted with ethyl acetate after addition of saline and acidification (with HCl) to pH 3.0 as described for platelets. The residue obtained was dissolved in 250 μl of methanol—ethyl acetate (4:1, v/v) in the case of aorta, and in 300 μl of methanol—ethyl acetate (2:1, v/v) in the case of lungs.

TLC separation of ^{14}C-labeled $PGF_{2\alpha}$, PGE_2, PGD_2, TxB_2 and 6-keto-$PGF_{1\alpha}$

This was achieved by two-dimensional TLC using solvents II and III as described for platelets. A relatively greater amount of 6-keto-$PGF_{1\alpha}$, as reference standard compared to other PGs was needed for being visualized on the plate in iodine.

RESULTS

Blood platelets

Unidimensional TLC. Table I gives the results for the separation of ^{14}C-labeled PGs and TxB_2 in 25 μl of the incubation extract. The three solvent systems II, III and IV were used. In solvent systems II and IV the PGs and TxB_2 were resolved, whereas solvent system III did not separate TxB_2 from PGE_2. Manual scanning of the entire length of the developed part of the plate showed that radioactivity was distributed along the entire length of the plate and even in areas between $PGF_{2\alpha}/TxB_2$, TxB_2/PGE_2 and PGE_2/PGD_2. High radioactivity areas, as expected, were located a little below the solvent front. Just above the application point, considerable radioactivity was found. In each solvent system, repeated separations of a certain incubation extract gave close values for the resolved PGs and TxB_2; the values, however, were different in different solvent systems.

TABLE I

TLC SEPARATION OF LABELED AA METABOLITES FORMED IN HUMAN PLATELETS FROM LABELED AA BY UNIDIMENSIONAL DEVELOPMENT IN THREE SOLVENT SYSTEMS*

Values are reported as cpm (mean ± S.D.) of three separations. Exactly equal amounts of the same incubation extract were resolved in different solvent systems (unidimensional).

	Sample 1		Sample 2		
Metabolite	II	III	II	III	IV
$PGF_{2\alpha}$	589 ± 81	467 ± 16	467 ± 50	370 ± 69	337 ± 39
TxB_2	19018 ± 388	19436 ± 1311	4522 ± 117	4664 ± 99	3511 ± 164
PGE_2	1308 ± 107		539 ± 20		384 ± 28
PGD_2	768 ± 68	1160 ± 175	351 ± 12	438 ± 17	630 ± 43

*II, chloroform—methanol—acetic acid (90:8:6, v/v); III, benzene—dioxane—acetic acid (40:20:2, v/v); IV, ether—methanol—acetic acid (90:1:2, v/v).

TABLE II

UNIDIMENSIONAL TLC SEPARATION OF HHT AND HETE FORMED IN BLOOD PLATELETS FROM LABELED AA

Solvent system I: chloroform—methanol (90:3, v/v).

Metabolite	cpm (mean ± S.D.)*
HHT	3198 ± 136
HETE	9776 ± 926
Application spot**	10983 ± 282

*Three separations.
**Due to classical PGs + TxB_2 + phospholipids + other known and unknown AA metabolites.

TABLE III

TLC SEPARATION OF LABELED AA METABOLITES FORMED IN HUMAN PLATELETS BY TWO-DIMENSIONAL DEVELOPMENT

Values are reported as cpm. For each sample, an exactly equal amount of the incubation extract was resolved in the different solvent systems.

	Sample I		Sample 2	
Metabolite	II and III*	II and IV**	II and III*	II and IV**
$PGF_{2\alpha}$	258 ± 22	204	130 ± 16	103
TxB_2	18055 ± 1045	17365	4266 ± 159	3972
PGE_2	443 ± 88	478	230 ± 28	198
PGD_2	484 ± 45	562	166 ± 24	167

*Three separations (mean ± S.D.).
**Two separations.

Separation of AA, HHT and HETE from PGs and TxB_2. Though the major part of the radioactivity was localized in AA, HHT and HETE and also in areas between HHT/HETE, and HETE/AA, a substantial amount of radioactivity (>1000 cpm per 1.2 cm × 2.5 cm area) was found distributed along the entire length, right from the application point to the HHT spot (Table II).

Two-dimensional TLC. Table III gives the separation results for the PGs and TxB_2 resolved by two sets of solvents. As can be seen, the values for TxB_2 are quite close to those obtained in unidimensional TLC. In the case of prostaglandins $F_{2\alpha}$, E_2 and D_2, the values were reduced by about 35—50% of those obtained in unidimensional TLC. The values for PGs in the two solvent sets were acceptably close.

Manual scanning after two-dimensional TLC. Besides TxB_2, the major radioactivity was found distributed in the top right-hand corner of the plate in areas 7.5 cm × 10.5 cm (solvents II and III), and 8.0 cm × 9.5 cm (solvents II and IV) (Fig. 1). The radioactivity contained in these areas should mainly be due to excess AA, HHT, HETE and other known and unknown metabolites of AA.

Autoradiography of TLC plates after two-dimensional development. Major radioactivity was localized in the spot due to TxB_2, and at the right-hand top corner area, confirming the results obtained by manual scanning (Fig. 2). Plates

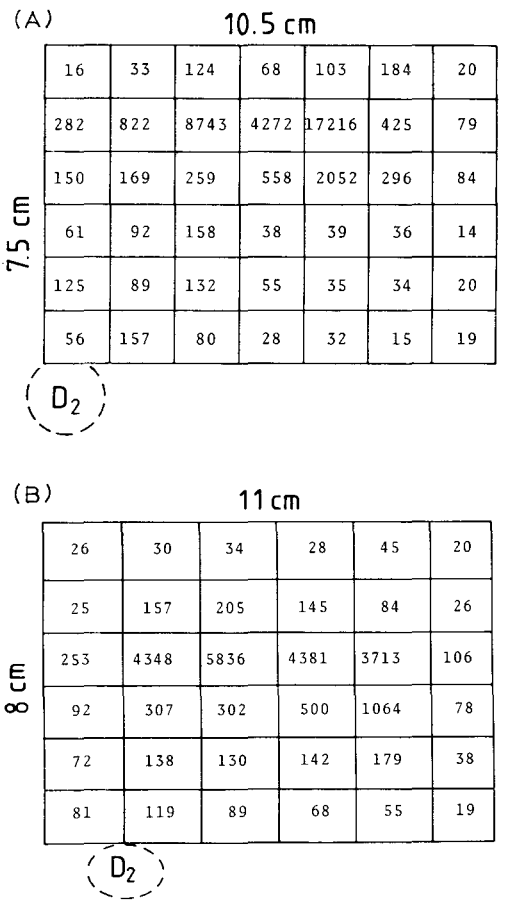

Fig. 1. Manual scanning of the top right-hand corner area of the TLC plate after two-dimensional development. (A) Developed successively in solvents II and III; area scanned 10.5 cm × 7.5 cm. (B) Developed successively in solvents II and IV; area scanned 11 cm × 8 cm. The values are cpm in the respective area. Location of PGD_2 is also shown.

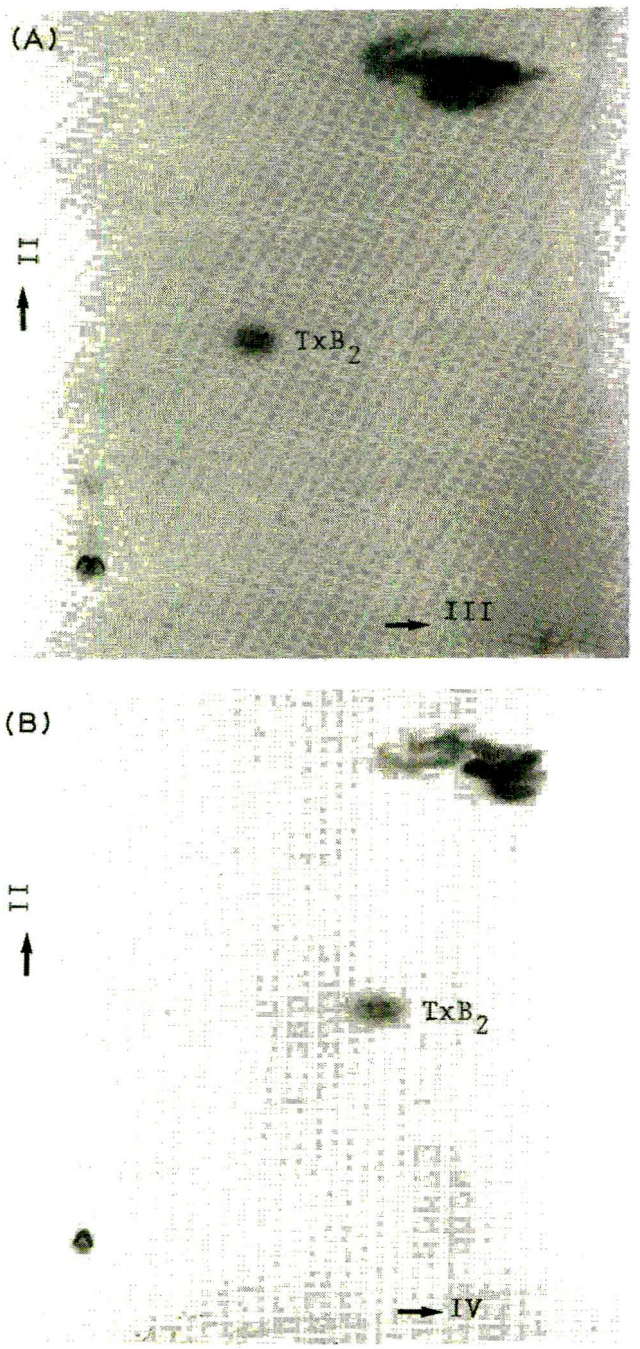

Fig. 2. Autoradiography of the TLC plate developed successively in solvents II and III (A), and solvents II and IV (B). The spot in the middle is due to TxB_2. Areas of high radioactivity are seen in the top right-hand corner of the plate.

developed unidimensionally in solvents II and III, were also subjected to autoradiography. The radioactivity distribution confirmed that ascertained by manual scanning.

Aorta and lung (rat)

Chromatographic separation of ^{14}C-labeled $PGF_{2\alpha}$, PGE_2, PGD_2, TxB_2 and 6-keto-$PGF_{1\alpha}$. Separation of these AA metabolites was achieved by two-dimensional TLC (Fig. 3) and their amounts were calculated after taking into consideration the recovery factors. Results are shown in Table IV.

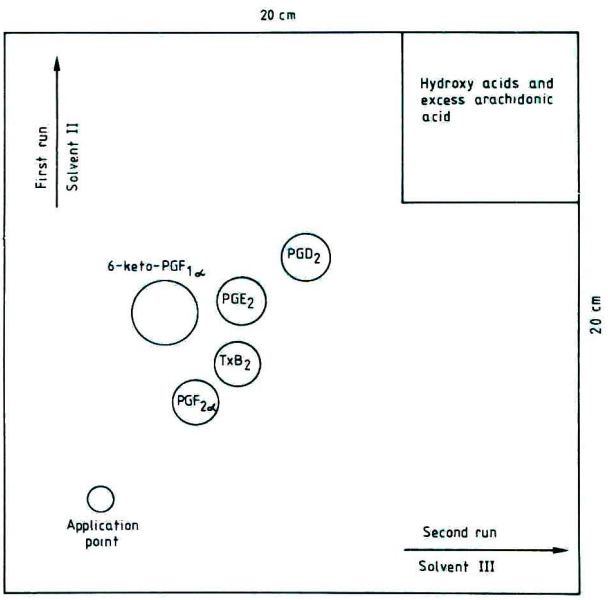

Fig. 3. Schematic presentation of the separation of prostaglandins, thromboxane, 6-keto-prostaglandin $F_{1\alpha}$ and hydroxy acids by two-dimensional TLC.

TABLE IV

SEPARATION OF LABELED AA METABOLITES FORMED BY AORTA RINGS AND LUNG HOMOGENATE OF RAT BY TWO-DIMENSIONAL TLC

Values (mean ± S.D.) are reported as picomoles per aorta piece (13.2 mg) and as picomoles per 500 μl of lung homogenate of three separations which were achieved by developing the plate in solvents II and III in succession.

Tissue	AA metabolite				
	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	TxB_2	PGE_2	PGD_2
Aorta	76 ± 5	5 ± 2	13 ± 3	5 ± 1	4 ± 2
Lung	214 ± 16	18 ± 3	148 ± 10	21 ± 2	18 ± 3

DISCUSSION

Several solvent systems have been described in the literature for the complete resolution of the PGs and TxB_2 by unidimensional development. Repeated separation of AA metabolites in an incubation extract by unidimensional development using a certain solvent system gives reproducible results. But separation results, with the exception of TxB_2, are not the same with other solvent systems in the case of classical PGs for which varying values are obtained. This necessitates a further resolution. Two-dimensional TLC was found suitable because separation by different sets of solvent systems yields comparable values for TxB_2 and classical PGs. While no appreciable change was observed in the TxB_2 counts obtained in the two-dimensional TLC when compared with those obtained in the unidimensional development, the same for the classical PGs were reduced by 35—50% of the values obtained with unidimensional TLC. Manual scanning of the plate after two-dimensional development showed that the radioactivity was mainly localized in the spot due to TxB_2, in the application spot and also in the spots of HHT, HETE and AA. Other areas of radioactivity were observed too. The chemical characterization of the unknown radioactivities evidently requires further study.

Although a three-solvent development of the same plate is suitable for the separation of AA, HHT, HETE, TxB_2 and PGs [24], one may best utilize the separation schedule described in this paper by a combination of unidimensional development (for the separation of AA, HHT, and HETE) and two-dimensional development (for the separation of PGs and TxB_2) if one can afford a little more material for the separation. Further, this combined procedure will require one extra TLC plate per 5 or 6 samples (for the separation of AA, HHT and HETE). Moreover, for the complete resolution of a mixture containing 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) with the other AA metabolites mentioned above, a combination of unidimensional and two-dimensional separation is suitable. For the separation of 6-keto-$PGF_{1\alpha}$ from other AA metabolites, it is necessary to acidify the incubation medium with hydrochloric acid (instead of the most frequently used citric acid) prior to extraction with ethyl acetate because acidification with citric acid leads to the development of multiple spots of 6-keto-$PGF_{1\alpha}$ on the TLC plate [25].

In prostaglandin research one is often interested in the endogenous synthesis of the biologically active AA metabolites which are usually determined by either bioassay or radioimmunoassay. These methods, though very useful for quantitative assay, have limitations in the costs involved if one would like to estimate several of the AA metabolites. Formation of AA metabolites from the labeled precursor may give an insight into the endogenous biosynthesis of all the known AA metabolites and more so of their relative proportions. This can be achieved by labeling the AA pool of the membrane phospholipids and subsequently subjecting them to hydrolysis by a phospholipase reaction [26]. This is possible only when a suitable separation schedule is available. For this purpose our method is convenient and reproducible giving reliable prostaglandin values which do not change on further TLC. Manual scanning of the TLC plate has shown that TxB_2 is the main cyclooxygenase product in the blood platelets,

and that areas of unknown radioactivity are localized close to those of the hydroxy acids (HHT, HETE).

ACKNOWLEDGEMENT

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DETECTION OF THE CHANGES IN PROTEIN DISTRIBUTION IN RAT SERUM AFTER PARTIAL HEPATECTOMY USING TWO-DIMENSIONAL ELECTROPHORESIS UNDER NON-DENATURING CONDITIONS

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SUMMARY

The changes in rat serum protein distribution after partial hepatectomy were examined using two-dimensional electrophoresis, utilizing isoelectric focusing in polyacrylamide gel in the first dimension and pore gradient polyacrylamide gel electrophoresis in the second dimension. Drastic decreases in amount of protein were observed at more than twenty spot positions, and drastic increases in amount or newly appeared proteins were observed at eight spot positions. The amounts of albumin, immunoglobulin M and α_2-macroglobulin did not change relatively after hepatectomy.

The time course of the changes was examined using a densitometer, and it was observed that almost all the serum proteins changed drastically at 24 h after hepatectomy.

INTRODUCTION

The factors that trigger the proliferation of liver cells after partial hepatectomy in rat are not yet known with certainty. A series of specific substances are said to effect the proliferation of liver cells, but metabolic changes after partial hepatectomy possibly also act as a contributory causative factor in the stimulation of those substances. In order to define this question, the changes in the serum concentration of various metabolites after partial

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hepatectomy have been studied [1—4]. However, as for the changes in serum protein distribution after partial hepatectomy, little is known except for some proteins [2—5], due to poor resolution of the analytical techniques.

Manabe et al. [6, 7] described a two-dimensional electrophoretic technique which did not employ denaturing agents, and showed that human plasma proteins could be resolved into about 250 spots. Since this technique does not employ denaturing agents such as sodium dodecyl sulfate or urea throughout the electrophoretic run, it is suited to the analysis of mixtures of soluble proteins, maintaining their native physicochemical properties [8, 9] and their biological activities [10]. They also showed that this electrophoretic technique could be suitable for clinical purposes [11].

In the present report we show that the drastic changes in rat serum proteins, which may play an important role for the proliferation of liver cells, after hepatectomy can be analyzed by means of the two-dimensional electrophoretic technique.

MATERIALS AND METHODS

Reagents

Ampholines (pH 3.5—10 and pH 4—6.5) were obtained from LKB Produkter (Bromma, Sweden). Acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (all special grade for electrophoresis), Tris base, glycine and ammonium persulfate were from Wako Pure Industries (Tokyo, Japan). Coomassie Brilliant blue R-250 was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Hepatectomy and serum samples

Male rats (Wistar strain, 150 g) were used. Partial hepatectomy was performed under ether narcosis according to the method of Higgins and Anderson [12]. After hepatectomy the animals were maintained in our laboratory for 24—240 h. Food and water were provided ad libitum. At scheduled time intervals blood was taken from the descending aorta of each rat with a disposable syringe. The blood was left to stand at 4°C for 2 h, and then centrifuged at 3000 *g* for 10 min. Sucrose was added to the supernatant serum to give a concentration of 10% (w/v), and the serum samples were stored at —20°C until use.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was mainly carried out according to the method of Manabe et al. [6, 7]. First-dimension isoelectric focusing was performed on a gel column (14.5 cm × 0.5 cm I.D.). A 4% acrylamide (0.2% bisacrylamide) solution containing 2% Ampholine pH 3.5—10, 0.2% Ampholine pH 4—6.5 and 0.05% ammonium persulfate, was poured into the gel column. After gelling, the bottom ends of the tubes were covered with dialysis membrane held in place by O-rings, and the glass tubes were placed in a gel electrophoresis chamber. The electrode solutions were 0.04 *M* sodium hydroxide (cathode) and 0.01 *M* phosphoric acid (anode). An overlay solution (2% Ampholine pH 3.5—10, 0.2% Ampholine pH 4—6.5 and 5% sucrose, 50 μl)

was layered on top of the gel column and then serum samples (50 μl) were applied under the overlay solution. Electrophoresis was run at 2 mA constant current per gel column for 40 min and then at 460 V constant voltage for 20 h at 4°C. After electrophoresis the gel was pushed out by a rubber bulb and placed on top of the second-dimension slab gel without equilibration.

Second-dimension gradient polyacrylamide gel electrophoresis was performed with a slab gel apparatus which forms four slab gels 12 cm long, 16 cm wide and 0.4 cm thick. A 4—21% acrylamide linear gradient (0.2% bisacrylamide) containing a 0—10% sucrose gradient and a 0.05—0.025% ammonium persulfate gradient was poured in about 50 min at 4°C. Gelling occurred in about 2 h in a water-bath at 30°C. The gradient gel buffer was 0.14 *M* Tris—HCl (pH 8.9) and the electrode buffer was 0.05 *M* Tris—0.38 *M* glycine (pH 8.3). Electrophoresis was run at 50 mA constant current per slab gel for 22 h at 4°C. During the second-dimension electrophoresis, the electrode buffers in the anode chamber and the cathode chamber were exchanged continuously with each other at 40 ml per min using a peristaltic pump.

Measurement of the pH gradient

The first-dimension isoelectric focusing gel was duplicated for each sample. One of the gels was cut into 10-mm sections and these sections were placed in individual vials containing 2 ml of distilled water. These vials were capped and kept for 24 h at 4°C, then the pH was measured for each vial on a Horiba pH meter (Tokyo, Japan).

Staining and destaining

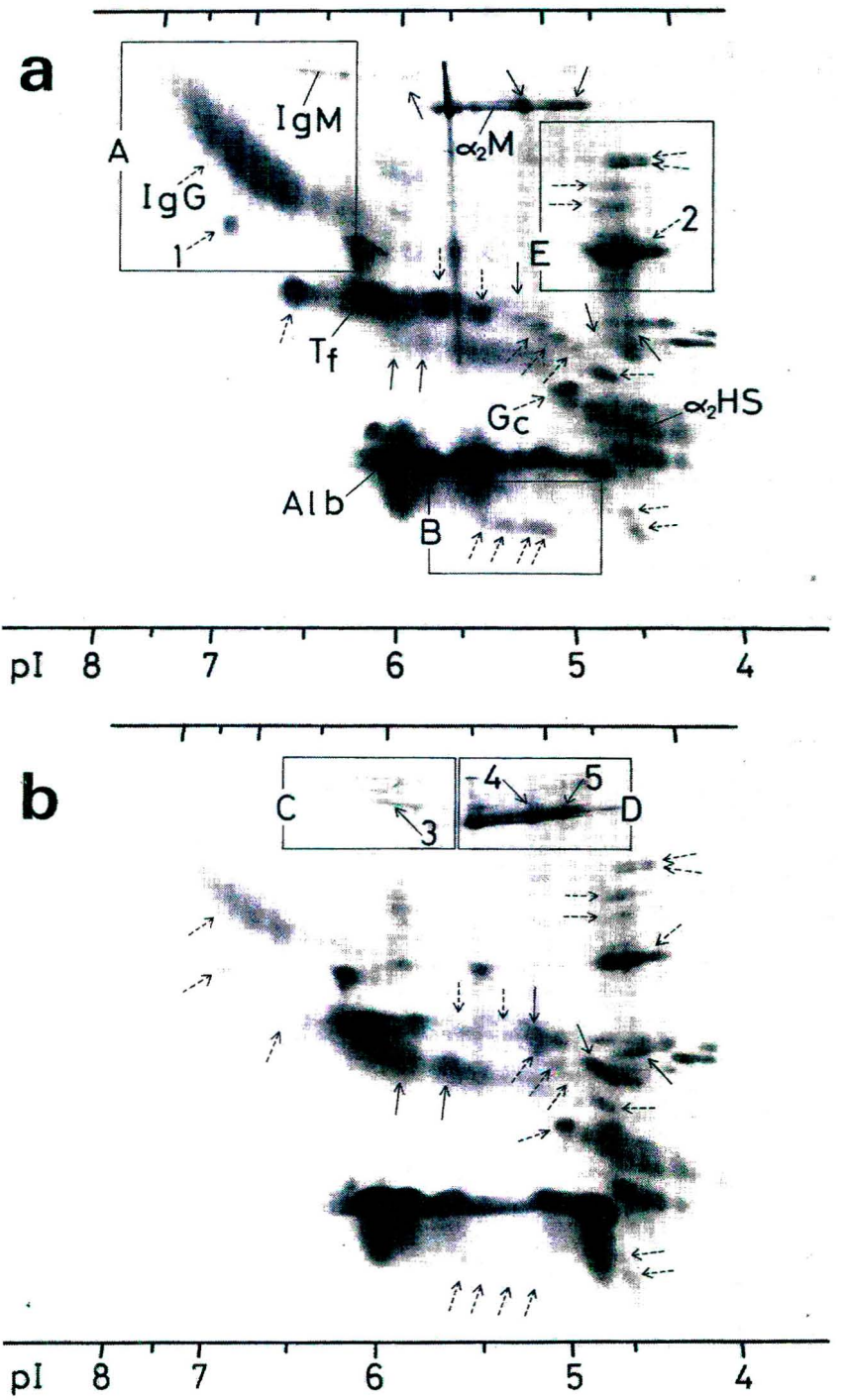
The gel was stained overnight in 0.025% Coomassie Brilliant blue R-250—7% (v/v) acetic acid—50% (v/v) methanol, and destained in 7% (v/v) acetic acid at 80°C for 4 h, then in two changes of 7% (v/v) acetic acid at room temperature for two days. During staining and destaining, the gel container was shaken gently.

Photography

Photography was carried out by placing the gel on top of a viewing box positioned under a 35-mm reflex camera. A Kenko Y-2 filter (Kenko Co., Tokyo, Japan) was attached to the camera and Fuji Neopan F 10D film (ASA 32, Fuji Photo Film Co., Tokyo, Japan) was used.

Densitometry

Densitometric quantitation of Coomassie blue-stained spots on the slab gel after two-dimensional electrophoresis was carried out using a Shimadzu dual-wavelength thin-layer chromatographic scanner CS-900 (Shimadzu Corp., Tokyo, Japan). Sample wavelength was set at 580 nm and reference wavelength was set at 750 nm. The densitometer was operated in "zig-zag scanning mode" and the protein amounts were quantitated by measuring the step height of the integrating signal.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A
IgM
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1
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Tf
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pI 8 7 6 5 4
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4
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D
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RESULTS

Fig. 1a shows one of the two-dimensional electrophoretic patterns of normal rat serum proteins, before partial hepatectomy. The protein distributions of several rat serum samples were compared and the positions of the serum proteins were reproducible. Major serum proteins were located on the gel by comparing the patterns with those of human plasma proteins [13], and some of them are shown by letters in the figure. Fig. 1b shows a pattern of the two-dimensional distribution of rat serum proteins which was taken at 24 h after partial hepatectomy. Drastic changes of protein distributions in quantity were observed. The locations of the proteins whose positions or spot areas apparently changed after partial hepatectomy, are shown by arrows in the figure. The proteins that apparently decreased after hepatectomy were observed at more than twenty spot positions containing immunoglobulin (Ig) G, transferrin and G_c-globulin, and those are indicated by dotted arrows. The proteins that apparently increased or newly appeared after hepatectomy were observed at eight spot positions, and those are indicated by solid arrows. Some of the major serum proteins, which were tentatively identified as IgM, α_2-macroglobulin and albumin did not change relatively after hepatectomy.

The time course of the changes in the two-dimensional pattern of serum proteins was examined. Serum samples which were taken at 24, 48, 72, 96, 168, and 240 h after hepatectomy were subjected to the two-dimensional electrophoresis and the protein distributions were compared with those of normal serum pattern. The time-dependent changes at the gel sections of areas A, B, C and D (indicated in Fig. 1) are shown in Fig. 2. As shown by arrows in Fig. 2A and B, IgG, spot 1 and spots in area B were rapidly decreased at 24 h after partial hepatectomy, then gradually increased and almost recovered their original level at 96 h. In contrast, the proteins which drastically increased nor newly appeared after hepatectomy are shown in Fig. 2C and D. The protein of spot 3 (indicated in Fig. 1b) which did not exist in normal rat serum, appeared at 24 h after hepatectomy, gradually decreased, and then almost disappeared at 96 h (Fig. 2C). The proteins of spot 4 and spot 5 (indicated in Fig. 1b) also increased at 24 h after hepatectomy, and almost decreased to their original level at 96 h (Fig. 2D).

Fig. 3 shows the time-dependent changes at the gel sections of area E (indicated in Fig. 1a). The proteins in area E also changed after partial hepatectomy, but the changes were extremely slow. These proteins were minimal at 96 h and did not recover their original level even at 240 h after hepatectomy.

Fig. 1. Two-dimensional electrophoresis of rat serum proteins, (a) before partial hepatectomy, and (b) 24 h after partial hepatectomy. Arrows with a dotted line indicate the spots that apparently decreased after hepatectomy. Arrows with a solid line indicate the spots that apparently increased or newly appeared after hepatectomy. The positions of major serum proteins were located on the gel by comparing the patterns of rat serum proteins with those of human plasma proteins [13]. IgM = immunoglobulin M; α_2M = α_2-macroglobulin; IgG = immunoglobulin G; Tf = transferrin; G_c = G_c-globulin; α_2HS = α_2HS-glycoprotein; Alb = albumin.

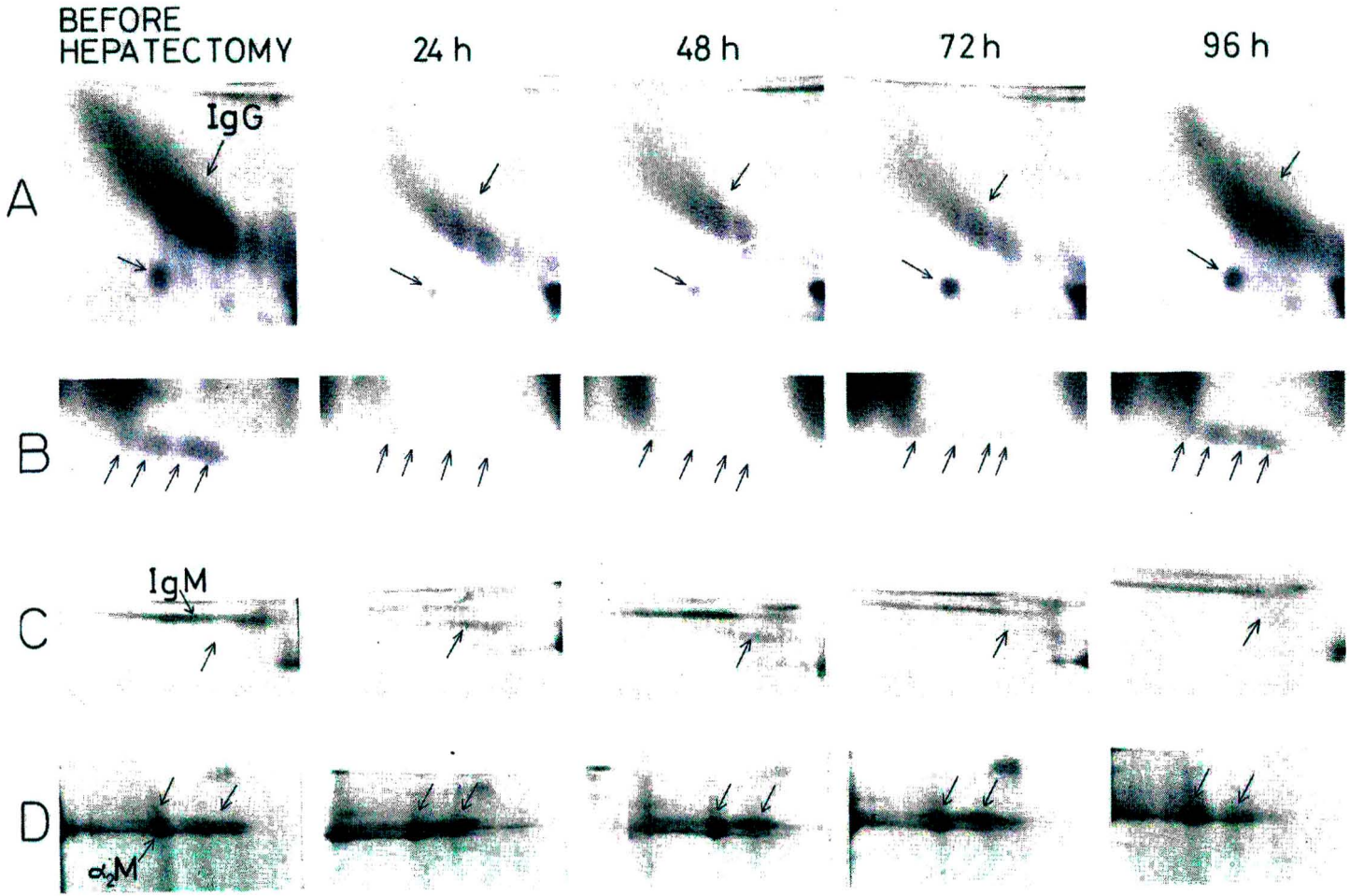

Fig. 2. Time course of the changes in the two-dimensional pattern of rat serum proteins after partial hepatectomy. Serum samples taken at 0, 24, 48, 72, and 96 h after partial hepatectomy were analyzed by two-dimensional electrophoresis. Time-dependent changes at the gel sections of areas A, B, C, and D (indicated in Fig. 1) are shown. Spot positions are indicated by arrows. IgG = immunoglobulin G, IgM = immunoglobulin M, α_2M = α_2-macroglobulin.

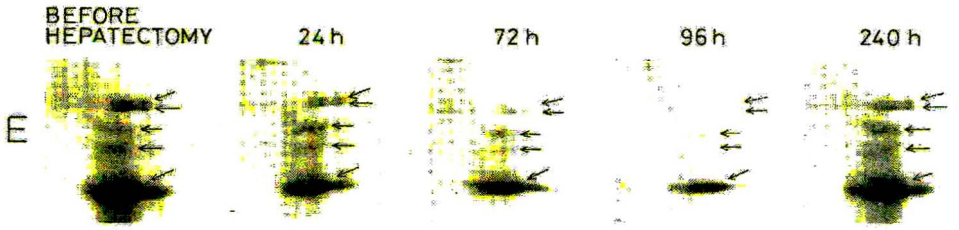

Fig. 3. Time course of the changes in the two-dimensional pattern of rat serum proteins after partial hepatectomy. Serum samples taken at 0, 24, 72, 96, and 240 h after partial hepatectomy were analyzed by two-dimensional electrophoresis. Time-dependent changes at gel sections of area E (indicated in Fig. 1a) are shown. Spot positions are indicated by arrows.

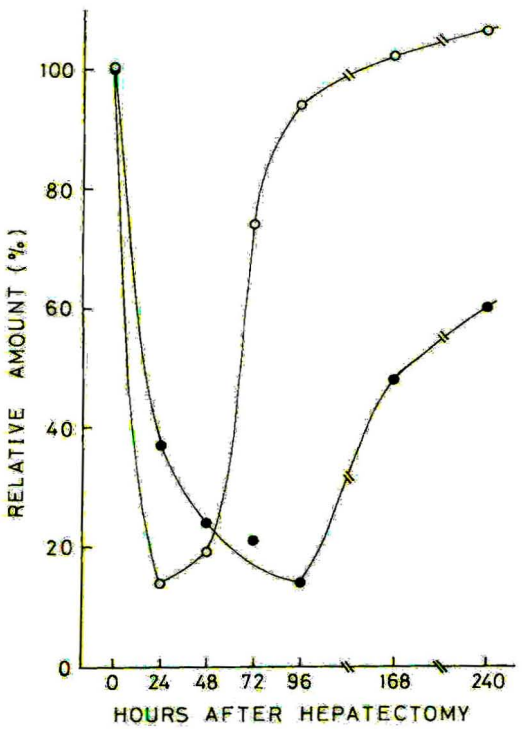

Fig. 4. Densitometric quantitation of spot 1 and spot 2 (indicated in Fig. 1a) after partial hepatectomy. Spots 1 and spots 2 on acrylamide slab gels were quantitated using a Shimadzu TLC scanner. (○—○), spot 1; (●—●), spot 2.

Densitometric quantitation of spots 1 and spots 2 on acrylamide slab gels was carried out with a Shimadzu TLC scanner. Fig. 4 shows the time courses of the quantity of spot 1 and of spot 2. A drastic fall in the amount of spot 1 at 24 h and of spot 2 at 96 h after hepatectomy was demonstrated.

DISCUSSION

Changes in serum proteins after partial hepatectomy have been studied by means of one-dimensional electrophoretic techniques, such as cellulose acetate membrane electrophoresis [2] and polyacrylamide gel electrophoresis [4]. However, these one-dimensional techniques could offer little information about the changes in the serum proteins which may be closely related to the proliferation of liver cells after partial hepatectomy. As shown in Figs. 1–3, the two-dimensional electrophoretic technique could detect the changes in serum protein distribution after hepatectomy. The time course of the decrease or increase of each protein spot also could be followed by means of the technique. The proteins which changed after hepatectomy can be divided into three types: type I includes proteins that drastically decreased in amount after hepatectomy (e.g. IgG, spot 1 and proteins in area B), type II includes those that newly appeared (e.g. spot 3) or drastically increased (e.g. spot 4 and spot 5), and type III includes those that did not change relatively (e.g. IgM, α_2-macroglobulin and albumin). We suggest that the proteins of type I and type II are closely related to the proliferation of liver cells after partial hepatectomy.

The electrophoretic technique employed isoelectric focusing in the first dimension and acrylamide pore gradient (4—21%) electrophoresis in the second dimension. The technique used no denaturing agent such as urea or sodium dodecyl sulfate throughout the run, thus equilibration of the first-dimension gel was not necessary. Further, when the isoelectric focusing gel was examined for the protein remaining after the second-dimension run, no Coomassie blue-stained band was observed. Therefore, comparison of spot area was possible since there was no loss of proteins during the course of the electrophoretic run. Densitometric quantitation of isolated spots (such as spot 1 and spot 2, see Fig. 1a) was readily performed using a TLC scanner. The quantitation will help to estimate the restoration of liver cells after partial hepatectomy.

Spot 3 was newly appeared protein after partial hepatectomy. It showed a relatively wide p*I* range (p*I* 5.5—6.5) and had an apparent molecular weight of about 900,000. The identification of this spot will not be easy since it can be either cellular protein or modified serum protein.

Spot 4 and spot 5 were rapidly increased proteins after partial hepatectomy. These proteins could also be found in AH-130 tumour-bearing abdominal ascites. Therefore, we suggest that these proteins play an important role in the proliferation of the cells. The molecular weights of these proteins were estimated to be about 800,000 and their isoelectric points were 4.9 (spot 5) and 5.1 (spot 4). Spot 4 and spot 5 were periodic acid—Schiff-positive proteins and this characterization may be useful for their purification.

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CHROMBIO. 1639

DETERMINATION OF THE α,β-ADRENOCEPTOR BLOCKER YM-09538 IN URINE BY GAS CHROMATOGRAPHY WITH A NITROGEN-SENSITIVE DETECTOR

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SUMMARY

A gas chromatographic method for the quantitative determination of the α,β-adrenoceptor blocker YM-09538 in urine is described. YM-09538 was extracted from alkalinized urine with ethyl acetate and converted to its cyclic methylboronate derivative. Analysis by gas chromatography using a nitrogen-sensitive detector allowed quantitation of the drug over a concentration range of 0.2—5.0 μg/ml. Urinary excretion of YM-09538 was determined in humans after oral administration of 50 mg.

INTRODUCTION

A combined α- and β-adrenoceptor blocker, 5-{1-hydroxy-2-[2-(*o*-methoxyphenoxy)ethylamino]ethyl}-2-methylbenzenesulphonamide hydrochloride (YM-09538), has been introduced as a dose-dependent antihypertensive agent [1,2]. Biopharmaceutical studies require estimation of urinary excretion of YM-09538. The concentration of this drug in plasma can be analyzed by high-performance liquid chromatography with fluorescence detection [3]. However, that method is not applicable to determine urinary YM-09538 since endogenous fluorescent materials in the urine interfere with the analysis; a gas chromatographic (GC) method was therefore investigated. YM-09538, being a

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multifunctional amino alcohol, requires derivatization of the reactive groups before it can be analyzed by GC. Aryl- or alkylboronic acids have been used as specific reagents for derivatization of various bifunctional amino alcohols for GC or gas chromatography—mass spectrometry (GC—MS) (for a review see ref. 4). Some attempts have been made to increase the sensitivity of the derivatives. Poole et al. [5,6] introduced electron-capturing groups into benzeneboronic acid and the reagents were used to analyze nanogram levels of β-blocking drugs in biological fluids by GC with electron-capture detection [7,8]. Recently, a series of β-blocking drugs in plasma was determined by GC with a nitrogen-sensitive detector after conversion to their *n*-butyl- or phenylboronic esters [9]. This present report describes a GC method of determining YM-09538 in urine using a nitrogen-sensitive detector and methylboronic acid as a reagent for derivatization.

EXPERIMENTAL

Chemicals and reagents

YM-09538 and 5-{1-hydroxy-2-[2-(*o*-methoxyphenoxy)ethylamino]ethyl} 2-methoxybenzenesulphonamide hydrochloride, used as the internal standard, were synthesized in our laboratory by the method of Imai et al. [1]. Methylboronic acid was purchased from Applied Science Labs. (State College, PA, U.S.A.) and *n*-butyl- and phenylboronic acid were from Tokyo Kasei (Tokyo, Japan).

The methylboronic acid solution was prepared by dissolving 3 mg of the boronic acid in 10 ml of ethyl acetate.

Instrumentation

A Hewlett-Packard Model 5730A gas chromatograph equipped with a nitrogen-sensitive detector Model 18789A was used. The column was a glass tube (90 cm × 1.8 mm I.D.) packed with 2% OV-7 on Chromosorb W (AW DMCS, 80—100 mesh). The injection port, column oven and detector were maintained at 350°C, 290°C and 300°C, respectively. The helium carrier gas was dried over molecular sieves and passed at a flow-rate of 30 ml/min. Hydrogen and air flow-rates to the nitrogen-sensitive detector were 4 and 100 ml/min, respectively. Mass spectra of cyclic methylboronate esters of YM-09538 and the internal standard were obtained with a JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) which was combined with a Hewlett-Packard Model 5710A gas chromatograph. The operating conditions were: accelerating voltage, 3.0 kV; ionizing potential, 70 eV; total emission current, 300 μA; separator and ion source temperature, 250°C.

Procedures

To 1 ml of urine in a 10-ml glass stoppered centrifuge tube were added 2 ml of an aqueous solution containing 3 or 10 μg of the internal standard and then about 0.5 g of sodium hydrogen carbonate. The mixture was extracted with 4 ml of ethyl acetate and, after centrifugation, the ethyl acetate layer was evaporated under reduced pressure. The residue was dissolved in 100 μl of the methylboronic acid solution and, after 5 min, 6 μl of the solution were in-

jected into the GC column. Ratio of the peak height of YM-09538 to that of the internal standard was used to calculate the amount of YM-09538 by referring to a standard curve. The standard curve was generally prepared by analyzing drug-free control urine spiked with 1—5 μg of YM-09538 using 10 μg of the internal standard. When the concentration of the drug in urine samples was low, we used control urine spiked with 0.2—1.5 μg of YM-09538 and 3 μg of the internal standard.

Human studies

Three male volunteers, aged 28—33 years, received 50-mg YM-09538 tablets after overnight fasting. Urine was collected at 2-h intervals up to 12 h and then one fraction between 12 and 24 h. The urine samples were stored frozen until taken for assay.

The excretion rate was calculated by dividing the amount of YM-09538 excreted into urine by the period of collection time. The elimination half-life was demonstrated by least-squares analysis from the linear terminal portion of the curve plotted on semilogarithmic graph paper.

RESULTS AND DISCUSSION

Extraction of YM-09538 from urine

YM-09538 can be considered to dissociate as in Fig. 1. The pK_1 value determined by potentiometry and the pK_2 value determined by UV spectrophotometry [10] at 25°C were 7.4 and 10.2, respectively. This result is a good explanation of the previous finding [3] that the compound was most efficiently extracted from plasma at pH 7.5—8.5. When control human urine containing 1 μg of YM-09538 was saturated with sodium hydrogen carbonate (pH was about 8.2) and extracted with ethyl acetate, extraction recovery determined by the GC method was 91.7 ± 3.4% (n=5).

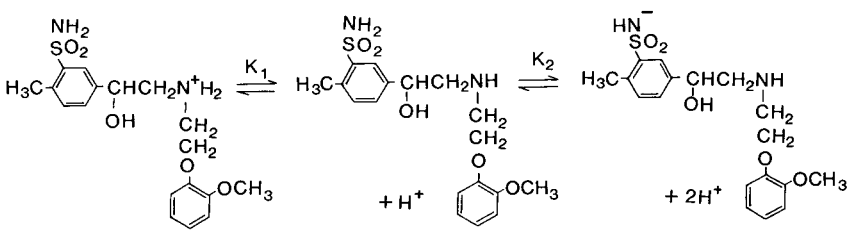

Fig. 1. Dissociation of YM-09538. K_1 and K_2 are dissociation constants for the secondary amino and sulphonamide groups, respectively.

Gas chromatography of YM-09538

YM-09538 has polar multifunctional groups which may render the drug unsatisfactory for GC analysis. Initial attempts to derivatize the compound with various silylating reagents were unsuccessful owing to incomplete silylation of the amino or sulphonamido group. For example, when N,O-bis-(trimethylsilyl)acetamide was used as a silylating reagent, a mixture of a major bis-trimethylsilyl (TMS) derivative with a minor tris-TMS derivative was obtained; the ratio of the tris-TMS derivative to the bis-TMS derivative increased with

the time course. After two days at room temperature, the product had changed to a mixture of tris-TMS and tetrakis-TMS derivatives.

To obtain a single derivative of YM-09538, aryl- or alkylboronic acids were very useful. Initially, the reaction of YM-09538 with the substituted boronic acids was studied by thin-layer chromatography (TLC) on silica gel 60 F_{254} plates (Merck, Darmstadt, G.F.R.) with the solvent system chloroform—tetrahydrofuran—methanol—28% ammonia solution (20 : 20 : 4 : 0.05). When YM-09538 and the internal standard were admixed with methylboronic acid and examined by TLC, YM-09538 and the internal standard, detectable by short-wave UV light at R_F 0.45 and 0.38, disappeared within 5 min at room temperature and was completely replaced by new compounds which appeared at R_F 0.25 and 0.21, respectively. MS analysis showed these compounds to be cyclic methylboronate esters of YM-09538 and the internal standard (Fig. 2). When the reaction mixture was injected into a GC column (OV-7, 2%, oven temperature 290°C) and monitored by a flame ionization detector (FID), well-shaped single peaks were obtained at retention times of 1.9 and 2.6 min, respectively. However, the large solvent peak disturbed the analysis and the sensitivity was relatively low. In order to increase the sensitivity, we examined selected ion monitoring using the base peaks at *m/z* 281 and *m/z* 297. However, both YM-09538 and the internal standard derivatives gave broad peaks. This was probably due to the adsorption of these derivatives on the separator or ion source. In fact, the shape of the peaks improved when the temperature of the separator and ion source was increased (up to 310°C); it was still insufficient for analytical purposes. The most satisfactory results, in terms of sensitivity and peak shape, were obtained with a nitrogen-sensitive detector. The two compounds gave sharp peaks as with FID and the sensitivity with the nitrogen-sensitive detector was 10—15 times higher than with FID.

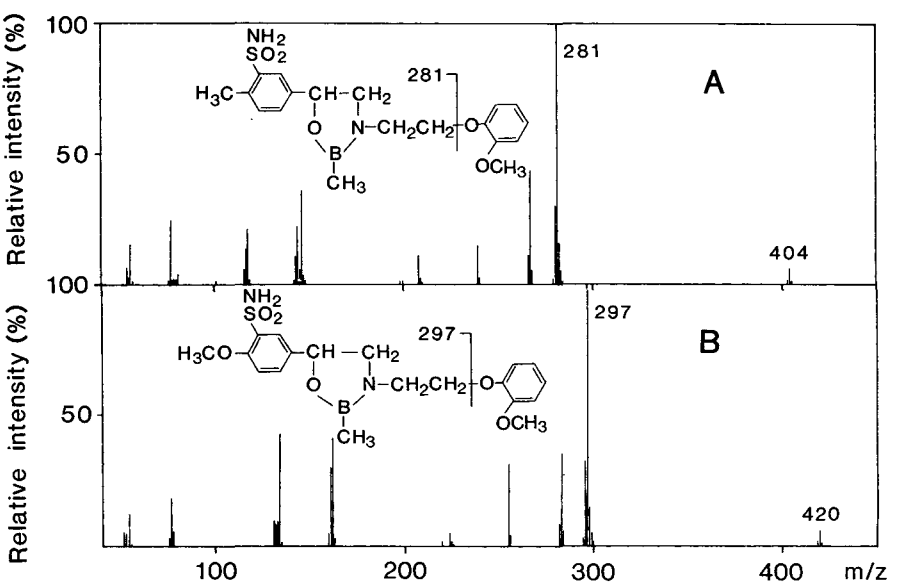

Fig. 2. Mass spectra of YM-09538 (A) and the internal standard (B) cyclic methylboronates.

TABLE I

RETENTION INDICES OF CYCLIC BORONATE ESTERS OF YM-09538 AND THE INTERNAL STANDARD

Boronate	YM-09538	The internal standard	ΔI
Methyl	3628	3798	170
Butyl	3849	3993	144
Phenyl	4274	4417	143

Among various boronic acid commercially available, *n*-butylboronic acid has most frequently been used [4]. Brooks and Maclean [11] have reported the relative merits of methyl-, *n*-butyl-, phenyl- and cyclohexylboronate derivatives of mandelic and salicylic acids and noted the advantage of the *n*-butylboronate derivatives regarding their relatively low retention indices and their stability. Table I shows comparative retention indices of methyl-, *n*-butyl- and phenylboronate derivatives of YM-09538 and the internal standard. To determine urinary YM-09538, we selected methylboronic acid because it gave the lowest retention indices and complete separation of the peaks of YM-09538 and the internal standard. Since methylboronic derivatives were reportedly the least stable [11], the stability of the methylboronates of YM-09538 and the internal standard was studied. When the sample analyzed by GC was stored at room temperature, no significant difference in response was found after two days.

Quantitative determination of YM-09538 in urine

The chromatograms obtained from control human urine and the urine to which 5 μg of YM-09538 and 10 μg of the internal standard had been added are shown in Fig. 3. Drug-free control urine gave no interfering peaks and the sepa-

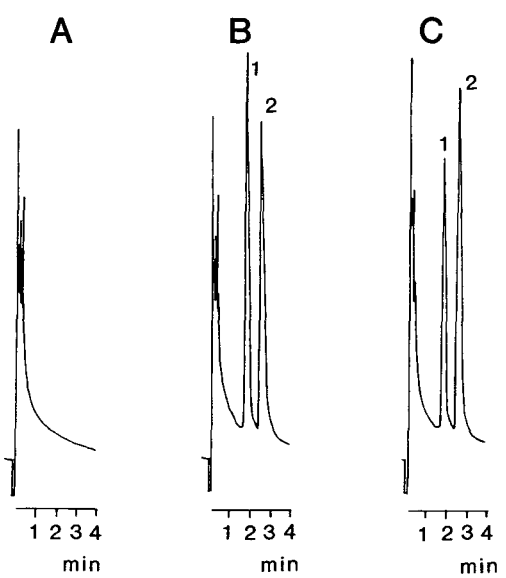

Fig. 3. Chromatograms of control human urine (A), human urine spiked with 5 μg of YM-09538 (B) and 0—2 h urine obtained from a volunteer who took 50 mg of YM-09538 orally (C). Peaks: 1, YM-09538; 2, internal standard.

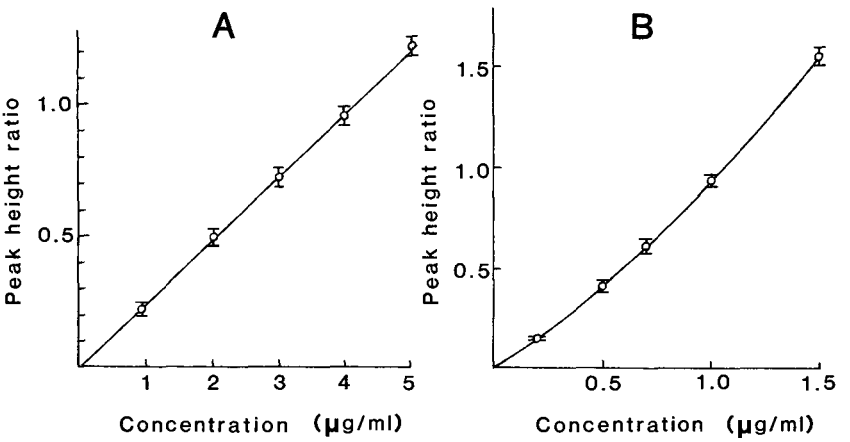

Fig. 4. Standard curves obtained from control human urine spiked with 1—5 μg of YM-09538 and 10 μg of the internal standard (A) and with 0.2—1.5 μg of YM-09538 and 3 μg of the internal standard (B). Each point represents the mean ± S.E.M. from four experiments.

ration of the two compounds added was complete. The standard curve prepared by subjecting control urine spiked with known amounts of YM-09538 (1—5 μg/ml) to the above procedure, after addition of 10 μg of the internal standard, is shown in Fig. 4A. A linear response was obtained for the drug over the concentration range examined; the equation of the line was $Y=0.244x - 0.012$ and the correlation coefficient was 0.984. For the analysis of the urine samples which contained a low concentration of YM-09538, we prepared another standard solution by adding known amounts of the drug (0.2—1.5 μg/ml) to control human urine. Fig. 4B shows the standard curve obtained by subjecting the standard solution to the above procedure using 3 μg of the internal standard. Although the graph did not show a linear response, the reproducibility at each concentration was good. For example, the intra- (n=4) and inter-assay (n=6) coefficients of variation at 0.5 μg/ml were 5.4% and 6.4%, respectively.

The sulphonamide function of YM-09538 and the internal standard has not been modified during the analysis. This polar function, which might cause adsorption of the derivative, was probably responsible for the standard curve being concave at low concentration as well as for extreme tailing of the peaks when the derivatives were analyzed by selected ion monitoring. If these problems are severe, further modification of the derivative, trimethylsilylation after reaction with alkylboronic acid [12], or some other derivatizations of sulphonamide [13] is necessary. However, the present method has good reproducibility as described above and offers high sensitivity obtained by introducing the nitrogen-sensitive detector. Because of its simplicity, this method is well suited for the routine analysis of a large number of samples.

Application of the method

Fig. 5 shows the excretion rate—time curve of YM-09538. The elimination half-life calculated from the data was 3.5 h and it agreed with the half-life of 3.5 h obtained from plasma concentration gained after oral administration of 50 mg of YM-09538 to humans [3].

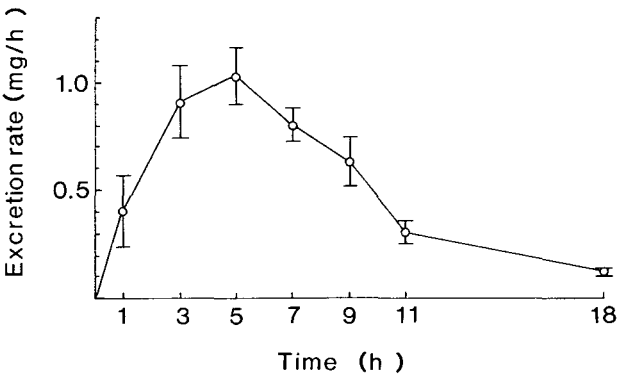

Fig. 5. Urinary excretion rate—time curve of the unchanged drug after oral administration of 50 mg of YM-09538 to humans. Each point represents the mean ± S.E.M. from three experiments.

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We are indebted to Mr. K. Takanobu for measuring the pK values of YM-09538.

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CHROMBIO. 1642

ANALYSIS OF D-PENICILLAMINE IN PLASMA BY FLUORESCENCE DERIVATISATION WITH N-[*p*-(2-BENZOXAZOLYL)-PHENYL] MALEIMIDE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic assay for penicillamine in plasma is described. The method is based on the derivatisation of penicillamine in acidified protein-free plasma supernatants with the sulphydryl-specific reagent N-[*p*-(2-benzoxazolyl)-phenyl] maleimide (BOPM) to give a stable fluorescent product. After separation of the penicillamine—BOPM derivative by reversed-phase high-performance liquid chromatography, fluorescence detection enables the quantitation of plasma penicillamine concentrations in the range 0.25—500 μmol/l. The method is selective and reproducible, and since chromatography time is less than 7 min the method is readily applicable to the analysis of the large number of samples associated with pharmacokinetic studies.

INTRODUCTION

Although D-penicillamine (β,β-dimethylcysteine) was originally introduced for the treatment of Wilson's disease, a rare disorder of copper metabolism, more recently it has found widespread acceptance for the treatment of rheumatoid arthritis. Despite the favourable effects of penicillamine in rheumatoid disease, use of the drug is associated with a high incidence of side effects. However, the relationship between plasma penicillamine concentration,

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clinical efficacy and the occurrence of adverse effects has still to be investigated.

To date, detailed studies on the pharmacokinetics of penicillamine in man have been impeded by the lack of suitable analytical methodology. Methods developed for the determination of penicillamine include radioimmunoassay [1], colourimetry [2, 3] and gas—liquid chromatography [4]. Separation and detection based on an automated amino acid analyser have also been described [5]. These procedures are all generally unsatisfactory for pharmacokinetic studies, however, since they are either non-selective, lack the required sensitivity or require complex sample manipulation. In recent years the convenience and versatility of high-performance liquid chromatography (HPLC) has led to its acceptance as one of the most useful techniques available for the analysis of drugs in biological fluids [6]. Recently assays for penicillamine using HPLC with electrochemical detection have been described [7, 8] but problems inherent in the use of this type of detector have precluded the widespread acceptance of such procedures. Similarly, a liquid chromatographic method based on post-column derivatisation of penicillamine with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] requires a specially constructed reaction cell [9].

Fluorescence derivatisation of the sulphydryl group of penicillamine provides a means of both preventing oxidation of the compound during analysis and enhancing assay sensitivity. An HPLC method for penicillamine based on formation of the fluorescent dansylaziridine derivative has been reported [10] but this procedure has been difficult to reproduce (see Results and discussion). Thus, we have developed a simple procedure for the analysis of penicillamine in plasma based on reaction of the sulphydryl group with N-[*p*-(2-benzoxazolyl)-phenyl] maleimide (BOPM) to give a stable fluorescent derivative which may be separated by reversed-phase HPLC. The assay is simple, specific, sensitive and reproducible, and readily applicable to pharmacokinetic studies.

EXPERIMENTAL

Reagents and standards

D-Penicillamine was purchased from Sigma (St. Louis, MO, U.S.A.) and N-[*p*-(2-benzoxazolyl)-phenyl] maleimide from Eastman-Kodak (Rochester, NY, U.S.A.). Other reagents and solvents were of analytical grade.

A penicillamine stock solution was prepared each day by dissolving the pure standard in 0.1% ethylenediamine tetraacetic acid (EDTA) to give a final concentration of 5 mmol/l. The stock solution was further diluted with 0.1% EDTA to give solutions containing 1000, 500, 250, 100, 50 and 10 μmol/l of penicillamine. Penicillamine calibration standards were then prepared by making a 10-fold dilution of the aqueous standard solutions with drug-free plasma to give final concentrations of 100, 50, 25, 10, 5 and 1 μmol/l, respectively. Upon preparation the penicillamine plasma standards were immediately treated according to the procedure described below under *Sample preparation.*

Chromatography

The HPLC system used consisted of a Model U6K injector, a Model 6000A solvent delivery system (both Waters Assoc., Milford, MA, U.S.A.) and a Model 970 fluorescence detector (Spectra-Physics, Santa Clara, CA, U.S.A.). The excitation wavelength of the fluorescence detector was set at 319 nm and emission was measured using a 360-nm cut-off filter. The chromatograph was fitted with a 30 cm × 3.9 mm I.D. 10-μm reversed-phase μBondapak C_{18} column (Waters Assoc.) and operated at ambient temperature. The mobile phase was methanol—sodium acetate, 0.1 m*M* (48:52) used at a flow-rate of 2.0 ml/min.

Sample preparation

After collection, patient blood samples were rapidly transferred into 1.5-ml Eppendorf microtubes and centrifuged at 6500 *g* for 30 sec (Eppendorf Model 5412 high-speed centrifuge) to separate the plasma. A 1-ml aliquot of plasma, from the patient samples or standards, was then immediately transferred into a second Eppendorf microtube containing 0.15 ml of 25% trichloroacetic acid. The acidified plasma was vortex mixed, cooled on ice for 10 min (to complete the plasma protein precipitation process) and the protein separated by centrifugation at 6500 *g* for 2 min. The penicillamine in plasma treated in this manner and stored at −20° C is stable for at least ten days.

For the derivatisation process, 0.5 ml of the plasma supernatant in a 5-ml glass culture tube was neutralised with 0.2 ml of 1% aqueous sodium hydroxide solution and the pH of the solution was adjusted to 5.0 by the addition of 0.25 ml of 0.5 mol/l sodium citrate (titrated to pH 5.0 with perchloric acid). To this buffered solution was added 1.0 ml of the derivatising agent (BOPM, 1 mmol/l in ethanol) and the mixture was incubated at 37° C overnight. A 0.05-ml aliquot of the reaction mixture was injected directly into the chromatograph.

Unknown concentrations were determined by comparison of the penicillamine peak heights obtained from the patient samples with those of the calibration curve.

RESULTS AND DISCUSSION

Sample preparation

Using the HPLC procedure reported here, we confirmed the previous results of Bergstrom et al. [11] that plasma samples must be acidified and deproteinised immediately upon collection to avoid the loss of reduced penicillamine. Thus, for an untreated 50 μmol/l plasma standard stored at room temperature the half-life for the rate of penicillamine loss, presumably by oxidation to the disulphide, was found to be only 15 min. However, when plasma samples were treated according to the procedure outlined in the Experimental section, minimal (< 5%) loss of penicillamine occurred when the acidified, protein-free supernatants were stored frozen for ten days. Although EDTA was used to prevent oxidation of penicillamine in the aqueous standard stock solutions, it was demonstrated that the presence of EDTA did not significantly improve the stability of penicillamine in the acidified plasma samples. Indeed, reaction mixture EDTA concentrations in excess of 5 mmol/l

adversely affected the chromatography of the penicillamine-BOPM derivative.

It was also shown by HPLC that minimal penicillamine loss occurred from plasma samples during the sample preparation procedure. Thus, the mean (± S.D.) peak heights obtained from quadruplicate plasma standards containing 50 and 5 μmol/l of penicillamine compared to stabilised aqueous standards of the same concentrations were 96.6 ± 2.3% and 96.0 ± 3.1%, respectively.

Derivatisation

Since penicillamine does not significantly absorb energy in the ultraviolet or visible region of the spectrum, the detection of the drug at the concentrations normally found in plasma requires derivatisation of one of the functional groups of penicillamine with a suitable chromophoric reagent or else utilisation of other physico-chemical properties of the molecule (e.g. electrochemical oxidation). The approach followed in developing the present assay was derivatisation of the sulphydryl group of penicillamine with a chromophoric reagent, thereby providing a means of both detecting the compound and preventing autoxidation during chromatography. Numerous reagents previously reported as being suitable for the derivatisation of sulphydryl-containing amino acids were investigated. These included: *o*-phthalaldehyde, dinitrolfluorobenzene, Dns-aziridine and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride). However, derivatisation of sulphydryl groups with each of these reagents requires a weakly alkaline reaction medium (pH 8—9), conditions known to catalyse penicillamine disulphide formation. Only the reaction of *o*-phthalaldehyde with penicillamine proceeded rapidly enough to minimise the problems of poor sensitivity and reproducibility arising from competing penicillamine autoxidation. Even then, due to the non-specificity of *o*-phthalaldehyde as a derivatising agent, the penicillamine—phthalaldehyde complex could not be adequately resolved from the large number of endogenous plasma peaks using reversed-phase HPLC.

The importance of pH control during the derivatisation process was confirmed in an experiment which determined the rate of penicillamine loss with increasing alkalinity. Here aliquots of a 500 μmol/l penicillamine aqueous standard solution (in 0.1% EDTA, see *Reagents and standards* section) were buffered to either pH 5.0, 7.0, 8.0 or 9.0 and the reduced sulphydryl concentration determined by Ellman's procedure [12] at known intervals after buffer addition. The results of this experiment are summarised in Fig. 1 and clearly demonstrate the instability of penicillamine in neutral and alkaline solutions at room temperature.

The reaction of maleimides with sulphydryl groups is known to proceed under mildly acidic conditions [13, 14] and the use of compounds of this class offers obvious advantages for the derivatisation of the penicillamine sulphydryl group. In particular, BOPM was investigated as a potential penicillamine derivatising agent since the compound is an established labelling reagent for sulphydryl-containing proteins [15, 16]. The reaction of penicillamine with BOPM at pH 5.0 readily formed a fluorescent product, with absorbance and emission maxima at 319 and 368 nm, respectively. At least a 4- to 5-fold excess of BOPM was shown to be necessary for the derivatisation

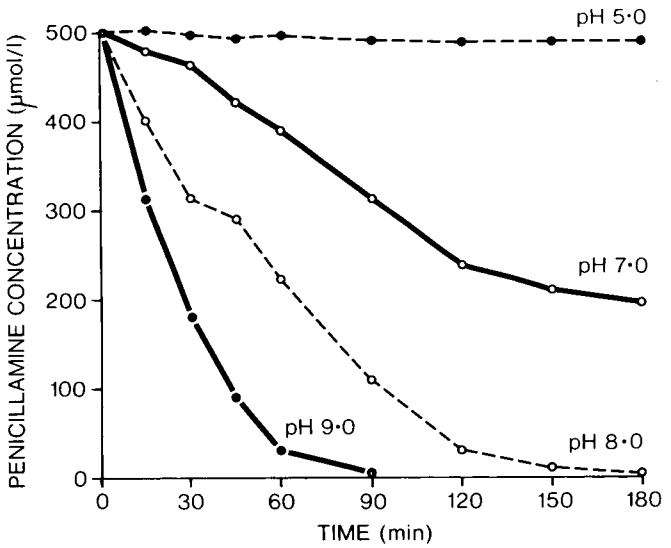

Fig. 1. Rate of loss of penicillamine from a 500 μmol/l aqueous standard solution buffered to pH 5.0, 7.0, 8.0 or 9.0.

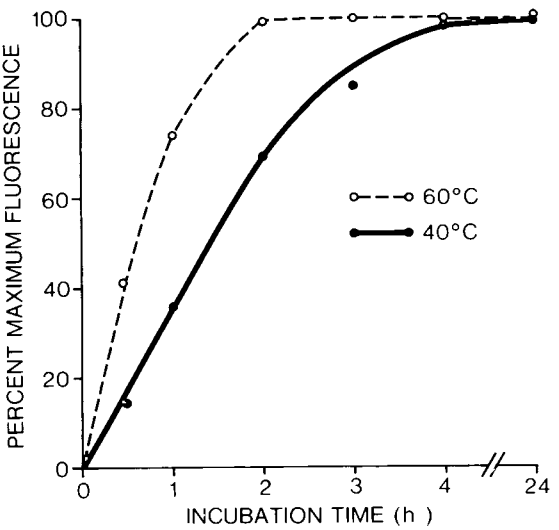

Fig. 2. Time course of the reaction of BOPM with a 50 μmol/l penicillamine plasma standard at 40°C and 60°C. Sample preparation and chromatography conditions as outlined in the Experimental section.

of penicillamine in deproteinised plasma samples. The time course of the extent of reaction of BOPM with a 50 μmol/l penicillamine plasma standard at 40°C and 60°C was followed by HPLC and indicated that the rate of formation of the penicillamine–BOPM complex was temperature dependent (Fig. 2). Although the reaction was complete within 2 h at 60°C or 4 h at 40°C, the normal procedure adopted in this laboratory has been to carry out the derivatisation overnight in a 37°C incubator. Once formed, the penicillamine–

BOPM complex is stable for 24 h (Fig. 2) and if stored in the dark less than 5% decomposition occurs over 60 h.

Since BOPM is a sulphydryl-specific reagent neither penicillamine disulphide nor penicillamine—cysteine disulphide, the major metabolites of penicillamine, interfere with the assay procedure. The extent of disulphide formation may, however, be determined by the method described here following electrochemical reduction of the disulphides [7] and measurement of total penicillamine. No endogenous plasma constituents interfere with the quantitation of penicillamine and the BOPM complexes formed by cysteine and glutathione have been shown to be unretained under the chromatography conditions employed. When the derivatisation is performed under alkaline conditions at least three fluorescent products were shown to be formed. The formation of one of these products is penicillamine-independent and may represent hydrolysis of BOPM to its maleamic acid derivative.

Chromatography

The penicillamine—BOPM derivative chromatographs well on reversed-phase HPLC with methanol—0.1 mmol/l sodium acetate as the mobile phase. The complex elutes as a sharp, symmetrical peak with a retention time of 5.5 min.

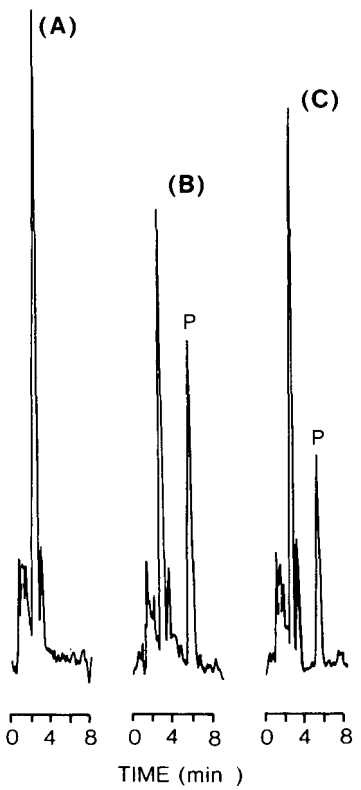

Fig. 3. Chromatograms of plasma samples. (A) Drug-free plasma; (B) plasma standard containing 10 μmol/l of penicillamine (P); (C) patient plasma sample containing 6.5 μmol/l of penicillamine. Detector sensitivity 0.05 μA. Sample preparation and chromatography conditions as outlined in the Experimental section.

Fig. 3B shows the chromatogram of a plasma standard containing 10.0 μmol/l of penicillamine. Similarly, Fig. 3C shows the chromatogram of a plasma sample, taken 6 h after a dose from a patient on chronic penicillamine therapy (250 mg, 12 hourly), containing 6.5 μmol/l of penicillamine. Drug-free plasma gave no interfering peaks under the chromatography conditions described (Fig. 3A).

The penicillamine–BOPM derivative exhibits a marked capacity for ion-pairing with carboxyl anions. Any masking of the charged amino function of the penicillamine–BOPM derivative by carboxyl ions leads to a profound reduction in the polarity of the complex. Low concentrations of sodium acetate are therefore routinely added to the mobile phase to avoid changes in chromatography during the course of analysis.

Unreacted BOPM is sufficiently lipophilic to be retained on the reversed-phase column with the mobile phase used for the assay. Retention of this compound is advantageous since it allows successive injections to be made without interference from later eluting peaks. The accumulation of BOPM on the column does not have any major adverse effects, although baseline drift may increase after approximately 80 injections. Thus, methanol should periodically be pumped through the column to elute retained BOPM.

Assay sensitivity and reproducibility

Optimal sensitivity was obtained when the excitation wavelength of the fluorescence detector is set at 319 nm and a 360 nm cut-off filter used. Under these conditions the limit of sensitivity (signal-to-noise ratio 5:1) for the detection of penicillamine is 0.25 μmol/l. The standard curve is linear for penicillamine plasma concentrations over the range 1–500 μmol/l and passes through the origin.

Although no internal standard is used in this procedure, assay reproducibility is nevertheless good. The mean (± S.D.) coefficient of variation for normalised peak heights (i.e. peak height/concentration) from 20 standard curves prepared over a period of three months was 6.3 ± 2.1%. While a number of sulphydryl-containing compounds were initially investigated as possible internal standards, the assay does not require extraction or quantitative transfer procedures and it soon became apparent that reproducibility was not compromised by the omission of an internal standard.

In summary, the HPLC method described here for the determination of penicillamine in plasma is sufficiently simple, rapid, sensitive and reproducible to readily enable the analysis of the large number of samples involved in pharmacokinetic studies. The method is currently being applied to a study investigating the relationship between plasma penicillamine concentration and the variability observed in therapeutic response and the occurrence of serious adverse effects.

ACKNOWLEDGEMENT

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENYTOIN AND HYDROXYPHENYTOIN IN HUMAN URINE

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SUMMARY

Simple and rapid quantitative analysis utilizing high-performance liquid chromatography was performed to determine the concentration of the antiepileptic agent phenytoin, and its main metabolite hydroxyphenytoin, in human urine. For the purposes of simple and rapid determination, the commercially available Extrelut® columns were used with diethyl ether and chloroform as extraction solvents. High-performance liquid chromatography was performed on a LiChrosorb RP-8 column, with a mobile phase of methanol—0.02% ammonium acetate (1:1). The internal standard was 5-(4-methylphenyl)-5-phenylhydantoin.

The method was applied to patients' urine, to examine the influence of concomitant drugs. Also, the results obtained using the commercially available enzymatic immunoassay method were compared with those from the present method, and it was concluded that a simple and rapid microanalysis is possible with a high extraction ratio.

INTRODUCTION

We have previously reported the extraction of blood phenytoin (DPH) and its main metabolite in humans, hydroxyphenytoin (HDPH), for the purposes of a simple and rapid quantitative analysis utilizing commercially available pre-packed columns, and their quantitative analysis by high-performance liquid chromatography (HPLC) [1].

In the present study, we examined the quantitative analysis of DPH and HDPH in human urine as a follow-up to the previous report. It has been reported that about 75% of the administered DPH is excreted as conjugated glucuronides of HDPH in the urine and that about 5% is excreted as unchanged

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DPH [2]. We have attempted to analyze quantitatively human urinary DPH and HDPH under conditions where combined drug therapy, including DPH, was undertaken for treating epilepsy.

EXPERIMENTAL

Reagents and solutions

The phenytoin (DPH) used was recrystallized from one standardized according to the Japanese Pharmacopoeia. Hydroxyphenytoin (HDPH) and 5-(4-methylphenyl)-5-phenylhydantoin (internal standard) were the products of Sigma, St. Louis, MO, U.S.A. Diazepam, phenobarbital, carbamazepine, and acetazolamide were those standardized according to the Japansese Pharmacopoeia. Methanol for HPLC was from Wako Junyaku, Co. Ltd., Osaka, Japan, and the other agents were commercially available extra-pure reagents.

Control urine used was the Teck Check™ Control Urine (No. 1) produced by Miles-Sankyo, Tokyo, Japan. The kit for quantitative analysis of DPH was Markit® Phenytoin, a product of Dai Nippon Seiyaku, Osaka, Japan, and the procedure was followed according to the manufacturer's instructions.

Conditions for high-performance liquid chromatography

A Hitachi chromatoprocessor Type 834 was connected to a Hitachi high-performance liquid chromatograph Type 635, and the area under the peak was measured. The column was LiChrosorb RP-8, from Merck, Darmstadt, G.F.R. (5 μm, 150 mm × 4.0 mm I.D., with built-in water jacket, adjusted to 40°C). The detector, Type UV-8 (250 nm), was a product of Toyo Soda Co. Ltd.; a.u.f.s. 0.08—0.16. The mobile phase was methanol—0.02% aqueous ammonium acetate (1:1), at a flow-rate of 0.8 ml/min.

Analytical method for DPH and HDPH

To 1 ml of urine, 200 μl of methanol solutions of DPH (0.3—1.5 mg/ml) and HDPH (0.03—1.5 mg/ml) were added, and 200 μl of a methanol solution of 5-(4-methylphenyl)-5-phenylhydantoin as the internal standard. Then, 20 ml of 0.01 *M* phosphate buffer solution at pH 6.8 were added and the solution was mixed. The solution was transferred to the top of an Extrelut® prepacked column for the extraction of these compounds and was adsorbed. Fifteen minutes later, to elute DPH, HDPH and internal standard, 30 ml of diethyl ether were added, followed by 30 ml of chloroform to elute the compounds.

The clear effluent was dried under reduced pressure at a temperature below 20°C using a concentrator with trap which was cooled to —70°C. After passing nitrogen gas for 1 min, the dried sample was dissolved in 100 μl of methanol, and 5 μl of that solution were injected onto the column. From the chromatogram obtained, the ratio of the areas under the peaks of DPH and HDPH to that of the internal standard was calculated, and the amounts of DPH and HDPH were obtained from the calibration curve prepared beforehand.

Quantitative analysis of DPH and HDPH in human urine

The application of the present method to patients' urine was made as fol-

lows. To 1.0 ml of urine was added 1.0 ml of 12 *N* hydrochloric acid and the container was loosely sealed. After boiling at 90°C for 120 min, the solution was cooled, and 1.0 ml of 12 *N* sodium hydroxide was added. After adjusting the pH to 7.0 with 20% sodium hydroxide, the analytical procedure described above was followed. The total amount of HDPH in a free state was calculated after hydrolysis according to the method of Dykeman et al. [3], since most of the HDPH is present as glucuronide conjugates in the urine [4—9].

RESULTS AND DISCUSSION

Among the reports on the measurement of antiepileptics in urine utilizing HPLC, Kabra and Marton [10], Dykeman and Ecobichon [2], Sawchuk and Cartier [11], and others used μBondapak C_{18} (30 cm × 3.9 mm I.D.) columns. The mobile phases they used were acetonitrile—water (37:63, v/v), methanol—phosphate buffer (0.025 *M*, pH 8.0) (40:60, v/v), and methanol—water (55:45, v/v), respectively, and the measurements were made at a wavelength of 254 nm. However, in these methods, the extraction ratios of DPH and HDPH were 41% and 79%, respectively [11], or the retention time in HPLC of DPH, HDPH and internal standard was long [3]. Therefore, we examined the simple and rapid quantitative analysis of urinary DPH and HDPH following the previously reported simple and rapid method [1] for blood DPH and HDPH in which the extraction ratio was good.

In addition, as the internal standard, we adopted 5-(4-methylphenyl)-5-phenylhydantoin, whose chemical structure is similar to that of DPH, because its retention time does not overlap that of DPH or other combination drugs.

In the next step, we examined the quantitative nature of DPH and HDPH. On the chromatogram of the mixture of DPH and HDPH to which 2 μg of internal standard were added, the ratio of the area under the peak was measured and plotted on the ordinate, which gave the analytical curve. There was good linearity within the range 3.0—15.0 μg for DPH ($Y = 0.340X + 0.027$). For HDPH in 0.3—15.0 μg amounts, all experimental data points are approximately on a straight line ($Y = 0.914X + 0.003$).

At the first step the influence of pH on the extraction ratio with the Extrelut® column was examined using healthy human urine in 1.0 ml of which 120 μg of DPH, 30.2 μg of HDPH, 40 μg of acetazolamide, 80 μg of diazepam, 80 μg of carbamazepine and 1.6 mg of phenobarbital were dissolved.. When comparing the three phosphate buffers of varying pH, the extraction ratio at slightly acidic pH (4.0) was 90.6% for DPH and 104.6% for HDPH. Under slightly basic conditions, pH 9.0, the extraction ratio was 92.9% for DPH and 81.2% for HDPH. In contrast, the extraction ratio was 92.2% for DPH and 95.3% for HDPH at neutral pH adjusted with phosphate buffer of pH 6.8; thus the extraction efficacy is excellent at neutral pH. We decided therefore to make the solution neutral (pH 6.8) at the time of extraction.

Before experiments on the recovery of added DPH and HDPH, the influence of urine components on the HPLC separation when performing the present extraction method was examined. Internal standard was added to the control urine and to healthy human urine and mixed; thereafter, each mixture was transferred to the top of an Extrelut® pre-packed column and extraction

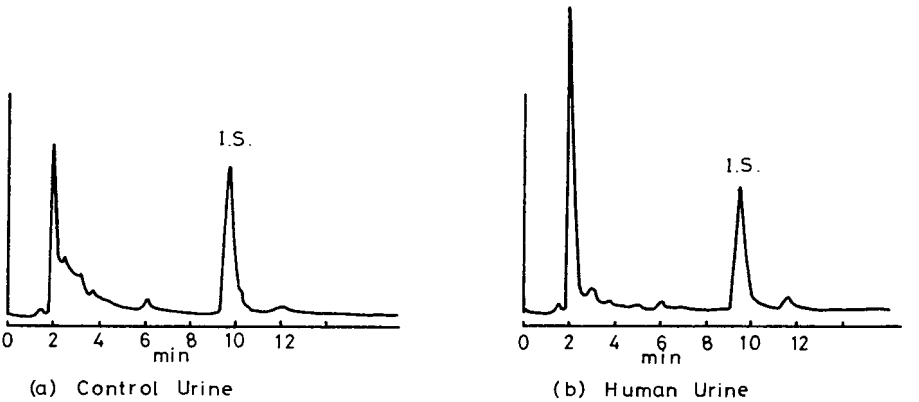

Fig. 1. HPLC of 5-(4-methylphenyl)-5-phenylhydantoin (internal standard, I.S.) added to control urine (a) and healthy human urine (b).

of these compounds was made (blank). The results of HPLC after using this procedure are shown in Fig. 1. As is seen in the chromatogram, a peak was observed at the position of HDPH (3.2 min) in the control urine, whereas the influence of this peak was small in the human urine. Therefore, for control urine, the ratio of this peak to the internal standard was subtracted, and the remainder taken as the amount of HDPH. As a result of this procedure, both DPH and HDPH were separated from human urine without interference by urine components.

We then performed experiments on the recovery of DPH and HDPH added to water, control urine and healthy human urine. As shown in Table I, the recovery was 98.5—107.7% for DPH in the range of 3.0—15.0 μg, and 89.2—107.6% for HDPH in the range 0.41—12.00 μg. On the other hand, the recovery for healthy human urine to which several comedicated drugs were added, was 92.2—93.8% for DPH and 95.3—118.9% for HDPH. Therefore, the results were reasonably satisfactory though the results for HDPH against water were slightly low. On the basis of these results, the quantitative analysis of DPH and HDPH was considered to become possible.

The limits for reliable quantitation of DPH and HDPH were 1.0 μg for DPH and 0.4 μg for HDPH.

The next examination concerned the influence of other antiepileptic agents administered concomitantly with DPH on the quantitative analysis of DPH and HDPH. Acetazolamide, phenobarbital, carbamazepine and diazepam were tested as the concomitant drugs. They were added to healthy human urine to which DPH, HDPH and internal standard were already added, and extraction was made using the Extrelut® column. It was found that DPH and HDPH were both separated by HPLC without interference from the added drugs (Fig. 2).

In the next step, to examine the possible application to the clinical field, the influence of the concomitant drugs on the amounts of DPH and HDPH excreted in the urine was examined using patients admitted to the hospital. Similarly to the quantitative analysis of DPH and HDPH described previously, the simultaneous analysis of acetazolamide, phenobarbital, carbamazepine

TABLE I

ANALYTICAL RECOVERIES OF DPH AND HDPH ADDED TO WATER, CONTROL URINE AND HEALTHY HUMAN URINE

	HDPH				DPH			
	Added (μg)	Found (μg)	Recovery (%)	C.V. (%)	Added (μg)	Found (μg)	Recovery (%)	C.V. (%)
Water	0.61	0.54 ± 0.07	89.2 ± 11.2	12.6	6.0	6.4 ± 0.1	106.1 ± 1.4	1.4
	0.81	0.76 ± 0.03	93.7 ± 4.1	4.4	8.0	8.6 ± 0.1	107.7 ± 0.8	0.7
Control urine	0.41	0.36 ± 0.02	91.1 ± 4.7	5.2	6.0	6.2 ± 0.1	102.6 ± 0.9	0.9
	1.01	1.02 ± 0.02	101.8 ± 1.2	1.2	8.0	8.2 ± 0.2	101.9 ± 2.2	2.1
Healthy human urine	0.91	0.95 ± 0.05	104.0 ± 5.2	5.0	3.0	3.0 ± 0.2	101.7 ± 5.2	5.1
	2.12	2.13 ± 0.06	100.2 ± 2.8	2.8	6.0	6.1 ± 0.1	101.4 ± 2.5	2.4
	3.03	3.26 ± 0.06	107.6 ± 2.1	2.0	12.0	12.3 ± 0.1	102.8 ± 0.6	0.6
	12.00	12.50 ± 0.42	104.2 ± 3.5	3.4	15.0	14.8 ± 0.6	98.5 ± 3.9	4.0
Healthy human urine*	0.61	0.72 ± 0.04	118.9 ± 6.4	5.4	3.0	2.8 ± 0.1	93.8 ± 2.6	2.8
	1.52	1.44 ± 0.03	95.3 ± 2.3	2.4	6.0	5.5 ± 0.1	92.2 ± 1.2	1.3
	2.12	2.18 ± 0.03	102.9 ± 1.5	1.4	9.0	8.3 ± 0.1	92.2 ± 0.6	0.7
	3.00	3.35 ± 0.06	111.7 ± 2.3	2.1	12.0	11.2 ± 0.1	93.5 ± 0.1	0.7
	7.33	8.27 ± 0.03	112.8 ± 0.4	0.4				
	14.66	16.10 ± 0.42	109.8 ± 2.9	2.6				

*These results show the values obtained by the addition of 40 μg of acetazolamide, 80 μg of carbamazepine, 80 μg of diazepam and 1.6 mg of phenobarbital as concomitant drugs of DPH and HDPH to 1 ml of healthy human urine. Following extraction and determination procedures were carried out as described, and 5 μl of the extracts (100 μl) were injected for HPLC.

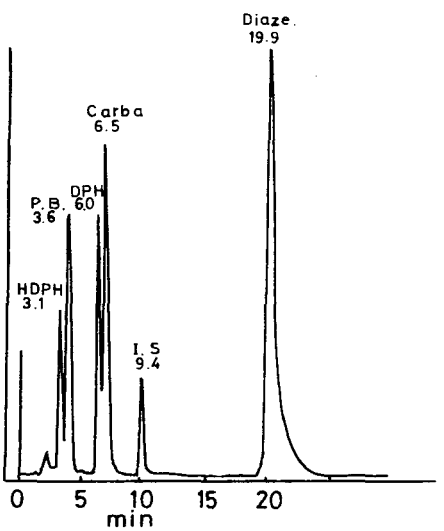

Fig. 2. HPLC of DPH, HDPH, acetazolamide, carbamazepine, diazepam and phenobarbital added to healthy human urine. I.S. = 5-(4-methylphenyl)-5-phenylhydantoin as internal standard. HDPH: 2.1 μg; DPH: 12 μg; I.S.: 2 μg; acetazolamide: 2 μg; carbamazepine (Carba): 4 μg; diazepam (Diaze.): 4 μg; phenobarbital (P.B.): 80 μg.

and diazepam with DPH and HDPH was tried. Firstly, with respect to each drug, the ratio of the area under the peak to that of 2 μg of internal standard was measured and the analytical curves were constructed (acetazolamide: $Y = 3.550X - 0.422$, $r = 0.995$; phenobarbital: $Y = 0.058X - 0.037$, $r = 0.990$; carbamazepine: $Y = 1.447X + 0.011$, $r = 1.000$; diazepam: $Y = 5.259X - 0.503$, $r = 0.990$).

Secondly, experiments on the recovery of the drugs added to water and freshly obtained healthy human urine were carried out. The recovery from healthy human urine was 83.3—88.3% for carbamazepine in the range 1.34—5.35 μg and 78.4—88.0% for diazepam in the range 2.58—10.30 μg, which was satisfactory, and thus the quantitative analysis of these drugs was possible. However, acetazolamide was not extracted by the organic solvent in the Extrelut® column, and was considered to remain adsorbed on the column. In addition, the extraction ratio of phenobarbital was low. Therefore, acetazolamide and phenobarbital did not interfere in the quantitative analysis of DPH and HDPH when phenobarbital was added to human urine in the usual dose.

Thirdly, DPH, HDPH and the concomitant drugs carbamazepine and diazepam were analyzed by the present method in the urine of nine patients 25—68 years old of both sexes admitted in the Department of Psychiatry and Department of Neurology who were administered carbamazepine concomitantly with DPH. Most of the main metabolites of HDPH are excreted in the urine as glucuronides [4—9]. Before hydrolyzing the glucuronides, the stability of DPH and HDPH was examined for the hydrolysis conditions by the method of Dykeman and Ecobichon [3]. To 1.0 ml of water, 150 μg of DPH, 60 μg of HDPH and 1.0 ml of 12 *N* hydrochloric acid were added. The mixture was boiled at 90° C for 120 min. The recoveries of DPH and HDPH were 104.3% and 115.2%, respectively. On the other hand, the recoveries without the boiling procedure at 90° C for 120 min were 108.7% and 109.2%, respectively. These data show that DPH and HDPH were stable at 90° C in 6 *N* hydrochloric acid (12 *N* HCl 1.0 ml + H_2O 1.0 ml).

The quantitative analytical method described above was performed after hydrolyzing the urine according to the method of Dykeman and Ecobichon [3]. The chromatograms of the urine of patients after DPH administration are shown in Fig. 3. It was found that the amount of HDPH excreted in the urine was small in the patients concomitantly administered carbamazepine compared to the patients without administered carbamazepine, the difference being statistically significant ($P < 0.05$). With respect to the reason for this, it was speculated first that the amount of HDPH, which is a metabolite of DPH, became smaller because the amount of DPH administered was smaller by about 100—150 mg due to the concomitant administration of carbamazepine, and, secondly, that the oxidation enzyme which converts DPH to HDPH acted increasingly on carbamazepine, leading to a decrease in the effect on DPH, which might result in a decrease in the amount of HDPH. The amount of DPH excreted in the urine was not different between the groups treated with carbamazepine and those without carbamazepine, independent of the dose of DPH. Therefore, it was suggested that the administration of carbamazepine might have some influence on the metabolism of DPH to HDPH.

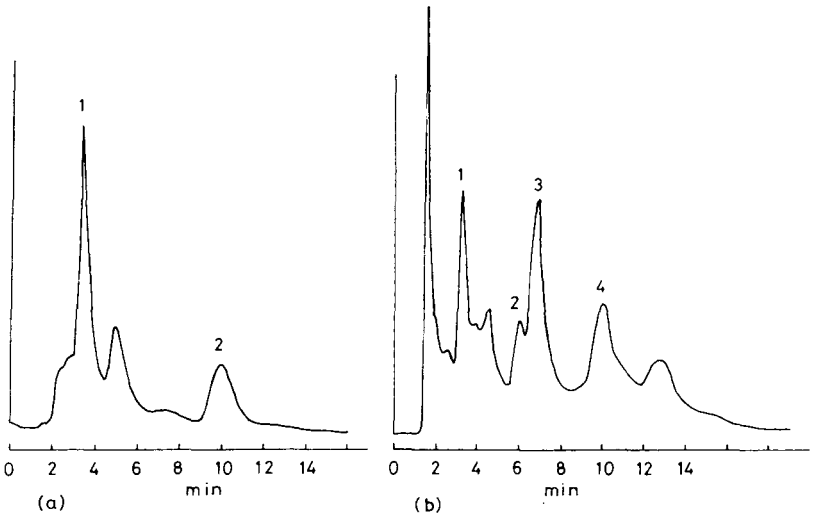

Fig. 3. HPLC of epileptic urine after anticonvulsant administration. (a) 150 mg of DPH were administered a day; peaks: 1 = HDPH, 2 = internal standard. (b) 100 mg of DPH and 600 mg of carbamazepine were administered a day; peaks: 1 = HDPH, 2 = DPH, 3 = carbamazepine, 4 = internal standard.

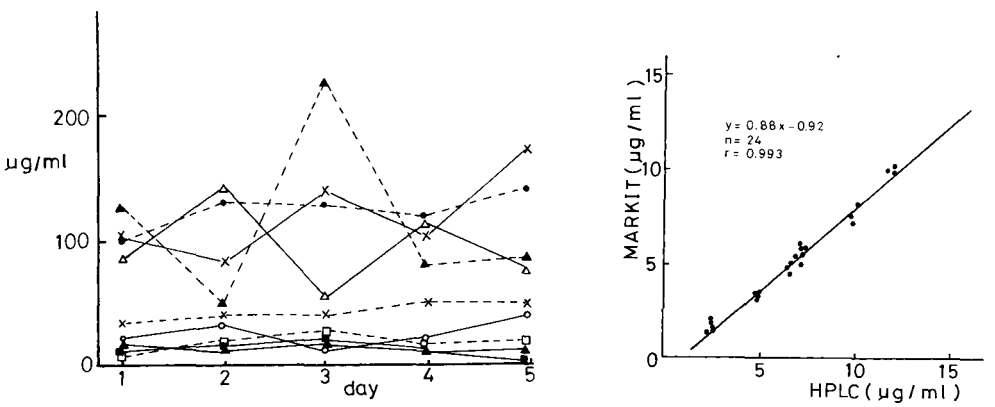

Fig. 4. Concentration of HDPH in urine of epileptics receiving DPH and other drugs. (▲ — — — ▲) DPH 150 mg; (▲———▲) DPH 100 mg, carbamazepine 600 mg; (△———△) DPH 250 mg; (× — — — ×) DPH 250 mg, carbamazepine 300 mg; (×———×) DPH 250 mg; (○———○) DPH 150 mg, carbamazepine 400 mg; (● — — — ●) DPH 200 mg; (□ — — — □) DPH 100 mg, carbamazepine 600 mg; (■———■) DPH 150 mg, carbamazepine 600 mg.

Fig. 5. Correlation between Markit® and HPLC determinations of DPH in human urine.

When 300—600 mg of carbamazepine and 100—250 mg of DPH were administered, the amounts of DPH, HDPH and carbamazepine were 0—229.9 μg/ml, 1.0—50.1 μg/ml, and 0.5—38.1 μg/ml, respectively. When 150—300 mg of DPH were administered without concomitant carbamazepine, the amounts of DPH and HDPH were 0—56.4 μg/ml and 50.5—227.6 μg/ml, respectively (Fig. 4). When comparing the results for the blood concentration of DPH and HDPH we reported previously, carbamazepine administration did not

produce a difference in the blood concentration of HDPH, but produced a clear difference in the amount of HDPH in the urine.

For comparing the present method with the other methods, we used the commercially available DPH assay kit which is based on the enzymatic immunoassay method. At first, the experiments on the recovery of DPH added to healthy human urine with concomitant drugs were performed using the kit and the same sample as for the present method. As a result, the values measured by the kit were slightly lower in the range 2.67—13.19 μg/ml, but an extremely high correlation was observed between the values obtained with the kit and by the present method, the correlation coefficient being $r = 0.993$ (Fig. 5). Therefore, the kit method was considered to be applicable to the quantitative analysis of urinary DPH, and it was applied to the analysis of patients' urine and compared with the results obtained by the present method utilizing HPLC (Table II). However, the DPH value assayed by the present method is slightly higher than that obtained with the kit. This phenomenon can be attributed to the small amount of urinary constituents, as shown at the retention time of DPH (6.0 min) in Fig. 1.

TABLE II

URINARY DPH LEVELS IN PATIENTS TAKING DPH

No.	Initials	Age	Sex	DPH taken (mg)	Markit® (μg/ml)	Column + HPLC (μg/ml)
1	F.S.	68	f	150	23.4	44.3
2	Y.N.	42	f	150	13.2	23.4
3	A.O.	46	f	250	17.8	29.8
4	R.K.	25	f	250	25.0	49.8
5	I.N.	37	f	100	5.1	17.2
6	K.T.	43	f	250	23.0	56.4
7	C.N.	47	f	200	14.2	27.5
8	S.K.	26	f	100	0.56	0.5
9	S.K.	43	m	300	11.0	11.0

From these results, the present method was found to be useful for urine analysis in addition to the measurements of the blood DPH. The advantages of the present method are as follows. Solvent extraction is simply performed by adsorption on the Extrelut® column, and the urinary components can be excluded. Concomitant drugs do not interfere with the measurements, and DPH and HDPH can be analyzed simultaneously; moreover, the quantitative analysis of the concomitant drug carbamazepine can be made simultaneously. In addition, the time for the measurements with HPLC is less than 25 min. Therefore, the present method can be considered to be a simple and rapid method with a high extraction ratio; by this method, microanalysis of an antiepileptic drug, DPH, and its metabolite, HDPH, becomes possible.

ACKNOWLEDGEMENT

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CHROMBIO. 1651

QUANTITATION OF 3'-HYDROXYPENTOBARBITAL IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative method for the determination of 3'-hydroxypentobarbital (3'HP) in serum has been developed using reversed-phase high-performance liquid chromatography. A sensitivity limit of 0.5 μg/ml in 100 μl serum was obtained by detection at 215 nm. Serum concentrations of 3'HP were measured in patients receiving high-dose pentobarbital therapy and pentobarbital:3'HP concentrations are discussed.

INTRODUCTION

Pentobarbital is a short-acting barbiturate used for a variety of sedative and hypnotic purposes [1]. Studies indicate that 3'-hydroxypentobarbital (3'HP) is the major metabolite in man [2, 3]; two additional metabolites are N-hydroxypentobarbital and 3'-carboxypentobarbital [4, 5].

Recently, high-dose pentobarbital therapy (HDPT) has demonstrated beneficial effects in the treatment of some of the consequences of severe brain insult [6—13]. Clinical HDPT usually results in steady-state serum concentrations of 20—50 μg/ml pentobarbital, inducing barbiturate coma and requiring artificial ventilation and continuous physiologic monitoring [14]. These consequences of HDPT, as well as the interpatient variability of pentobarbital pharmacokinetics [15], necessitate the availability of therapeutic drug monitoring procedures.

Gas chromatography (GC), high-performance liquid chromatography (HPLC), and ultraviolet spectroscopy (UV) have been used to quantitate pentobarbital [16—18]. More recently, this laboratory modified a homogeneous enzyme immunoassay to allow pentobarbital quantitation in 50 μl of serum [19, 20]. Although EMIT® serum barbiturate reagents have

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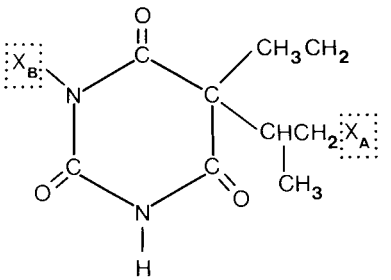


COMPOUND	X_A	X_B
PENTOBARBITAL	CH_3CH_2	H
3′-HYDROXYPENTOBARBITAL	$CH_3CH(OH)$	H
N-HYDROXYPENTOBARBITAL	CH_3CH_2	OH
3′-CARBOXYPENTOBARBITAL	CH_2COOH	H

been modified to quantitate pentobarbital previously, cross-reactivity towards other barbiturates or metabolites were not reported [21, 22]. Therefore, as part of the methodologic evaluation, the quantitation of 3′HP was necessary in order to investigate the potential analytical interference (bias) due to immunoassay antibody—enzyme cross-reactivity towards 3′HP. Additionally, the quantitation of 3′HP and pentobarbital in patients receiving HDPT allowed comparison between low dose/high dose drug:metabolite concentration ratios for pentobarbital as compared with other barbiturate analogues [23—25].

Hydroxylated barbiturate metabolites have been quantitated by radioimmunoassay (RIA), gas chromatography—mass spectrometry (GC—MS), thin-layer chromatography (TLC), and gas chromatography (GC) [23, 24, 26—29]. While all of these methods are considered to be selective, disadvantages have been recognized: RIA requires antibody production and the use of radioisotopes; GC—MS requires expensive instrumentation; TLC requires a large specimen volume; and GC requires the methylation of barbiturate compounds, which has been subject to criticism because of incomplete derivatization and shortened GC column life. HPLC has been used to quantitate other barbiturates and their metabolites and has proven to be sensitive, selective, and rapid [30—32]. This report describes a reversed-phase HPLC assay for the selective quantitation of 3′HP at concentrations greater than 0.5 μg/ml in 100 μl of plasma.

EXPERIMENTAL

Instrumentation

Analyses were carried out with a Perkin-Elmer Series 3 high-performance liquid chromatograph equipped with a Whatman Partisil PXS-10/25 ODS-2 column (250 mm × 4.6 mm I.D., 10 μm particle size) and guard column (45 mm × 5.0 mm I.D.) packed with CO:Pell ODS (Whatman, Clifton, NJ, U.S.A.), and a Model LC-55 variable-wavelength detector (Perkin-Elmer, Norwalk, CT,

U.S.A.) at 215 nm. Chromatographic conditions were as follows: mobile phase, tetrahydrofuran—water (5:95); flow-rate, 2.5 ml/min; column temperature, 50°C.

A Beckman Microfuge B centrifuge (Beckman, Palo Alto, CA, U.S.A.) was used to centrifuge serum extracts.

Reagents

Analytical grade 3′-hydroxypentobarbital was verified by GC—MS and TLC. 3′HP can be synthesized to obtain analytical material [33]. A 10 μg/ml stock standard solution of 3′HP was prepared in drug-free human serum. Serum standards (2, 4, 6, and 10 μg/ml) were prepared by diluting a stock solution of 3′HP with drug-free serum.

Tetrahydrofuran (UV) was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ethyl acetate, pesticide grade, was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A., Cat. No. E-191.). Hydrochloric acid used was ACS grade. Water was deionized and passed through a 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

Procedure

To 100 μl of standard or patient sample, 50 μl of 3 *N* hydrochloric acid and 1.0 ml of ethyl acetate were combined in a 1.5-ml polypropylene microcentrifuge tube, and vortexed for 30 sec. The extraction tubes were centrifuged for 10 min at 11,000 *g*; 0.8 ml of organic layer was transferred to a glass conical tube and the organic layer was evaporated under nitrogen at 35°C. The residue was reconsituted with 40 μl of methanol—water (50:50) and 30 μl were quantitatively injected into the chromatograph.

Pentobarbital was quantitated by HPLC using a modified procedure reported by Salvadori et al. [17]. Measurements were made at 239 nm and chromatographic conditions were as follows: mobile phase methanol—water (45:55); flow-rate 2.5 ml/min; column temperature 50°C.

RESULTS AND DISCUSSION

Chromatographic conditions allowed the separation of endogenous coextractables from 3′HP (Fig. 1). The HPLC pentobarbital method, using a more polar mobile phase, resulted in the apparent elution of 3′HP in the void volume of the column. HPLC analysis also suggested the presence of an additional metabolite (P1) in all samples containing 3′HP. This peak was not present in drug-free serum or in serum spiked with 3′HP. Assuming that 3′HP and P1 have the same analytical recovery and molar absorptivity at 215 nm, peak area data for P1 suggested that the concentration of P1 and 3′HP were grossly equivalent. P1 was not identified.

Analytical recovery of 1—10 μg/ml 3′HP added to serum was 85 ± 7%. The analysis of drug-free patient sera indicated that nonselective detector response (due to HPLC system fluctuation or endogenous coextractables) was equivalent to less than 0.025 μg/ml of 3′HP. However, the distortion of 3′HP peak shape prohibited accurate integration at concentrations less than 0.5 μg/ml (equivalent to 30 ng of 3′HP injected on-column).

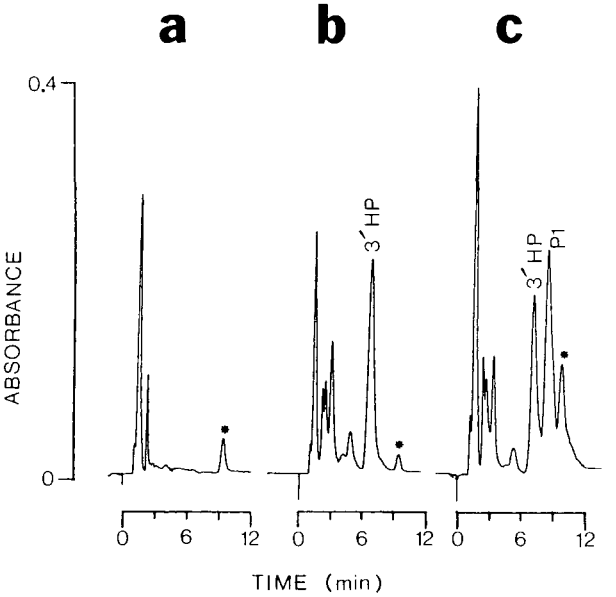


Fig. 1. HPLC chromatograms illustrating: (a) blank serum extract, (b) drug-free serum spiked with 5 μg/ml of 3′-hydroxypentobarbital (3′HP), (c) patient serum extract containing 3.5 μg/ml of 3′HP and unidentified metabolite P1. * designates serum interference.

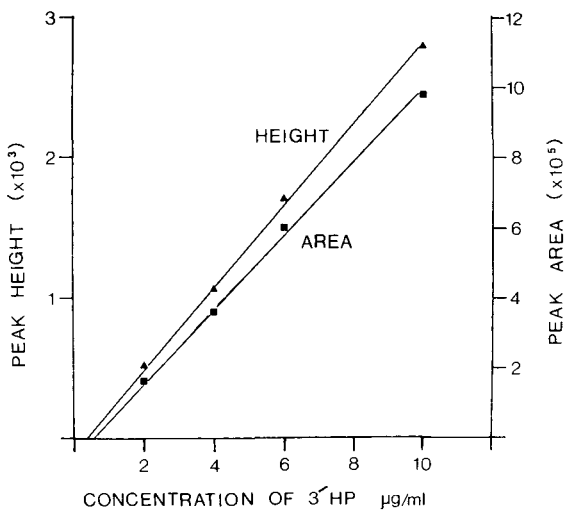


Fig. 2. Calibration curve for 3′-hydroxypentobarbital quantitation using peak area (▪) and peak height (▲) calculations.

A standard curve obtained by linear regression of concentration versus peak height or peak area was used to quantitate 3′HP from 0.5—10 μg/ml (Fig. 2). Between-day variation was examined by analysis of variance of slopes and intercepts from four standard curves (Table I). The slopes for these standard curves had coefficients of variation (C.V.) of 15.4% and 10.6% for calculations based on peak area and peak height, respectively. Such variance is not unusual and

TABLE I

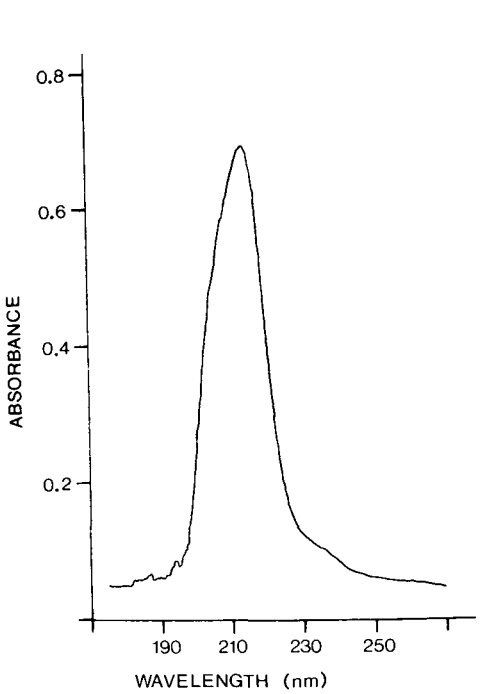
ANALYSIS OF VARIANCE ON SLOPES AND INTERCEPTS FOR THE 3'HP STANDARD CURVES

Analysis	Slope				Intercept	
	r	Value	C.V.	S.E.	Value	S.E.
Peak area						
1	0.999	1.034	3.86	0.039	—0.388	0.249
2	0.998	0.963	7.83	0.075	—0.145	0.412
3	0.996	0.920	16.57	0.152	0.390	0.835
4	0.998	0.708	13.06	0.092	1.693	0.507
Peak height						
1	0.999	0.299	0.54	0.002	—0.086	0.010
2	0.999	0.255	4.23	0.010	0.035	0.059
3	0.998	0.243	11.08	0.026	0.153	0.147
4	0.999	0.239	7.28	0.017	0.267	0.095

appears to be related to changes in column performance due to reversible deactivation of the C_{18} column associated with solvent bonding or accumulation of impurities from samples and/or mobile phase [34, 35]. Statistical analysis of the variance of each standard curve, using the general linear test approach, indicated equal regression parameters for slope and intercept, with $p < 0.05$ [36].

The UV absorption of 3'HP in the HPLC mobile phase was determined (Fig. 3). The λ_{max} at 217 nm is due to $\pi \rightarrow \pi^*$ transition of C=C in the barbiturate ring. Analysis of 100-μl sample volumes required a high degree of sensitivity from our method. The relatively low molar absorptivity of unionized 3'HP in neutral (or acidic) mobile phase at 254 nm ($\eta \rightarrow \pi^*$ transition) did not allow detection below 10 μg/ml of 3'HP. Alternatively, the use of alkaline buffers (> pH 7.6) to increase the spectrophotometric selectivity and sensitivity of barbiturates will slowly dissolve silica columns causing loss in efficiency [34]. Numerous reported procedures for the analysis of barbiturates using reversed-phase HPLC have utilized wavelengths < 220 nm when neutral mobile phases have been employed for chromatographic separation [37—39]. Differences in absorbance from 254 nm to 215 nm indicated a 75-fold theoretical increase in spectroscopic sensitivity for 3'HP. While a corresponding decrease in analytical selectivity was experienced, the procedure was designed to minimize interference from biological coextractable constituents. No interference from other commonly prescribed barbiturates was noted; however, the HPLC behavior of other barbiturate metabolites was not investigated.

3'HP was quantitated in sera from 26 patients receiving HDPT (Fig. 4). Pentobarbital concentrations, determined by separate HPLC analysis, ranged from 3—36 μg/ml (mean = 19 μg/ml); while 3'HP was < 4 μg/ml (mean = 1.4 μg/ml). Even in patients (n = 8) treated for between seven and ten days, the highest 3'HP concentration recorded was 3.6 μg/ml. Regression analysis of pentobarbital versus 3'HP suggested no significant relationship between the concentration of drug and metabolite (Fig. 5). However, samples were randomly



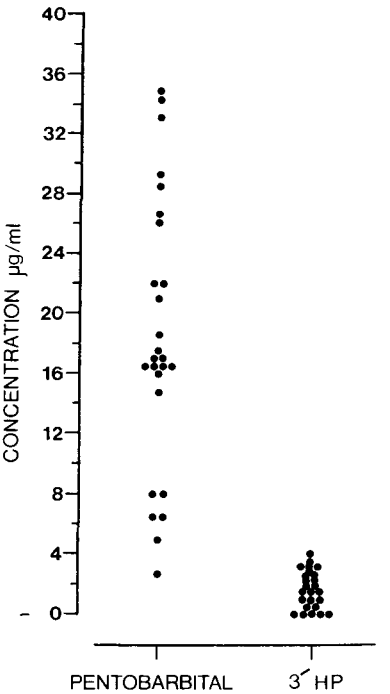


Fig. 3. UV spectrum of 3′-hydroxypentobarbital in tetrahydrofuran—water (5:95).

Fig. 4. Serum concentrations of pentobarbital and 3′-hydroxypentobarbital in patients receiving high-dose pentobarbital therapy. Analysis by HPLC. n = 26.

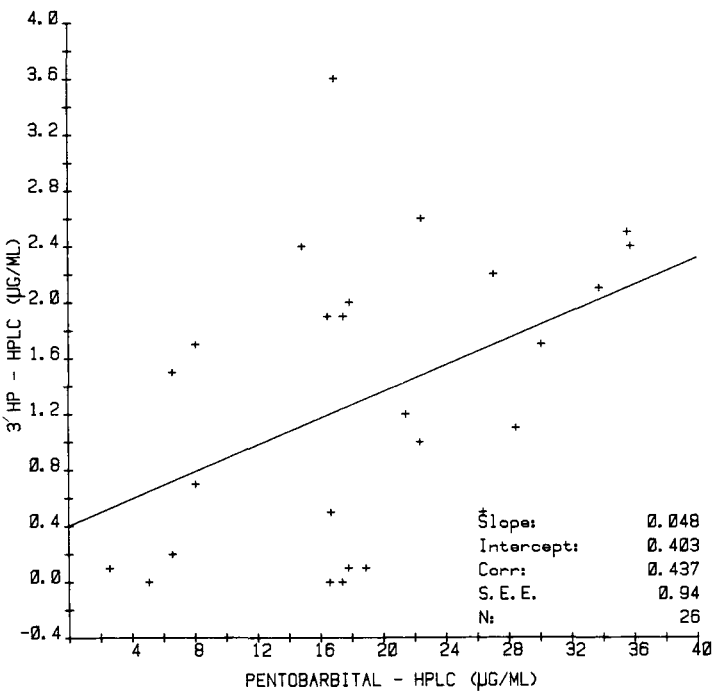


Fig. 5. Concentrations of pentobarbital and 3′-hydroxypentobarbital in high-dose pentobarbital patients. Analysis by HPLC.

acquired and were not selected for patients presumed to be at steady-state.

While data on blood concentrations of hydroxylated pentobarbital are limited, Robinson and McDowall [25] reported concentrations of hydroxypentobarbital from four pentobarbital overdoses at $\leqslant 4$ μg/ml. Additionally, their results for other, high-dose, short-acting barbiturates indicate drug/hydroxylated metabolite ratios much greater than one with poor correlation, which is consistent with our findings. Other studies involving low dose barbiturate administration also indicate that serum barbiturate:hydroxylated metabolite concentration ratios are > 1.0 and are variable [23, 24].

Although 3'HP is considered biologically inactive [40] its quantitation in serum may prove helpful in the investigation of pentobarbital biotransformation. Additionally, the ability to quantitate 3'HP and other hydroxylated barbiturates is useful in assessing the metabolite cross-reactivity of non-specific barbiturate procedures.

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CHROMBIO. 1652

TRACE ANALYSIS OF SULFAQUINOXALINE IN ANIMAL TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Sulfaquinoxaline (in combination with diaveridine as a potentiating agent) was administered orally to broilers, 4—5 weeks old, and sulfonamide residues were determined in muscle and liver at 0, 1, 2, 4, 6, 7, 8, and 10 days post-treatment, using ion-pair extraction followed by high-performance liquid chromatography with UV detection. Improved recoveries (ca. 80%, at the 10 ppb* level) were obtained after liquefaction of the tissues by the addition of 8 *M* urea and sodium hydroxide, prior to ion-pair extraction. A withdrawal period of seven days was found necessary in order to reduce drug residues in muscle and liver to 10 ppb, a level without hazard to humans.

INTRODUCTION

Sulfaquinoxaline and other sulfa drugs are widely used in feeds and premixes to prevent various intestinal disorders of domestic fowl and cattle. As the drugs are stored in tissue and can be ingested by humans, drug manufacturers have since 1973 been required to submit data on tissue residues of sulfonamides following their use in food-producing animals.

In order to quantitate no-effect sulfaquinoxaline (Sq) levels in tissues, methods of marked sensitivity and selectivity are needed. The earlier analytical methods, such as those based on the Bratton-Marshall procedure [1] and subsequent modifications [2—4], failed to meet these requirements due mainly to lack of specificity [5].

Of the methods with improved selectivity, high-performance liquid chromatography (HPLC) proved to be superior to either thin-layer or gas chromatography [6] in terms of simplicity and efficiency. Much work has been carried out on the HPLC separation [7, 8] and quantitative evaluation of various sulfonamides [9—11], but only one paper has been published for the

*Throughout the article the American billion (10^9) is meant.

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determination of Sq in tissues with remarkably low (30 ppb) detection limits [12]. The extraction procedure is, however, rather laborious and running a chromatogram may require over 150 min.

This paper reports the optimization of the extraction procedure of Sq from poultry tissues (muscle and liver), the selection of the appropriate internal standard, and the optimization of HPLC system parameters, with a view to sensitivity. The rate of tissue-residue depletion of Sq is also presented.

EXPERIMENTAL

Materials

Acetone, acetonitrile, *n*-hexane and isoamyl alcohol (each Uvasol), methanol (Selectipur) and ethyl acetate (LiChrosolv) were purchased from Merck (Darmstadt, G.F.R.); chloroform (Chrom AR) was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Ethyl methyl ketone was the product of Reanal (Budapest, Hungary). The sources of the compounds examined were as follows: Sq (Chinoin, Budapest, Hungary); sulfadimethoxine (Sd) (Hoffmann-La Roche, Basel, Switzerland); sulfaguanidine (Sgd), sulfadimidine (Sdd), sulfamethoxidiazine (Smd) (Alkaloida, Tiszavasvári, Hungary); diaveridine (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.). Bis-(2-ethylhexyl)-hydrogen phosphate (DEHP) and trioctylmethylammonium chloride (Adogen 464) were supplied by Serva (Heidelberg, G.F.R.); sodium lauryl sulfate (SLS) was obtained from Schuchardt (München, G.F.R.); tetrabutylammonium hydrogen sulfate (THS) was the product of Sigma (St. Louis, MO, U.S.A.). Other chemicals used were of the highest grade available.

Stock solutions of Sq and Sd were prepared at 0.1 mg/ml in acetone and stored in the refrigerator. For direct calibrations (solutions only, no tissue) varying aliquots of Sq stock solution (10—150 μl) were combined with 100-μl aliquots of Sd stock solution and diluted to 8.0 ml with the eluent. For the residue assay procedure 500 μl and 1000 μl aliquots of Sd stock solution were diluted to 40.0 ml with distilled water containing 1 ml of 25% (w/v) ammonia per 100 ml (solutions A and B, respectively).

Alkaline urea solution (AUS): solutions of 8 *M* urea and 1 *N* NaOH were mixed before use, in a volume ratio of 10:1. Tetrabutylammonium hydrogen sulfate solution (TBAHS): 0.5 g of THS was dissolved in a mixture of 2 ml of isoamyl alcohol and 498 ml of freshly distilled ethyl methyl ketone. The solution was further diluted with 500 ml of freshly distilled ethyl acetate. Trichloroacetic acid solution (TAS): 83 g of trichloroacetic acid were dissolved in 250 ml of distilled water. Borate buffer solution (BBS): 40 g of boric oxide were suspended in 500 ml of distilled water and titrated to pH 11.5 with 10 *N* NaOH. The solution was diluted to 600 ml with distilled water and filtered.

Procedures

Twenty-eight 4—5-week-old broilers were assigned to two groups. Four chickens were fed the standard growing chick mash as a basal control diet (control group). Twenty-four chickens were given the drug as a commercial premix (containing 8 g of Sq and 4 g of diaveridine in 100 kg of premix) for

two weeks before they were given the control diet only (post-treatment). Three chickens from the treatment group were killed on days 0, 1, 2, 4, 6, 7, 8, and 10 post-treatment by decapitation. The control group was sacrificed on the third day of this period. Tissue samples were analyzed without delay.

Residue assay procedure for muscle (procedure A) (see Fig. 1). The muscle was cut into small pieces and 20 g were weighed into the beaker of the homogenizer (MSE, with 100-ml vortex beakers and stainless-steel blades; Measuring and Scientific Equipment Ltd., London, Great Britain). Following the addition of 2.0 ml of solution A, the sample was thoroughly mixed with a glass stirring rod and allowed to stand for 5—10 min. After the addition of 45 ml of AUS, the mixture was homogenized for 5 min, then transferred to a centrifuge bottle and centrifuged at 6000 *g* (MSE, Mistral 4L) for 15 min. Next, the clear liquid was decanted into a 500 ml separating funnel, while the residue in the centrifuge bottle was replaced into the homogenizer, homogenized with another 45-ml portion of AUS and centrifuged as before. The clear liquid was decanted into the 500 ml separating funnel.

The extraction procedure was as follows. The viscous liquid in the separating funnel was mixed with 120 ml of TBAHS and, with frequent shaking, 12 ml of TAS were added in small portions. The resulting suspension was thoroughly shaken for 3—5 min, allowed to stand for 10—15 min and shaken again. The precipitate was removed by filtering the suspension through a suction filter. The filtrate was placed into a 350 ml separating funnel and, after separation, the lower phase was collected in a beaker. The precipitate in the filter was resuspended in the lower phase, the suspension was replaced into the 500-ml separating funnel and, with another 120-ml portion of TBAHS but without additional TAS, the extraction and the filtering processes were repeated.

The cake in the filter was washed with 15—20 ml of TBAHS and the whole filtrate was combined with the upper phase in the 350-ml separating funnel. After separation, the aqueous lower phase was discarded. The organic upper phase was washed by adding 35—40 ml of distilled water, shaking for 3 min and discarding the lower phase. The organic phase was placed in a 250-ml round-bottomed flask and about nine-tenths of the organic solvent was evaporated on a rotating evaporator at a temperature of about 90°C. Using some ethyl acetate, the residue in the flask was quantitatively transferred to a small separating funnel and washed again by the addition of 10—12 ml of distilled water, as described above. The organic phase was placed in a 50-ml round-bottomed flask and evaporated to dryness.

Residue assay procedure for liver (procedure B). The liver was pulped with a knife and 20 g were weighed into the beaker of the homogenizer. Following the addition of 2.0 ml of solution B, the sample was thoroughly mixed with a glass stirring rod and allowed to stand for 5—10 min. After the addition of 70 ml of AUS the mixture was vortexed for 5 min, then transferred to a centrifuge bottle and centrifuged at 6000 *g* for 15 min. The clear liquid was decanted into a 500-ml separating funnel.

The extraction procedure was the same as in procedure A, but instead of 12 ml of TAS only 10 ml were used. Having been washed, the organic phase in the 350-ml separating funnel was placed in a 250-ml round-bottomed flask

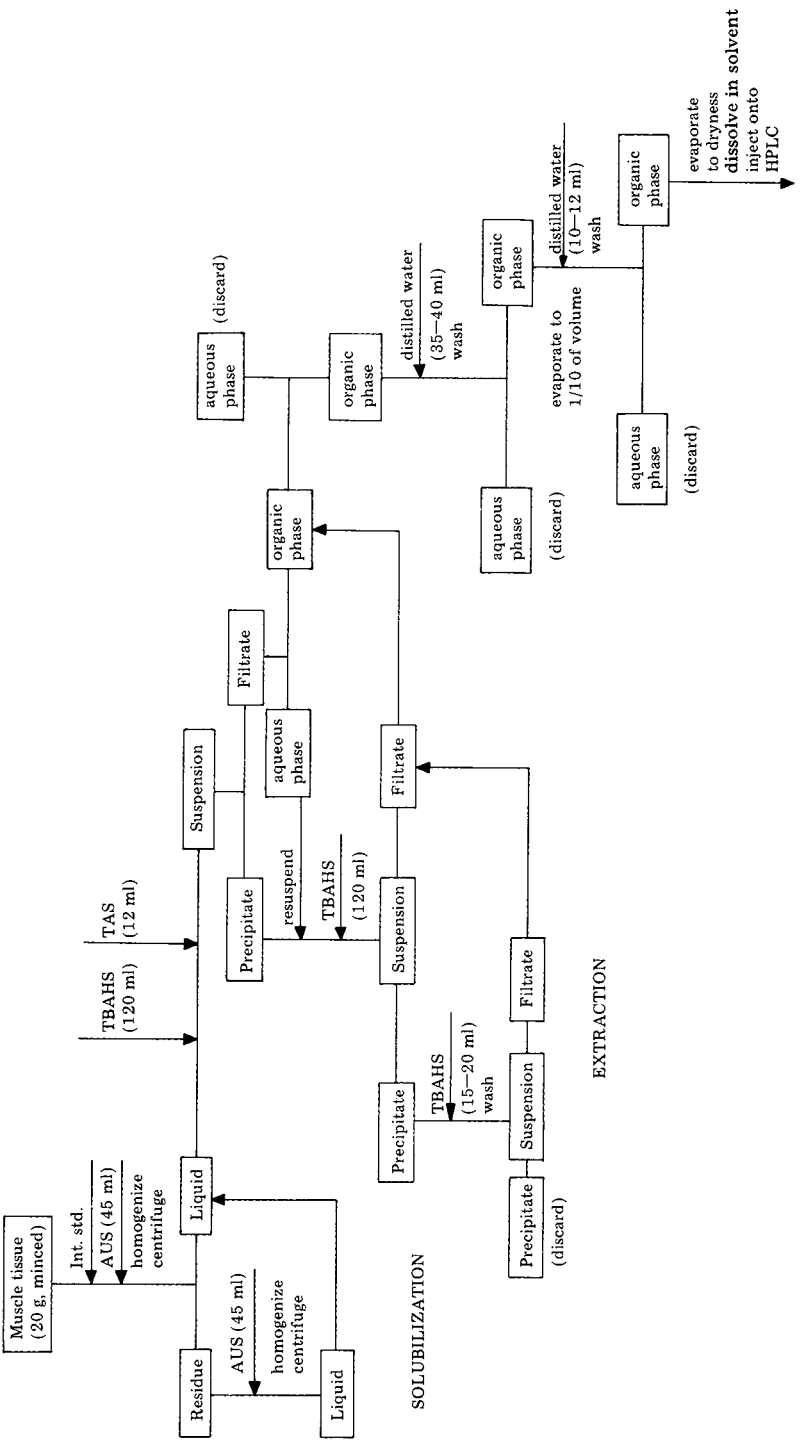


Fig. 1. Flow chart for the isolation procedure (Procedure A).

and the brown solution was evaporated to dryness. The residue in the flask was quantitatively transferred to a 100-ml separating funnel, using about 25—30 ml of hexane and two 10-ml portions of BBS. The contents of the separating funnel were thoroughly shaken and, after separation, the lower phase was collected in a small beaker; the organic phase was discarded. The lower phase was replaced into the separating funnel and the extraction of the pigments from it was repeated twice with 25-ml portions of hexane. The organic phase portions were discarded.

Next, the pH of the lower phase was adjusted to 4.5 ± 0.2 with concentrated hydrochloric acid. The solution was then transferred to a separating funnel and extracted twice with 40-ml portions of TBAHS. The combined organic phase was placed into a separating funnel and thoroughly shaken with 30 ml of distilled water. The resulting emulsion was centrifuged, then the organic phase was drawn off, placed in a 50-ml round-bottomed flask and its volume was reduced to 10 ml on a rotating evaporator. Using some ethyl acetate, the contents of the flask were carefully transferred to the separating funnel and shaken again with 10 ml of distilled water. The emulsion was centrifuged and the organic phase was drawn off, placed in a small round-bottomed flask and evaporated to dryness.

The dry residue obtained in procedure A or procedure B was redissolved, using about two 0.3-ml portions of chloroform—acetonitrile—methanol (15:15:5) and four small portions of *n*-hexane—chloroform—acetonitrile—methanol (80:12:7:1). The portions were collected in a previously calibrated, glass-stoppered test tube to make a total volume of 2.00 ml. Of the homogeneous solution 5—40 μl were injected onto the column.

For calculations, calibration graphs were prepared in such a way that sulfonamide-free muscle and liver samples were used for procedures A and B, respectively, but solutions A and B also contained increasing quantities of Sq, in series, in addition to the fixed amount of Sd. Peak height ratios (Sq/Sd) were plotted against the Sq concentration in the respective tissue.

HPLC conditions

Analyses were performed with a Varian 8500 pump equipped with a stop-flow septumless injector (Varian, Palo Alto, CA, U.S.A.) and an ISCO Model UA-5 (Type 6) absorbance monitor (254 nm, with peak separator and built-in recorder; Instrumentation Specialties Company, Lincoln, NE, U.S.A.). Separations were effected on a Spherisorb S10 W (10 μm), 250 × 3 mm I.D. column (Chrompack Nederland B.V., Middelburg, The Netherlands). The eluent was *n*-hexane—*n*-hexane (water saturated)—chloroform—acetonitrile—methanol—25% (w/v) ammonia (36:30:15:14.5:4.5:0.05) for procedure A or (37:30:15:14.5:3.5:0.05) for procedure B.

RESULTS AND DISCUSSION

Optimization of the extraction procedure

Although HPLC with UV detection offers good possibilities for the sensitive determination of sulfonamides (as most of these drugs exhibit strong UV

absorption between 240 and 270 nm), trace analysis of sulfa drugs in complex biological matrices also requires highly efficient clean-up techniques.

The amphoteric nature of sulfonamides is well established. At a pH below 2.8 the dominant form of Sq is cationic, while at a pH higher than 6.2 its preferred state is anionic, as shown in Fig. 2. The non-ionic form is dominant between pH 3.5 and 4.5, which is the optimal pH range for simple organic extractions of Sq (see Fig. 3).

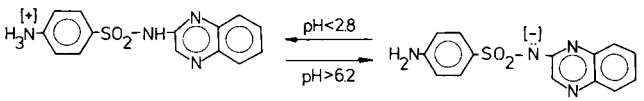


Fig. 2. The amphoteric nature of sulfaquinoxaline.

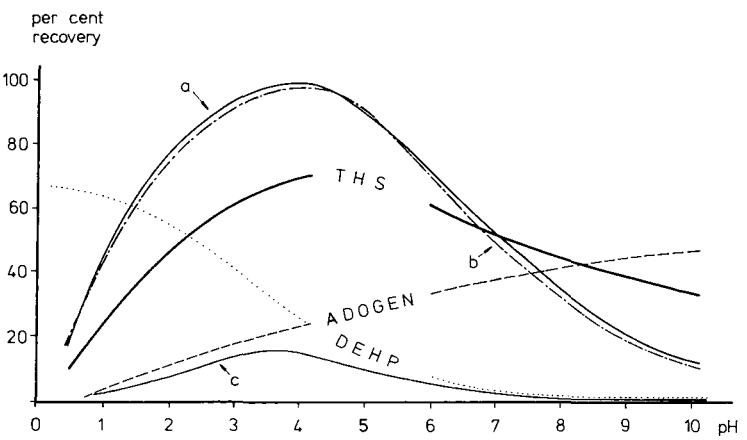


Fig. 3. The efficiency of the extraction of Sq from an aqueous solution (a), urine (b), and an aqueous solution mixed with pulped muscle tissue (c), as a function of pH. For the extraction conditions see Optimization of the extraction procedure. DEHP, ADOGEN and THS curves show the efficiency of the extraction method (average efficiencies from solubilized muscle) involving the use of the respective ion-pairing agent, as a function of pH. Twenty grams of tissue containing a known amount of Sq (in the 0.01—0.10 ppm range) were solubilized by alkaline urea as described in procedure A. The liquid was extracted with 120 ml of ethyl methyl ketone—ethyl acetate (1:1) containing the respective ion-pairing agent in a concentration of 1.5×10^{-3} *M*. The pH of the mixture was adjusted with TAS. Concentrations were all determined by the HPLC method for procedure A (see HPLC conditions).

First, we investigated the extraction of Sq from various aqueous media, using organic solvents only. From a number of solvents and solvent mixtures ethyl methyl ketone—ethyl acetate (1:1) was chosen for the extraction of Sq (and ion-pairs formed with Sq). Fig. 3 shows the efficiency of the extraction (percentage recovery) from a simple aqueous solution, urine and an aqueous solution mixed with pulped muscle tissue, as a function of pH (curves a, b and c, respectively). For curves a and b the concentration of Sq in the aqueous solution was 50 ng/ml and the volume ratio was 1:1. For curve c, 10-ml portions of an aqueous solution of Sq (100 ng/ml) were, after pH ad-

justment, thoroughly mixed with 20-g portions of pulped muscle tissue. The mixtures were allowed to stand for 10 min. The volume of the organic extractant was 100 ml. The extractions were all carried out by shaking the mixtures at about 24°C for 15 min.

As shown, the efficiency of the extraction of Sq from a simple aqueous solution and urine is roughly the same, and at a pH of about 4 it is very high. However, if the aqueous medium also contains a large amount of tissue the efficiency drops considerably, obviously because Sq is strongly bound to tissue proteins (which may account for the poor recoveries obtained at this level with earlier extraction methods).

Next, we attempted to improve the efficiency of the extraction by treating the tissue with 8 *M* urea. Urea is known to be a good protein denaturant, which solubilizes tissue proteins lowering the free energy of the denatured state relative to that of the native structure [13]. Consequently, denaturation by urea should reduce the ability of proteins to bind Sq and improve the recovery of the drug. Although it did, further improvement was needed and was achieved after combining alkaline urea treatment with the technique of ion pairing [14].

Cationic and anionic species (Adogen 464, THS and DEHP, SLS) were tested as counter-ions in a concentration range of 1×10^{-3} to 5×10^{-3} *M*. The efficiency of the ion-pair extraction with three of the counter-ions, as a function of pH, is shown in Fig. 3. As can be seen, the best recovery from tissue was achieved using the cationic counter-ion, THS (1.5×10^{-3} *M*) at a pH of 4.1—4.2. The shape of the THS curve at pH values higher than 6 can be explained by assuming a mixed (non-ionic and ion-pair) extraction mechanism. In the pH range 4.3—6.0 extraction was impossible due to the gel-like structure formed by the proteins precipitated.

Recovery was further improved slightly by the addition of a good adduct-forming agent, isoamyl alcohol, to the organic phase. Thus, the overall recovery with two replicate extractions was 85 ± 8% from muscle and 77 ± 11.2% from liver (average values in the 0.01—0.1 ppm Sq concentration range, see also Table II). Lower recovery data with liver are due to the need for a more laborious extraction procedure aimed at removing tissue pigments from the organic phase.

In the search for a suitable internal standard procedures A and B were also used for the extraction of tissues spiked with other sulfa drugs (Sd, Smd, Sdd, Sgd). The extraction properties of Sd were identical with those of Sq.

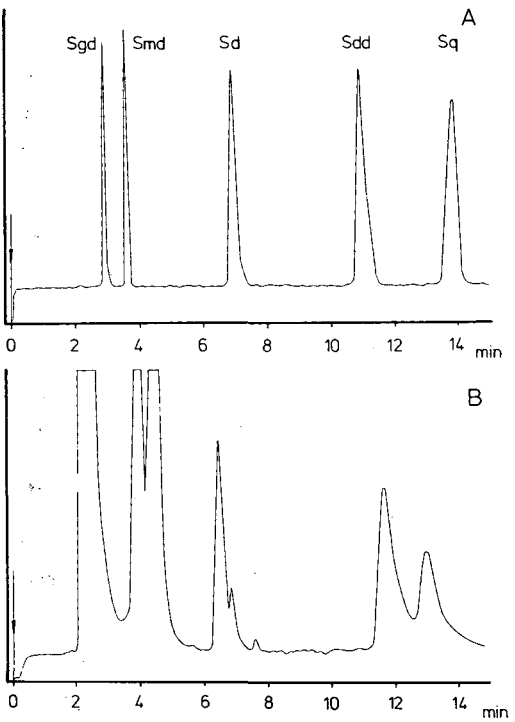
Optimization of HPLC parameters

When optimizing chromatographic parameters from the point of view of the lowest possible detection limits, we relied mainly on the work of Karger et al. [15]. Tissue extracts were chromatographed in a number of reversed-phase and normal-phase systems. In each case, naturally occurring and potentially interfering components of tissue made it difficult to optimize separation in such a way that the capacity factor, k', for Sq was in the optimal range of $0.5 < k' < 2.5$. Background interference was a more serious problem with reversed-phase systems. A comparison of chromatograms A and B in Fig. 4 demonstrates the difficulty in achieving a reversed-phase separa-

tion of Sq as well as an additional sulfa drug (to be used as internal standard) from interfering tissue components, if k'_{Sq} is to be kept low.

Figs. 5 and 6 present normal-phase chromatograms obtained on the analysis of tissue samples from control animals (Figs. 5A and 6A) and those obtained from feed-medicated ones (Figs. 5B and 6B). Based on its extraction and retention behaviour Sd was chosen as internal standard. As is shown, interference was eliminated, but, for greater sensitivity, the resolution (R_s) of the compounds of interest was kept to the lowest adequate value.

Following the analysis of 20—30 samples, the column was washed with 50 ml of methanol, then reequilibrated with the eluent. Column performance and calibration graphs were checked by the injection of solutions of Sq and Sd (direct calibration). In the system presented in Fig. 5 (k'_{Sq} = 7; theoretical plate number, N_{Sq} = 4800 per 25 cm, linear velocity, u = 2.5 mm/sec) the detection limit for Sq (twice the noise) was 1.2 ng per injection. With k'_{Sq} at the theoretical optimum, i.e. k' = 1.0, the same system would have permitted a detection limit of 250 pg.



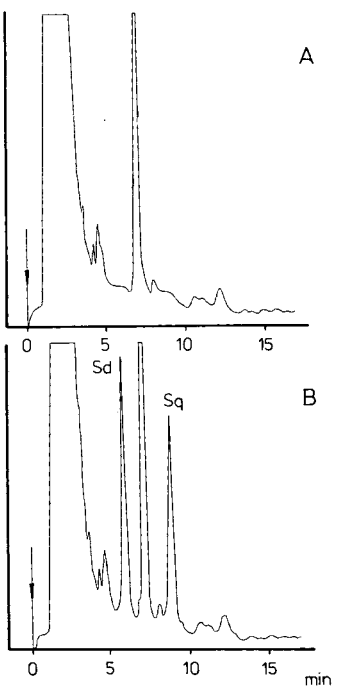


Fig. 4. Reversed-phase chromatography of (A) some sulfonamides and (B) an extract obtained with procedure A from sulfonamide-free muscle tissue (without internal standard). Column, Micro-Pak CH-10 (Varian) (10 μm) 300 × 4.0 mm I.D.; eluent, 0.01 M $(NH_4)_2CO_3$—methanol (90:10); flow-rate, 0.83 ml/min; temperature, ambient.

Fig. 5. Normal-phase chromatography of (A) an extract obtained with procedure A from the muscle tissue of a control animal (without internal standard) and (B) an extract obtained with procedure A from the muscle tissue of a feed-medicated animal. The peaks Sd and Sq represent 50 ng and 37 ng of the respective sulfonamide. The HPLC method for procedure A (see HPLC conditions) was used. The flow-rate was 0.83 ml/min, the temperature was ambient.

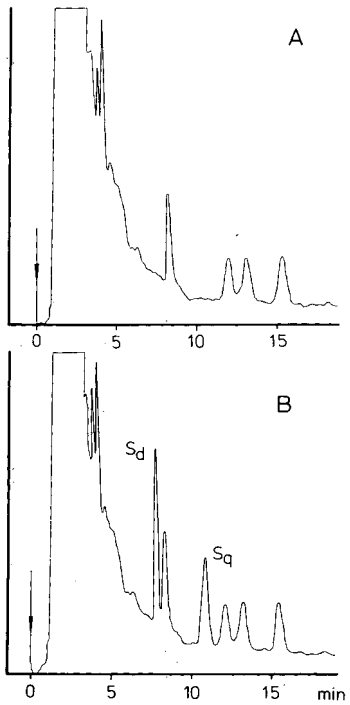


Fig. 6. Normal-phase separations of (A) an extract obtained with procedure B from the liver tissue of a control animal (without internal standard) and (B) an extract obtained with procedure B from the liver tissue of a feed-medicated animal. The HPLC method for procedure B (see HPLC conditions) was used. The flow-rate was 0.83 ml/min, the temperature was ambient.

Linearity, precision and accuracy

No interference originating from diaveridine was noted since the extraction and UV properties of this drug are quite different from those of Sq. The best signal-to-noise ratio was obtained with a fixed-wavelength UV detector. (Sq exhibits an absorption maximum at 252 nm in the eluent.) Linearity of the calibration graphs in the 10 ppb to 10 ppm range was good and fulfilled the criterion suggested by Fowlis and Scott [16].

Precision and accuracy studies were performed at three different concentrations of Sq in spiked control tissues. The results are tabulated in Table I. Coefficients of variation (C.V.) indicate the within-run precision of the quantitative results (samples from the same portion of tissue, on the same day).

Table II summarizes the results obtained on Sq-depletion patterns. Depletion was rapid, especially from liver, during the first two days of withdrawal. A withdrawal period of seven days was found safe enough for achieving no-effect Sq levels in the tissues examined. C.V. values incorporate day-to-day as well as inter-assay (samples originating from different animals) variability.

TABLE I

ACCURACY AND PRECISION OF THE HPLC ASSAY OF Sq IN TISSUES
$n = 8$.

Tissue	Amount added (ng Sq per g tissue)	Amount found (ng Sq per g tissue; mean ± S.D.)	C.V. (%)
Muscle	20	19.2 ± 2.02	10.5
	200	204 ± 11.85	5.8
	1000	988 ± 34.60	3.5
Liver	20	19.3 ± 2.61	13.5
	200	196 ± 14.10	7.2
	1000	1010 ± 49.50	4.9

TABLE II

DEPLETION OF Sq FROM MUSCLE AND LIVER DURING THE WITHDRAWAL PERIOD

Hours of withdrawal (post-treatment)	Sq content (ng/g tissue)							
	Muscle				Liver			
	ng found*			Mean C.V. (%)	ng found*			Mean C.V. (%)
0	956	1070	1010	1010	2210	2150	1750	2015
	1045	980	1050		1900	1830	2320	
	995	975	1010	3.80	2030	2140	1810	10.00
24	631	710	692	668	1010	896	1070	958
	697	628	705		945	1042	937	
	654	683	615	5.50	897	955	870	7.20
48	485	490	505	461	436	412	417	452
	422	418	440		488	501	407	
	462	454	478	6.64	442	465	503	8.46
96	218	251	229	223	108	120	97	99
	240	207	234		96	85	88	
	203	212	209	7.50	95	102	100	10.60
144	76	79	64	69	32	35	25	29
	63	62	74		27	26	31	
	71	70	63	9.22	28	30	27	11.10
168	10	6	7	8	8	10	6	9
	8	9	10		12	8	9	
	6	8	6	15.40	9	7	10	20.00
192	Below the detection limit				Below the detection limit			

*Triplicate samples per chicken.

CONCLUSIONS

The determination of no-effect sulfonamide levels in edible animal tissues poses a complex trace analytical problem due to the fact that sulfa drugs are

strongly bound to tissue proteins. Solubilization of tissues by 8 *M* alkaline urea was found to be effective in improving the efficiency of the trace enrichment procedure. Best recoveries from the solubilized tissues were obtained by the application of an extraction method of mixed (ionic and non-ionic) mechanism.

In the optimization of HPLC parameters from the viewpoint of sensitivity, a compromise had to be struck. In a sufficiently selective normal-phase system (see Fig. 5) the attainable detection limit for Sq was 1.2 ng per injection, almost five times the theoretical value.

The procedures as described above provide reproducible and quantitative methods for the determination of trace amounts of Sq in animal tissues.

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QUANTITATIVE DETERMINATION OF PRAZIQUANTEL IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple method for the quantitation of praziquantel by high-performance liquid chromatography is described. This method has a lower limit of sensitivity of 2.5 ng of drug per ml of human serum and a relative standard deviation of 2.6% at concentrations of 5 ng/ml.

INTRODUCTION

Praziquantel (PZQ), a pyrazino isoquinoline derivative with activity against all five species of schistosomes infecting man [1—5], is the current drug of choice for mass chemotherapy of schistosomiasis. Advantages of this agent include high efficacy after oral administration, low toxicity and a 1-day treatment regimen. Praziquantel also is effective against a variety of hermaphroditic flukes and cestodes of clinical importance to humans [6—8].

Clinical investigation of the pharmacokinetics and toxicity of such a clinically useful drug as PZQ requires the availability of a sensitive and specific assay for the routine measurement of drug levels in blood and other body fluids. Presently, radiometric [9], fluorometric [10], gas—liquid chromatographic [11], and bioassay techniques [12] are available for the measurement of this compound. However, the use of radioactive drug would not be suitable for routine clinical studies in patients. The other methods are limited by requirements for complex sample preparation or derivatization.

We now report the development of a simple, sensitive, and quantitative procedure to measure PZQ levels in serum by high-performance liquid chromatography (HPLC). This method is clinically applicable, readily validated, and eliminates the need for involved sample preparation or derivatization.

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MATERIALS AND METHODS

Standards and solvents

Praziquantel, 2-cyclohexylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-*a*]isoquinoline, and the internal standard, 2-cycloheptylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-*a*]isoquinoline, were provided by E. Merck (Darmstadt, G.F.R.). The structures of these compounds are shown in Fig. 1A. Acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and ethyl acetate (Fisher Scientific, Pittsburgh, PA, U.S.A.) were HPLC grade. The ethyl acetate was washed three times with equal volumes of distilled water before use.

Serum assay

Reagents. (1) Solvent, 38% aqueous acetonitrile; (2) water-saturated ethyl acetate; (3) praziquantel solution, 1 mg of praziquantel dissolved in 10 ml of 38% aqueous acetonitrile; (4) internal standard solution, 0.8 mg of internal standard dissolved in 10 ml of 38% aqueous acetonitrile.

Sample treatment. A 10-μl (0.8-μg) aliquot of internal standard solution is added to 1 ml of mouse, human, or fetal calf serum and mixed vigorously for 15—30 sec. The sample is extracted three times with 2 ml of water-saturated ethyl acetate. Each sample is mixed for 1 min after addition of organic solvent and phase separation is achieved by centrifugation at 1600 *g* for 5 min. The three ethyl acetate phases from each sample are combined and transferred to a 10-ml pear-shaped flask for removal of solvent by rotoevaporation (Buchler Rotovapor R110). Each sample residue is resuspended in 250 μl of 38% aqueous acetonitrile and aliquots of 25 μl are injected into the HPLC apparatus.

Apparatus

Analyses are carried out with a Model M-6000A high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model SF-770M-A2 variable-wavelength UV detector (Schoeffel Instrument Company, Westwood, NJ, U.S.A.).

An octadecyl silica gel RP-18, 5 μm (Spheri-5), 10 cm × 4.6 mm MPLC™ analytical cartridge and an RP-18, 5 μm (Spheri-5), 3 cm × 4.6 mm precolumn both from Brownlee Labs. (Santa Clara, CA, U.S.A.) are used as the stationary phase. The mobile phase is 38% aqueous acetonitrile.

Operating conditions

A flow-rate of 1.5 ml/min and a detector wavelength of 210 nm at ambient temperature are employed throughout. After 15—20 analyses, the column is cleaned with 100% acetonitrile for 20 min, using a Waters Assoc. Model 660 solvent programmer (flow program No. 6).

Quantitation of PZQ

The formula for calculation of the PZQ concentration in serum is as follows: $C_2 = (A_2 \cdot C_1)/(A_1 \cdot 1.13)$, where C_1 = concentration of internal standard added to serum, C_2 = concentration of PZQ in serum, A_1 = area under the

internal standard peak in the HPLC chromatogram, and A_2 = area under the PZQ peak in the HPLC chromatogram.

After extraction into ethyl acetate, recoveries of four different known amounts of PZQ and internal standard added to 1 ml of human serum varied from 91.7 to 95.3% (average 93.1%) for PZQ and from 91.3 to 97.5% (average 93.2%) for the internal standard. Thus, no difference in the extraction efficiency of PZQ and internal standard was noted. However, a difference in the molar extinction coefficients at 210 nm was found for PZQ and the internal standard. The molar extinction coefficient of PZQ in 38% aqueous acetonitrile was 31,746 whereas the comparable value for the internal standard was 28,199. The calculated ratio of the two extraction coefficients at 210 nm is 1.13; this ratio is included in the formula for calculating PZQ concentrations.

UV spectroscopy

The UV spectra of PZQ and the internal standard (not shown), recorded on a Gilford 2400-2 spectrophotometer (Gilford Instruments, Oberlin, OH, U.S.A.), were identical except for the difference in extinction coefficients.

Mass spectrometry

Mass spectral analysis was performed with a Kratos MS-30 dual-beam mass spectrometer equipped with a Kratos DS-50 S computer system. Samples were introduced by direct probe and spectra obtained by electron impact at 4 kV accelerating voltage, an ionizing potential of 70 eV, and a resolving power of 3000.

Animals and pharmacokinetic studies

CF-1 female mice (22—25 g) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and maintained on rat chow and water ad libitum in the animal facilities at Case Western Reserve University School of Medicine. Non-fasted mice were given 200 mg/kg of PZQ dissolved in 0.4 ml of 2% Cremophor EL (Sigma, St. Louis, MO, U.S.A.) by gastric intubation. Blood samples were drawn from the retroorbital sinus. Pharmacokinetic studies of PZQ in mice were performed in CF-1 mice exposed to 120 cercariae of *Schistosoma mansoni* by tail immersion for 30 min [13].

RESULTS

Standards and recoveries

PZQ and the internal standard (Fig. 1A) were well resolved from each other under the chromatographic conditions selected (k' values of 6.0 and 9.3, respectively). Moreover, when human or mouse serum, free of added standards, was extracted with ethyl acetate and the extracts were analyzed, no extraneous peaks were found which might interfere with the analysis of either PZQ or the internal standard (Fig. 1B).

When 0.05, 0.1, 0.2, 0.4 and 0.8 μg of PZQ and internal standard were added to 1 ml of human serum, extracted, and aliquots analyzed by HPLC at 210 nm, the amounts of PZQ and internal standard detected were linear with

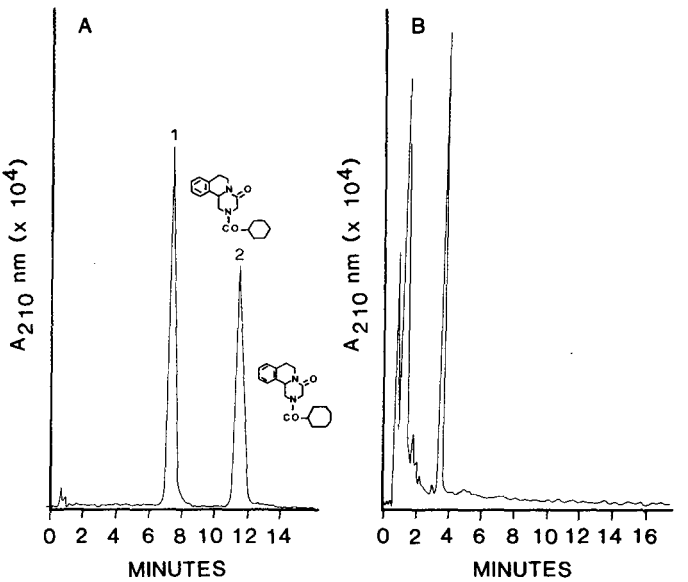

Fig. 1. (A) HPLC chromatogram of a mixture of praziquantel (PZQ), 1, and the internal standard (IS), 2. The concentration of each compound is 0.15 μg. The ratio of areas under the two peaks is 1.15 (PZQ:IS). (B) HPLC chromatogram of an ethyl acetate extract of normal human serum.

concentration. The linear regression equation of the line for the PZQ standard curve was $y = 1204.7x - 0.43$, correlation coefficient 0.99; for the internal standard $y = 976.7x + 1.24$, correlation coefficient 0.99. Identical results were obtained when the same amount of PZQ was added to either mouse serum or mouse plasma.

The lower limit of sensitivity for PZQ detection in either human or mouse serum is 2.5 ng/ml. The reproducibility of the method was checked by adding 5 ng of PZQ to three separate 1-ml serum samples, resuspending the residues in 100 μl of column solvent and injecting 50 μl into the HPLC apparatus. The variation in peak areas between these determinations was 2.6%. Repetitive injection of a sample from a human serum extract containing 5 ng of PZQ showed a variation of 2.9% ($n = 5$) in the calculated area (mm^2) of the PZQ peak.

Stability

The stability of PZQ in serum samples during storage was tested by adding 0.1 μg of the drug to five separate 1-ml serum samples obtained from two different people. One sample from each person was analyzed immediately. The others were frozen at −20°C and analyzed after 1, 2, 3, and 4 weeks of storage. The same concentrations were found after storage for up to 4 weeks.

Clinical application

Previous work [14] indicated that after oral administration of PZQ to schistosome infected mice, peak serum levels of PZQ were reached at 30 min and by 240 min most of the PZQ had disappeared from the serum. In order

to test the validity of the method, four mice, after oral administration of PZQ at a dose of 200 mg/kg, were bled at each of these time points and samples of approximately equal volume were pooled. The blood was allowed to clot at room temperature and the serum removed after centrifugation at 1600 *g* for 10 min. Aliquots of 1 ml of serum were processed and analyzed (see Materials and Methods).

The serum PZQ concentration was found to be 12.3 μg/ml in the 30-min sample and 0.2 μg/ml in the 240-min sample (Fig. 2A and B). In order to confirm that the absorbance peak designated as PZQ in Fig. 2A was due to PZQ, the fraction corresponding to this peak was collected and analyzed by mass spectroscopy after evaporation of the eluting solvent. The mass spectrum of the material in the peak fraction was found to be identical to the mass spectrum of authentic PZQ.

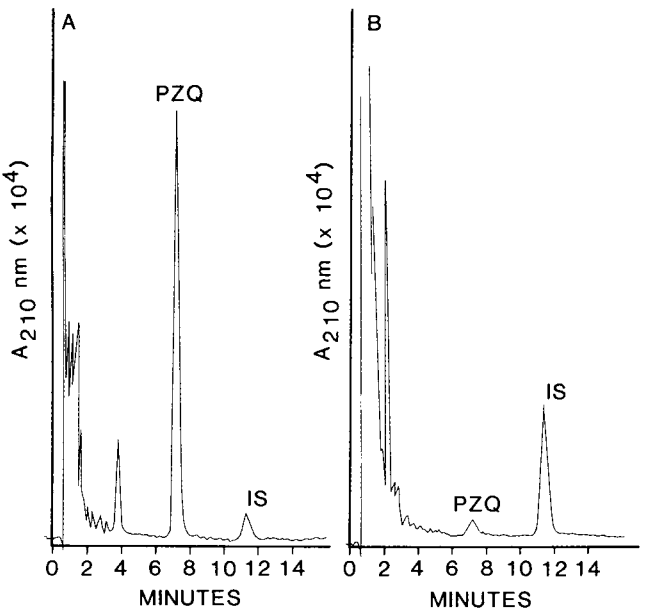

Fig. 2. PZQ concentration in serum of *S. mansoni* infected mice after oral administration of a single drug dose of 200 mg/kg. (A) At 30 min after drug administration. Due to the high concentration of PZQ present in serum, the injection volume was decreased to 5 μl which contained 0.016 μg of internal standard (IS). (B) at 240 min after drug administration. The injection volume was 25 μl which contained 0.08 μg internal standard.

DISCUSSION

The HPLC method described here combines simplicity of sample preparation and analysis with the selectivity and sensitivity required for clinical pharmacokinetic studies. Excellent separation of PZQ from internal standard was achieved and no extraneous peaks from serum components were noted in the HPLC chromatogram. Although PZQ is known to be metabolized extensively, predominantly to hydroxylated derivatives [15], more polar metab-

olites should either elute before the parent compound or remain in the aqueous phase. We found no mass spectral evidence for extraneous substances such as PZQ metabolites in the PZQ region of the HPLC trace of sera from PZQ treated mice.

This HPLC method is sufficiently sensitive to permit pharmacokinetic studies of PZQ in humans. Previously published data indicate that a peak level of 1 μg of PZQ per ml of serum was reached within 70—120 min after oral administration of 46 mg of PZQ per kg body weight [9]. This level is about 200-fold higher than that which can be determined with good accuracy by the present method.

ACKNOWLEDGEMENTS

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SYSTEMATIC APPROACH TO THE DETERMINATION OF CEPHALOSPORINS IN BIOLOGICAL FLUIDS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The chromatographic behaviour of some cephalosporins as a function of pH and ionic strength of the mobile phase was studied on 10-μm LiChrosorb RP-18. Acidic cephalosporins were retained longest in their neutral form with an acidic eluent. Amphoteric cephalosporins were retained longest in their protonated form with an acidic eluent of low ionic strength. Cefotiam was retained longer with an alkaline mobile phase. LiChrosorb RP-18, Nucleosil C_{18} and μBondapak C_{18} gave rise to different selectivities when an acidic eluent, methanol—water (25:75) containing 0.2% of 1.8 *M* H_2SO_4 was used. This may be related to interactions with residual silanol groups. The studied cephalosporins (with the exception of cefotiam and cefsulodin) were separated from compounds present in biological fluids on 5-μm LiChrosorb RP-18 using the mobile phase 0.2% of 1.8 *M* H_2SO_4 in a mixture of methanol and water with various methanol contents. The determination of cefotiam in biological fluids was performed with an alkaline mobile phase. The preparation of the sample was simple and rapid: precipitation of plasma proteins or dilution of urine. The method was applied to the determination of ceftizoxime in human plasma and urine. Concentrations down to 0.2 μg/ml of plasma and 25 μg/ml of urine could be determined with good reproducibility and accuracy.

INTRODUCTION

In recent years, many liquid chromatographic methods have been reported for the determination of different cephalosporins in plasma and urine. The reversed-phase mode is most commonly used, with precipitation of plasma proteins [1—7] or extraction from plasma with Sep-Pak cartridges [8] prior to injection. The nature and the concentration of the components of the mobile phases used differ arbitrarily from one method to another although cephalosporins have a common 7-aminocephalosporanic acid nucleus. Recently, Brisson and Fourtillan [9] determined seven cephalosporins in plasma in the

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reversed-phase mode by changing only the content of methanol or acetonitrile in the mobile phase. Cephalosporins were extracted and back-extracted in order to obtain clean plasma blanks. However, orally absorbed cephalosporins such as cefroxadin and cephalexin, which possess an α-amino group in their C-7 side-chain, cannot be extracted with the proposed method.

A study of the chromatographic behaviour of cephalosporins on reversed-phase packings may be useful to ascertain whether cephalosporins can be quantitatively determined in biological fluids under similar chromatographic conditions. In a previous paper [10] the influence of the ionization of some cephalosporins was studied with a non-polar octadecylsilyl stationary phase. In the present paper, this influence is examined with mobile phases of lower ionic strengths. On the basis of the results obtained, conditions for the determination of cephalosporins in human plasma, urine, bile and milk, with a rapid sample-preparation step prior to injection, are described.

EXPERIMENTAL

Materials and reagents

The structures of the investigated cephalosporins are given in Table I. Ceftizoxime, cefotiam, cefsulodin and cefroxadin were obtained from Ciba-Geigy (Basle, Switzerland), cefotaxime and its desacetyl metabolite from Roussel Uclaf (Paris, France), cefuroxime and cephalexin from Glaxo (Paris, France), cefamandole and cephalothin from E. Lilly (Saint-Cloud, France), and cefazolin from Allard (Paris, France).

Phosphoric acid (85%), sodium dihydrogen phosphate, disodium hydrogen phosphate, sulphuric acid (95—97%), sodium hydroxide (32%), sodium sulphate and trichloroacetic acid (TCA) were purchased from E. Merck (Darmstadt, G.F.R.). Methanol was purchased from Prolabo (Paris, France). All chemicals were of analytical reagent grade and were used without further purification.

The following reversed-phase materials were used: Co:Pell ODS, 30—38 μm particle size (Whatman, Clifton, NJ, U.S.A.); LiChrosorb RP-18, 5 and 10 μm particle sizes (E. Merck); μBondapak C_{18}, 10 μm particle size (Waters Assoc., Milford, MA, U.S.A.) and Nucleosil C_{18}, 5 μm particle size (Macherey-Nagel, Düren, G.F.R.).

Apparatus

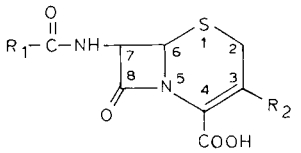
Chromatography was performed on a Hewlett-Packard Model 1081 B instrument equipped with a fixed-wavelength UV detector (254 nm) and an automatic sample injector. The detector was connected to a Hewlett-Packard data system (Model 3353 E).

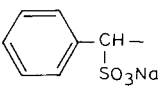
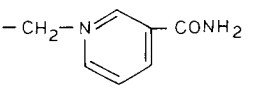
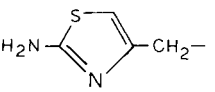
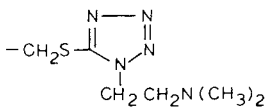
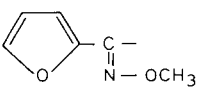
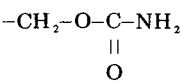
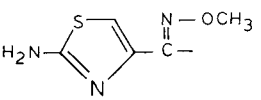
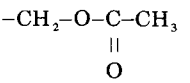
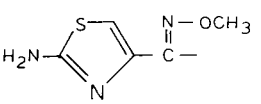
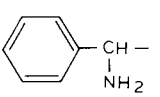
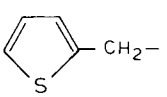
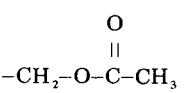
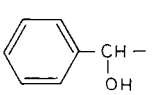
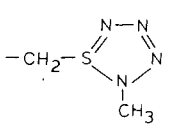
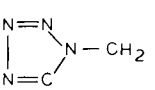
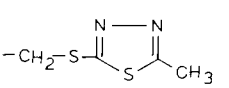
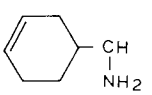
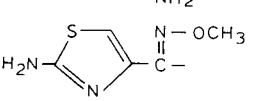
A pre-column (stainless-steel tube, 10 cm × 4.7 mm I.D.), tap-filled with Co:Pell ODS, was used to protect the analytical column. The analytical column (stainless-steel tube, 4.7 mm I.D.) was 15 cm long when filled with LiChrosorb RP-18 and Nucleosil C_{18} and 25 cm long when filled with μBondapak C_{18}. The reversed-phase material was suspended in 10^{-2} mol/l sodium acetate in water—methanol (20:80, v/v) in a ratio of 17:100 (w/v). The filling pressure was set at 420 bars with a pneumatic Haskel pump. Methanol was used as the pressurizing solvent.

TABLE I

CHEMICAL STRUCTURE OF THE INVESTIGATED CEPHALOSPORINS

Cephalosporins



	R_1	R_2
Cefsulodin		
Cefotiam		
Cefuroxime		
Cefotaxime		
Desacetylcefotaxime		$-CH_2-OH$
Cephalexin		$-CH_3$
Cephalothin		
Cefamandole		
Cefazolin		
Cefroxadin		$-O-CH_3$
Ceftizoxime		$-H$

The flow-rate of the mobile phase was 2 ml/min for the 10 μm particle size materials and 1.2 ml/min for the 5 μm particle size materials.

The pH of the aqueous part of the mobile phase was measured with a pH meter (Beckman digital pH meter) and adjusted with NaOH or H_2SO_4.

Sample preparation

Plasma samples were prepared as follows, unless otherwise specified: 150 μl of plasma, 50 μl of TCA (10:100, w/v) aqueous solution, and 15 μl of aqueous solution of the studied cephalosporin, were introduced into a 10-ml conical glass tube, mixed on a Vortex mixer for 15 sec and centrifuged for 3 min. A 20—40 μl volume of the clear solution was injected onto the column.

Human milk and bile samples were prepared as those of plasma, and 20 μl of the supernatant were injected after a two- to ten-fold dilution.

For urine samples, 100 μl of urine, 100 μl of standard solution of the cephalosporin and 800 μl of water were introduced into a 10-ml glass tube and mixed on a Vortex mixer; 10—20 μl of the solution were injected onto the column.

RESULTS AND DISCUSSION

Effect of pH and ionic strength of the mobile phase on the retention of cephalosporins

The effect of pH and ionic strength of the mobile phase was investigated on 10 μm LiChrosorb RP-18.

Cephalosporins contain one or more groups ionizable in the pH range studied (pH 2—8), and the retention of these compounds will depend on the extent of dissociation of these groups. All cephalosporins possess a carboxylic group and some have another acidic function (cefsulodin) or an amino group (cefroxadin, cephalexin and cefotiam). Furthermore, some new cephalosporins (cefotiam, cefotaxime and ceftizoxime) possess an aminothiazolyl moiety with an amino group which exhibits weak basic properties. The pK_a values which relate to the 4-carboxylic group and to the 7-side-chain amino group have been reported to be about 3—4 and 7—7.5, respectively, at 20°C and ionic strength 0.15 [10]. The three pK_a of cefotiam were reported to be $pK_{a_1} = 2.6$, $pK_{a_2} = 4.6$, $pK_{a_3} = 7.0$ in aqueous solution [11].

At pH 8, all cephalosporins are mainly present in anionic form. When the ionic strength of the mobile phase was increased by addition of sodium phosphates at this pH, the retention of all the investigated cephalosporins also increased, as illustrated in Fig. 1A. This is in agreement with the findings of Van de Venne et al. [12], who stated that with an increasing concentration of cations from the buffer, the adsorption of ionized acids on the organic silica is increased owing to compensation of their negative charges by these cations.

The ionic strength was further increased at pH 2.7 by addition of sodium sulphate. The dissociation of carboxylic groups is partly suppressed at this pH. The effect of the ionic strength was low for cefuroxime and cefazolin, whereas the retention decreased with increasing salt concentration for cephalosporins having an amino group. This is illustrated in Fig. 1B. Such a decrease in retention of protonated amino compounds on octadecyl-silicas with increasing salt

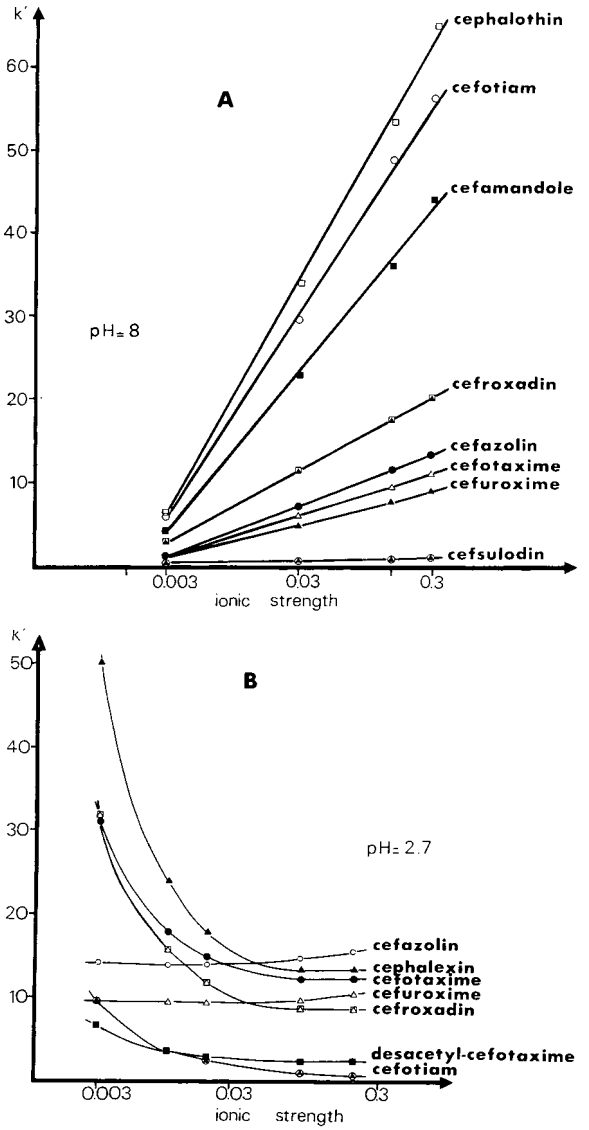


Fig. 1. Plots of capacity factors against ionic strength. Column: LiChrosorb RP-18, 10 μm. Methanol content in the mobile phase: 15% (v/v).

concentration was previously reported [13—15]. This decrease may be interpreted by the dual-retention model [13], which involves both solvophobic interactions with the hydrocarbonaceous layer and silanophilic interactions with unreacted silanol groups that remain on the surface of the stationary phase. With increasing concentration of cations from the buffer, an attenuation of silanophilic interactions may result from a competitive effect of these cations on residual silanols. Cephalexin and cefroxadin are fully protonated at this low pH and their retention is more markedly reduced than that of cefotaxime, which exhibits weak basic properties.

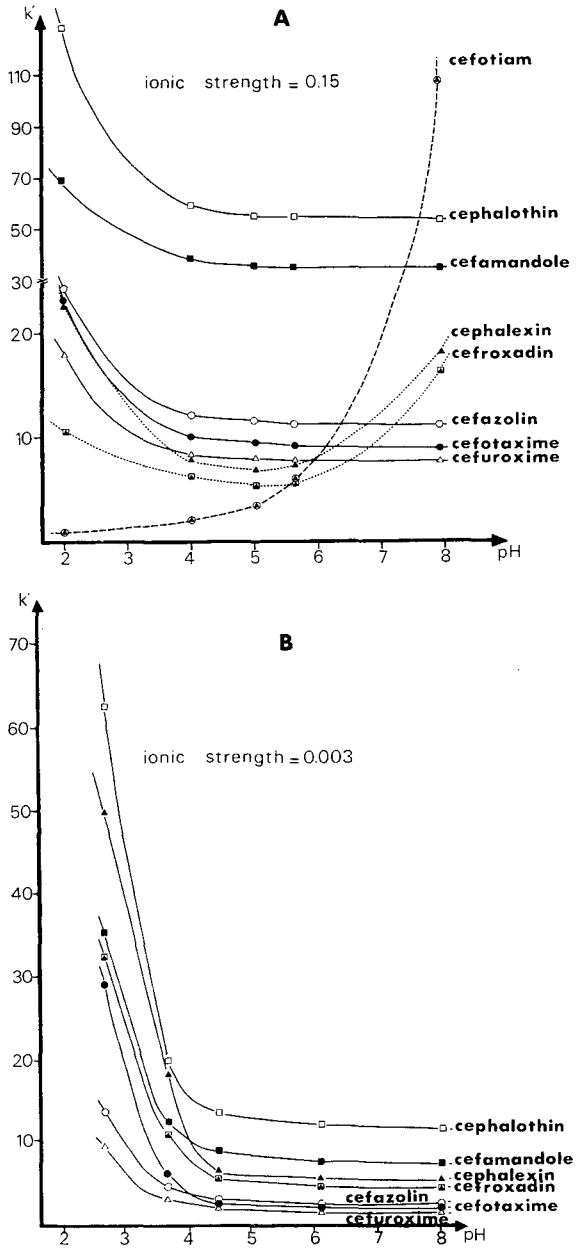


Fig. 2. Plots of capacity factors versus mobile phase pH. Column: LiChrosorb RP-18, 10 μm. Methanol content in the mobile phase: 15% (v/v).

The pH was increased at constant ionic strength (I = 0.15). The k'—pH profiles obtained (Fig. 2A) reflect the ionization of the compounds, as previously reported for some cephalosporins [10]. This indicates that the retention occurs essentially by way of solvophobic mechanisms at the studied ionic strength. As the pH increases, the decrease in retention is due to gradual formation of

anionic or zwitterion compounds, which are less retained on the hydrocarbonaceous surface than neutral or cationic compounds, respectively. A minimum retention is observed at the isoelectric point for the two amphoteric compounds cephalexin and cefroxadin where they have two opposite charges. This does not appear for cefotiam and cefotaxime, probably because their pK_a are too close. Cefotiam, which has two basic functions, behaved as a basic compound, in spite of its carboxylic group: its retention gradually increased with pH.

When pH was increased at a lower ionic strength (I = 0.003), the retention decreased and then tended to become constant for all investigated cephalosporins (Fig. 2B). The k'—pH profiles obtained for cephalexin and cefroxadin did not exhibit a minimum at a pH corresponding to their isoelectric point. This may be related to the fact that retention occurs by way of both solvophobic and silanophilic interactions at this low ionic strength.

Comparison of reversed-phase packing materials

The retention of cefuroxime, cefazolin, cephalexin, and cefotaxime was examined on 5 μm LiChrosorb RP-18 and 5 μm Nucleosil C_{18} with the mobile phase methanol—water—H_2SO_4 (1.8 M) (25:75:0.2, v/v). The results are shown in Table II. The retention of cephalexin, which is present in its protonated form, is the most modified by the nature of the solid phase. The retentions of cefuroxime and cefazolin, which are predominantly present in their neutral form, are similar on both packing materials. The elution order on μBondapak C_{18} was similar to that obtained on Nucleosil C_{18}. The different behaviour of the cephalosporins cannot be explained on the basis of a single retention mechanism. Such selectivity differences between reversed-phase materials were reported by Melander et al. [16]. They were attributed to silanophilic interactions. This result thus supports the hypothesis that silanophilic interactions may be partly responsible for the retention of cephalosporins having an amino group when acidic eluents of low ionic strength are used. LiChrosorb RP-18, which may have more residual silanol groups than the two other solid phases, was shown to give more peak tailing than μBondapak C_{18} [17] for protonated amines. This was not observed for cephalosporins.

TABLE II

RETENTION (k' VALUES) OF SOME CEPHALOSPORINS ON TWO PACKING MATERIALS

Eluent: methanol—water—H_2SO_4 (1.8 M) (25:75:0.2). Flow-rate: 1.2 ml/min. Ambient temperature.

Cephalosporin	Packing material	
	5-μm Nucleosil C_{18}	5-μm Lichrosorb RP-18
Cefuroxime	6.9	6.4
Cefazolin	7.9	7.6
Cephalexin	3.9	12.7
Cefotaxime	4.1	9.0

Determination of the investigated cephalosporins in biological fluids

Interactions with residual silanol groups have previously been shown to be useful to obtain an adequate selectivity for the separation of peptides [13]. A good retention is obtained with mobile phases of low pH and low ionic strength, not only for amphoteric cephalosporins (with the exception of cefotiam) but also for acidic ones. Silanophilic interactions may consequently also be useful for the determination of cephalosporins in biological fluids with closely related mobile phases, assuming that the specific separation of the cephalosporin from endogenous compounds will be facilitated if one selects a mobile phase that allows a good retention of the cephalosporin. An acidic eluent containing only a low concentration of H_2SO_4 was used to chromatograph the cephalosporins. When the mobile phase methanol—water—H_2SO_4 (1.8 *M*) (18:82:0.2, v/v) was used with 5 μm LiChrosorb RP-18, cefroxadin was the least retained of the investigated cephalosporins, and it was well separated from endogenous plasma and urine components, as illustrated in Fig. 3. LiChrosorb RP-18, 5 μm, was chosen as the packing material instead of the 10-μm one, in order to obtain better column efficiency. The methanol content in the above-mentioned mobile phase was increased, in order to decrease the retention of the other cephalosporins. A convenient separation could be obtained for each of the studied cephalosporins. The different methanol contents, which afford a compromise between adequate separation and rapid analysis, are indicated in Table III. Cefsulodin, the most polar compound, was not sufficiently retained with such conditions to obtain a separation allowing good sensitivities for the determination in plasma and urine.

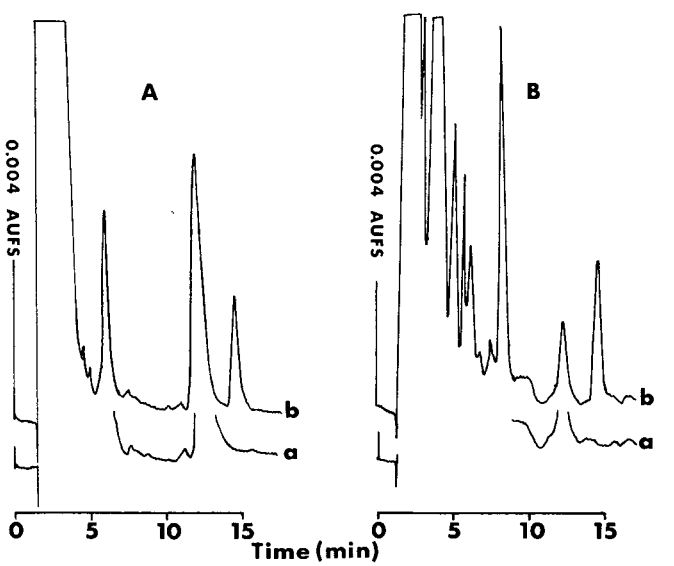


Fig. 3. (A) Chromatograms of blank plasma (a) and plasma containing 2 μg/ml cefroxadin (b); injection volume = 20 μl. (B) Chromatograms of blank urine (a) and urine containing 20 μg/ml cefroxadin (b); injection volume = 20 μl. Column 15 cm × 4.7 mm LiChrosorb RP-18, 5 μm; mobile phase, methanol—water—H_2SO_4 (1.8 *M*) (18:82:0.2, v/v); ambient temperature; flow-rate, 1.2 ml/min; UV detection at 254 nm.

TABLE III

METHANOL CONTENT USED FOR THE DETERMINATION OF CEPHALOSPORINS IN PLASMA AND URINE WITH A MOBILE PHASE OF METHANOL—WATER CONTAINING 0.2% (v/v) OF H_2SO_4 (1.8 *M*)

Column: 5 μm LiChrosorb RP-18. Flow-rate: 1.2 ml/min. Ambient temperature.

	Methanol content (%)
Desacetylcefotaxime	15
Cefroxadin	18
Cefuroxime	20*
Cefazolin	21.5
Cefotaxime	25
Cephalexin	28
Cefamandole	30*
Cephalothin	36

*The methanol contents are 19% for cefuroxime and 28% for cefamandole for the determination of these compounds in urine.

When the eluents described in Table III were used, cephalosporins could also be determined in human milk and bile, after appropriate dilution of the sample, as described above.

A convenient separation for cefotiam, which is retained longer on reversed-phase material in alkaline medium, was obtained with the alkaline mobile phase NaH_2PO_4 (1.4×10^{-3} *M*) and Na_2HPO_4 (2.5×10^{-2} *M*)—methanol (76:24, v/v), as previously reported [18]. This method was used for pharmacokinetic studies with sensitivity limits of 0.2 μg/ml of plasma and 5 μg/ml of urine. The method was also found adequate for the determination of the amphoteric compounds cephalexin and cefroxadin in plasma and urine with slightly lower methanol contents than for cefotiam.

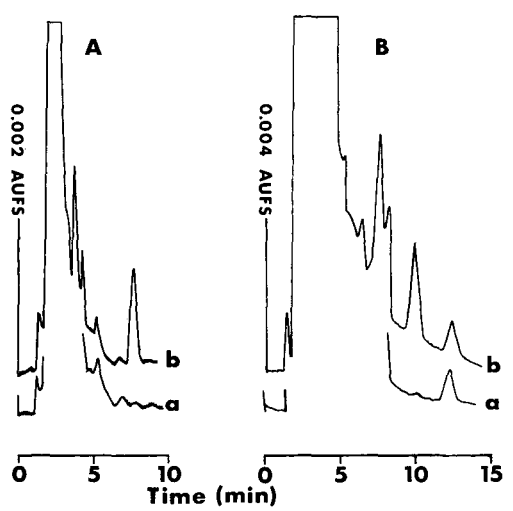


Fig. 4. (A) Chromatograms of blank plasma (a) and plasma containing 1 μg/ml cephalothin (b); injection volume = 30 μl. (B) Chromatograms of blank urine (a) and urine containing 20 μg/ml cephalothin (b); injection volume = 10 μl. Column 25 cm × 4.7 mm, μBondapak C_{18}, 10 μm; mobile phase, methanol—water—H_2SO_4 (1.8 *M*) (28:72:0.2, v/v, for plasma and 26:74:0.2, v/v, for urine); temperature, 35°C; flow-rate, 2 ml/min; UV detection at 254 nm.

Such separations were also found convenient on 10-μm μBondapak C_{18}, the retention times being generally shorter than on 5-μm LiChrosorb RP-18. The methanol contents in the mobile phase were also lower. An example of separation is given for cephalothin in Fig. 4. Cephalothin is bound to serum proteins to the extent of 65—79% [19] and the recovery of the drug was small (about 35%) after precipitation of plasma proteins with TCA. Therefore, proteins were precipitated with methanol for this drug: 150 μl of plasma, 150 μl of methanol, 15 μl of standard solution and 30 μl of H_2SO_4 (6 *M*) were mixed and centrifuged; 30 μl of the supernatant were injected. The recovery of cephalothin was then 65 ± 4%, as compared with that obtained upon direct injection of an aqueous solution.

Application: determination of ceftizoxime in human plasma and urine

Ceftizoxime is a new parenteral cephalosporin that presents structural similarities to cefotaxime (Table I). Consequently, an acidic mobile phase as mentioned above should be adequate to obtain a good retention of this compound.

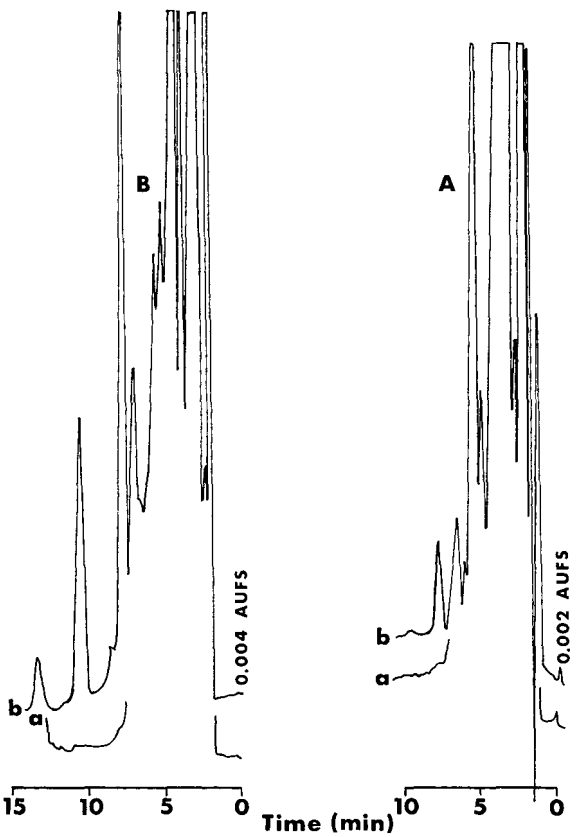


Fig. 5. (A) Chromatograms of blank plasma (a) and plasma containing 0.5 μg/ml ceftizoxime (b); injection volume = 20 μl. (B) Chromatograms of blank urine (a) and urine containing 38 μg/ml ceftizoxime (b); injection volume = 10 μl. Column 25 cm × 4.7 mm, μBondapak C_{18}, 10 μm; mobile phase, methanol—water—H_2SO_4 (1.8 *M*) (15:85:0.2, v/v); temperature, 35°C for plasma, ambient temperature for urine; flow-rate, 2 ml/min; UV detection at 254 nm.

TABLE IV

EVALUATION OF ASSAY REPRODUCIBILITY AND ACCURACY FOR CEFTIZOXIME

Fluid	Concentration added (μg/ml)	Mean* concentration found (μg/ml)	Coefficient of variation (%)
Plasma	0.20	0.20	4.8
	0.50	0.51	4.8
	5.0	4.9	2.0
	10.0	10.0	0.7
	100	98.5	1.8
Urine	25.0	25.0	6.0
	2000	1997	4.9

*Mean of six replicates.

A convenient separation from endogenous compounds of plasma and urine was obtained with the eluent methanol—water—H_2SO_4 (1.8 *M*) (15:85:0.2, v/v), as exhibited in Fig. 5. Plasma proteins were precipitated with TCA, as ceftizoxime is only 31% bound to proteins [20]. The mean recovery of ceftizoxime from plasma was 89 ± 2% in the 0.5—25 μg/ml range. Calibration curves were obtained by plotting peak height (in the range 0.2—2 μg/ml of plasma) or peak area (in the range 0.5—100 μg/ml of plasma and 25—2000 μg/ml of urine) against concentration of ceftizoxime on log—log graphs. The correlation coefficients were better than 0.9990 in all cases. Results of replicate analyses of plasma and urine samples are presented in Table IV. Concentrations as low as 0.2 μg/ml of plasma and 25 μg/ml of urine can be determined with good reproducibility and accuracy.

In routine analysis, more than 500 injections could be performed with the same column. As the column ages, the methanol content in the mobile phase has to be slightly decreased. With such chromatographic conditions, cefroxadin exhibits the same retention time as ceftizoxime, and it can be determined in plasma and urine with the method described for ceftizoxime without modification of the methanol content in the mobile phase. The retention time of cefroxadin is 8 min instead of 15 min on LiChrosorb RP-18.

CONCLUSION

Most cephalosporins are strongly retained on octadecylsilyl stationary phase with an acidic eluent of low ionic strength. It was shown that, with an eluent containing 0.2% (v/v) of H_2SO_4 (1.8 *M*) and various methanol contents, they can be determined in biological fluids after a simple and rapid sample preparation: precipitation of plasma proteins or urine dilution. Short retention times can be obtained when using μBondapak C_{18} material.

The method was checked for ceftizoxime. It allows adequate sensitivity for pharmacokinetic studies.

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DETERMINATION OF SPARSOMYCIN IN PLASMA AND URINE OF THE DOG BY MEANS OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FIRST PHARMACOKINETIC RESULTS

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SUMMARY

Sparsomycin — an antibiotic with marked cytostatic activity — was the subject of a clinical Phase I study in 1964. The structure of sparsomycin was elucidated in 1970 and its first total synthesis was reported in 1981. Here we describe a sensitive high-performance liquid chromatographic method of determination. The detection limit was found to be 10 ng/ml of plasma and 20 ng/ml of urine. With this procedure sparsomycin and isosparsomycin can readily be separated.

In addition, we performed a first pharmacokinetic study in the dog and found a half-life time $t_{1/2}$ β of 1.1 h. Only 25% of the administered dose could be recovered in the urine as sparsomycin.

We consider that by now many prerequisites for further preclinical studies have been achieved, and the results of these studies will determine whether sparsomycin deserves reintroduction into clinical use.

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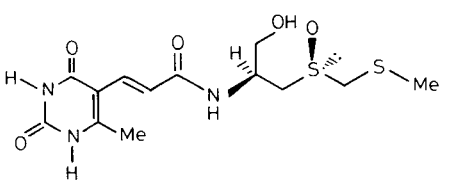
INTRODUCTION

Sparsomycin, an antibiotic, has been known since the early sixties as an in vitro and in vivo protein-synthesis inhibitor [1—5]. It has been found to block peptide-bond formation and thus to prevent the transfer of amino acids from RNA to the nascent polypeptide chains [3]. In addition, sparsomycin has shown cytostatic activity in a number of in vivo tumor systems [1, 6] as well as activity in a cell tissue culture of KB human epidermoid carcinoma [1]. Therefore, it was introduced in 1964 in a clinical Phase I study. Two of the five patients of this study developed an ocular toxicity, probably caused by sparsomycin, and so this Phase I study was stopped prematurely [6, 7].

Until recently, sparsomycin was only available from natural sources; it was isolated as a fermentation product of *Streptomyces sparsogenes* [8]. In 1970 Wiley and MacKeller [9] succeeded in elucidating the structure of sparsomycin and in 1981 two of us [10, 11] reported its synthesis (Fig. 1). In the course of its synthesis it was noticed, that under the influence of UV light the alkene double bond isomerises easily to a *cis* configuration, yielding isosparsomycin. In an in vitro clonogenic cell assay with L 1210 cells, sparsomycin was found to be the cytostatic active compound, whereas isosparsomycin was devoid of activity in this assay [12]. These findings are noteworthy, as sparsomycin from natural sources was found to contain appreciable amounts of isosparsomycin.



Sparsomycin

Fig. 1 Structure of sparsomycin.

Knowledge of the mechanism of action of sparsomycin, which is different from that of the cytostatics in common use, together with detailed knowledge of its structure and synthesis, which allows, for example, the synthesis of sparsomycin analogues, and the possibility to obtain highly purified sparsomycin, justify a preclinical reinvestigation of this antibiotic. Such an approach has also been proposed by the authors of the Phase I study [6]. To be able to undertake pharmacokinetic studies of its distribution, metabolism and excretion we report here a new, sensitive high-performance liquid chromatographic (HPLC) method for the determination of sparsomycin. The detection limit of our method is 10 ng/ml of plasma and 20 ng/ml of urine; and the method was applied in a first pharmacokinetic study in the dog. Until now, sparsomycin could only be detected in the microgram range, as by the HPLC method described by Chan et al. [13].

MATERIALS AND METHODS

Sparsomycin concentrations were determined by a two-column HPLC back-

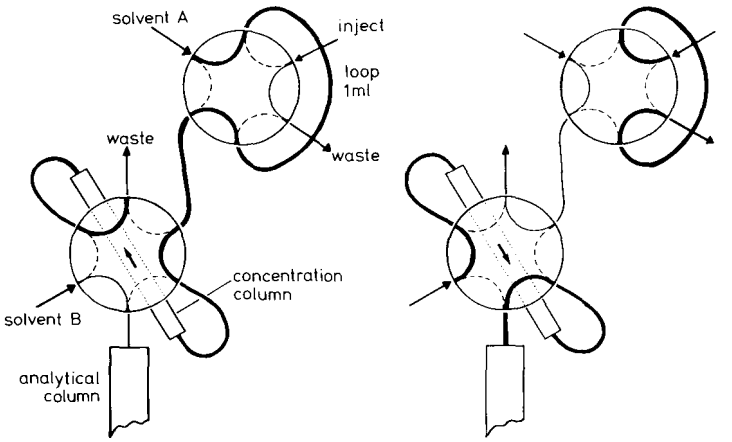


Fig. 2. Left: valves in concentration position. Right: valves in injection position [14].

flush method. By using a concentration column, the samples could be cleaned from interfering peaks and concentrated (Fig. 2) [14].

Equipment

The chromatography equipment consisted of a double head solvent pump for solvent A and solvent B (Orlita, DHP 1515, Bakker & Co., Zwijndrecht, The Netherlands). Two sampling valves (Valco, Houston, TX, U.S.A.) were used as in Fig. 2: left side = concentration position, right side = injection position. The concentration column (5 cm × 3.0 mm I.D.) was filled with LiChrosorb RP-8, 10 μm; the analytical column (15 cm × 4.6 mm I.D.) was packed with reversed-phase material Cp^{tm} Spher C8, spherical particle size 8 μm (both from Chrompack, Middelburg, The Netherlands). A spectrophotometric detector was used at a wavelength of 300 nm (Spectroflow monitor SF 770 + UV monochromator GM 770, Schoeffel, The Netherlands).

Solvents and concentration method

Solvent A, demineralized water, was flushed via the injection loop through the concentration column with a flow-rate of 2 ml/min. Plasma samples were flushed with 9 ml of water, urine samples with 11 ml of water. After 4.5 min and 5.5 min, respectively, the concentration column was flushed back with solvent B, methanol—demineralized water (15:85), onto the analytical column with a flow-rate of 1.3—1.5 ml/min.

Drugs

Sparsomycin was synthesized as described earlier [10]; an aqueous solution, which was protected from light, was used for calibration and for the animal study.

Dog

A beagle dog (No. 686), bred by the central animal laboratory of the University of Nijmegen, was kept under anaesthesia for the first 8 h. A continuous infusion (800 ml of 5% glucose) was administered over this period

of time via a catheter placed in the front leg to achieve a higher urine flow. Sparsomycin (0.7 mg/kg body weight) was injected via this catheter as a bolus injection. Plasma samples were drawn from an indwelling catheter in the jugular vein at 5, 15, 30, 45, and 60 min and subsequently each hour into heparinized tubes, which were immediately centrifuged and frozen (-20°C). For the next two days the dog was kept in a metabolic cage, to obtain further spontaneously voided urine samples.

Sample preparation

All samples were kept protected from light.

Plasma. To 1 ml of plasma, stirred on a vortex mixer, 1 ml of 10% trichloroacetic acid in water was added. After centrifugation at 1500 *g* for 5 min, 1.5 ml of the supernatant (protein-free) were transferred into a glass tube; 1 ml of chloroform was added and the resulting mixture was shaken on a vortex mixer. After a second centrifugation at 1500 *g* for 5 min, 1 ml of the upper (aqueous) layer was injected into the sampling loop.

Urine. Samples were diluted with demineralized water and also "cleaned" with chloroform. The early urine samples could be diluted 1 in 5, the late ones only 1 in 2. Samples of 1.5 ml of these dilutions were washed by mixing with 1 ml of chloroform. After centrifugation at 1500 *g* for 5 min, 1 ml of the upper layer was injected into the sampling loop.

Calibration curves. These were constructed by adding known amounts of sparsomycin to plasma or urine and the resulting solutions were treated as described above.

RESULTS

Detection of sparsomycin and isosparsomycin

UV absorption maxima. Sparsomycin shows absorption maxima at 300 nm and 188 nm. At 188 nm the extinction coefficients of isosparsomycin and

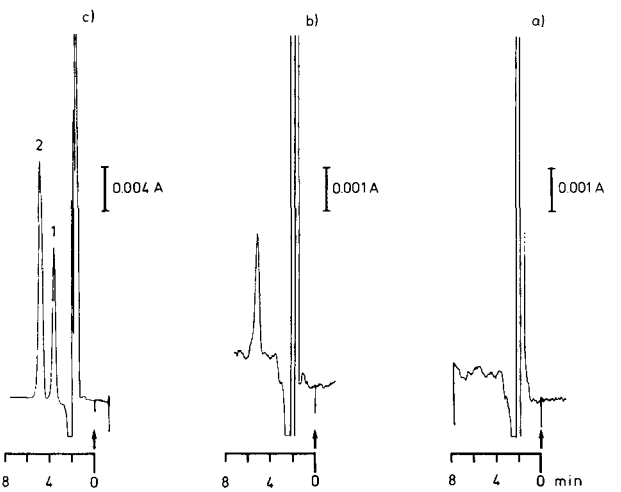


Fig. 3. HPLC of (a) blank plasma, (b) blank plasma spiked with 40 ng/ml sparsomycin, (c) sparsomycin (2) and isosparsomycin (1) in water.

sparsomycin are identical. However, at 300 nm the extinction coefficient of isosparsomycin is about one third that of sparsomycin. Therefore the ratio sparsomycin/isosparsomycin could be determined at 188 nm (see below and Fig. 4).

Chromatogram. Fig. 3 shows HPLC chromatograms detected at a wavelength of 300 nm; the flow rate of solvent B was 1.5 ml/min. In Fig. 3c it can be seen that sparsomycin (peak No. 2) and isosparsomycin (peak No. 1) can easily be separated by the applied procedure. The retention times are 4.8 min and 3.6 min, respectively. Chromatogram b is from a plasma sample spiked with 40 ng/ml sparsomycin. In comparison, chromatogram a is from blank plasma.

Detection limit. The lowest concentrations of sparsomycin that could be determined with the described method were 10 ng/ml of plasma and 20 ng/ml of urine; with urine samples there were more interfering peaks in the chromatogram.

Stability of sparsomycin during the work-up procedure

Towards light. To study the influence of light upon sparsomycin, we exposed an aqueous solution of sparsomycin (4 μg/ml) in a glass container, covered with parafilm, to normal laboratory light conditions. Another sample of the same solution was stored, protected from light, in the refrigerator. The concentrations of sparsomycin and isosparsomycin were determined daily and compared to the reference solution. In Fig. 4 the light-induced isomerisation of sparsomycin (*trans*) into isosparsomycin (*cis*) as a function of time is shown. Under the described conditions 50% of isosparsomycin was formed from sparsomycin in 3.6 days.

Towards thawing and freezing. To determine the stability of sparsomycin in frozen plasma, samples were spiked with 200 ng/ml and kept frozen for two weeks. Samples were then thawed and refrozen one to three times. After each thawing HPLC analysis was done in triplicate and compared with freshly

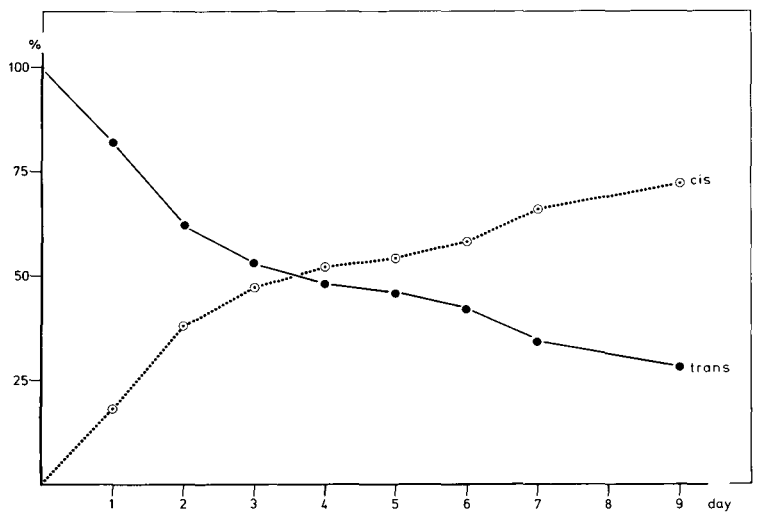


Fig. 4. Light-induced isomerisation of sparsomycin (*trans*) into isosparsomycin (*cis*); 50% isomerisation occurred within 3.6 days.

spiked plasma samples. The amount of sparsomycin determined in the samples that had been kept frozen was 92 ± 5% of the amount of sparsomycin determined in the samples that had been freshly spiked. No relation could be detected between the number of times that samples were frozen and the degradation of sparsomycin.

At room temperature. Samples of an aqueous sparsomycin solution, which had been kept protected from light for three days, showed no decrease in concentration of sparsomycin.

Recovery and reproducibility

The recovery of sparsomycin added to plasma in the concentration range 10–3500 ng/ml was found to be 70 ± 4% S.D.; in urine the recovery was 100 ± 2% S.D. (triplicate analyses). As expected from its polar structure, sparsomycin is not extracted into the chloroform phase during the clean-up procedure for plasma and urine. Repeated injections of samples with the same concentration showed a change in peak hight of ± 2% S.D. in the concentration range 10–3500 ng/ml.

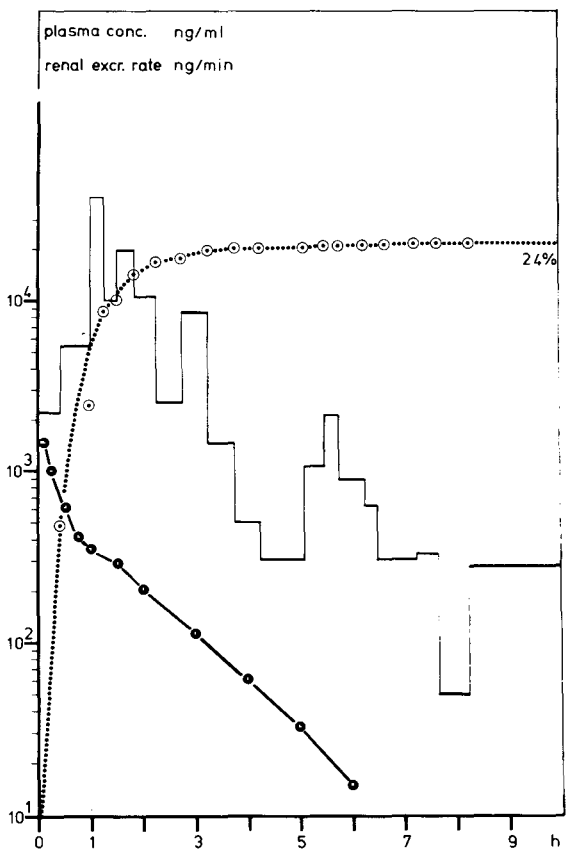


Fig. 5. (●——●), plasma concentration versus time after 0.7 mg/kg sparsomycin as intravenous bolus injection in the dog. (——), renal excretion rate per urine portion. (⊙···⊙), cumulative renal excretion of sparsomycin.

Results of the pharmacokinetic study in the dog

Plasma concentrations. In Fig. 5 the plasma concentrations of sparsomycin versus time are shown after an intravenous bolus injection of 0.7 mg/kg body weight in the dog. Two phases can be discriminated: (1) 5 min to 45 min with a half-life time $t_{1/2}$ α of 0.13 h; (2) 45 min to 6 h with a half-life time $t_{1/2}$ β of 1.1 h.

Renal excretion rate and recovery in urine. In Fig. 5 renal excretion rates per urine portion versus time are shown. The cumulative excretion of sparsomycin (dotted line) accounts for only 24% of the administered dose in the period of 10 h, as shown. (A total of 25% could be recovered after 24 h of urine collection.)

Further pharmacokinetic results. Based on the two-compartment model, the following parameters from the pharmacokinetic profile of the dog have been calculated: volume of distribution of the central compartment = 4.7 l; area under the plasma concentration curve = 1.4 mg h/l; total body clearance = 117 ml/min; renal clearance, based on total plasma concentration = 48 ml/min.

In Fig. 6 the flow dependency of the renal clearance of sparsomycin is shown in the range 0.2—4.9 ml/min urine flow (r = 0.87).

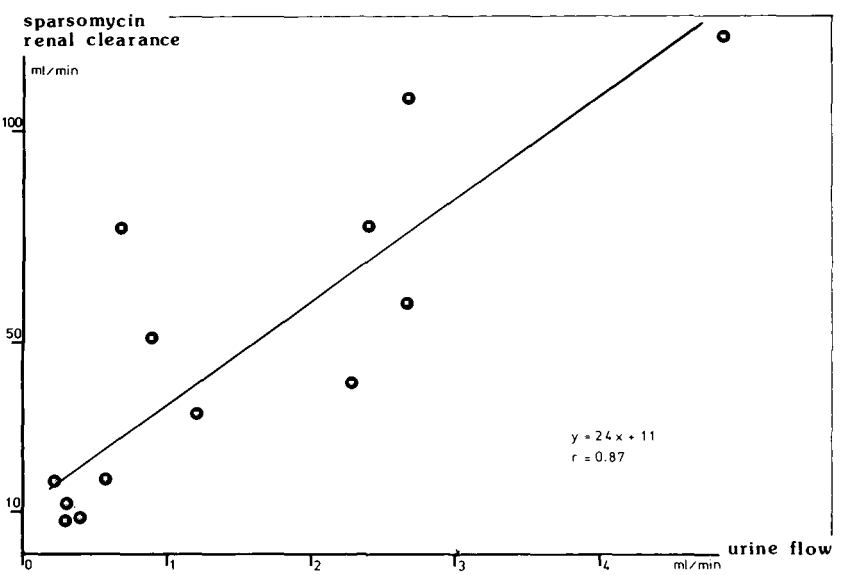


Fig. 6. Renal clearance of sparsomycin in the dog in relation to urine flow.

Clinical observations in the dog

Based on the reported side-effects of sparsomycin in animal studies [6], this pharmacokinetic study in the dog was accompanied by blood chemistry studies, in search of renal, hepatic and bone marrow toxicity. The following changes were observed:

(1) A drop of total serum protein from 65 to 50 g/l on the second day after sparsomycin administration. After one week serum protein was back to 58 g/l.

(2) A marked drop in the thrombocyte count from 300 $\times$ 10^9 per l towards 139 $\times$ 10^9 per l at the first day after sparsomycin administration and a recovery within a week.

(3) Absence of hepatic and renal toxicity as controlled by means of alkaline phosphatase, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), blood urea nitrogen (BUN) and serum creatinine.

(4) The dog showed a high degree of tiredness during the first week after the administration of sparsomycin.

DISCUSSION

Method of determination

As we have shown, the described method of determination is sensitive enough to be used in further preclinical pharmacokinetic studies. In addition, this method may also be of value on a preparative scale, to separate sparsomycin from isosparsomycin in the reaction mixture of the synthesis of sparsomycin.

Previously used doses in patients

In Table I we list the doses of sparsomycin that have been used in patients. Two patients, nos. 2 and 3, of the Phase I study developed ocular toxicity after 13 and 15 consecutive days of treatment with sparsomycin (the natural fermentation product). The pharmacokinetics of its distribution, metabolism and excretion after this consecutive way of administration could differ markedly from the usual single-dose administration used in most of the test systems.

TABLE I

PREVIOUSLY USED IN VIVO DOSAGES OF SPARSOMYCIN

Subjects studied	Total dose (mg/kg)	Reference
11 of 20 different tumors in animals significantly inhibited	0.25—1.0	1962 [9]
Pat. 1 10 consecutive days of administration	0.085	1964 [3]
Pat. 2 13 consecutive days of administration	0.24	Ocular toxicity [3, 7]
Pat. 3 15 consecutive days of administration	0.15	Ocular toxicity [3, 7]
Pat. 4 10 consecutive days of administration	0.136	[3]
Pat. 5 17 consecutive days of administration	0.154	[3]
Dog 868 one intravenous bolus	0.7	Present paper

Side-effects from sparsomycin, and its pharmacokinetics in the dog

The retinopathy [6, 7] is of course the most serious side-effect of sparsomycin that needs to be further evaluated. Furthermore, liver function impairment is a likely result of sparsomycin treatment; elevation of SGOT and/or alkaline phosphatase was reported in four of the five patients after treatment with sparsomycin had started [6].

Our first study in the dog was designed to obtain a pharmacokinetic profile of sparsomycin. It was found that sparsomycin has a very short half-life time of 1.1 h; and only 25% of the administered dose could be recovered in the urine as

the parent compound. Consequently, there may be other routes of excretion, and/or metabolites of sparsomycin may be formed which can not yet be detected by our procedure. Although no major emphasis was put on clinical issues in this study, hepatic toxicity was not observed in the dog.

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Note

Isotope cluster chromatography to locate isotopically labeled species

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The metabolic fate and pharmacokinetics of drugs are conveniently studied by gas chromatography—mass spectrometry (GC—MS). However, because body fluid samples are complex mixtures, it is frequently difficult to confidently identify metabolites versus non-metabolites. Many investigators have resolved this problem by the use of stable-isotope labeled analogues in conjunction with the non-labeled drug [1]. The incorporation of ^{2}H, ^{13}C, ^{15}N or ^{18}O into the parent drug and subsequently into its metabolites reliably distinguishes products derived from the drug from normal constituents of body fluids. No radioactivity is involved, and there is no break in the administration of the drug, so risks to the patient or experimental animal are minimal. If a mixture of stable isotope labeled and non-labeled drug is administered, the mass spectra derived from such a mixture, or from metabolites of such a mixture, will display characteristic isotopic doublets, unambiguously identifying compounds which originated from the drug.

Deuterium, because it is relatively inexpensive and easy to incorporate into molecules, has been the most widely used of the stable isotopes. Deuterium labeled analogues have aided in studies of the metabolism of propoxyphene [2], phencyclidine [3], warfarin [4], pyrazole [5], and alprenolol [6], to name a few. Compounds discovered in blood or urine samples which display mass spectra with the appropriately spaced isotope doublets are presumed to have come from the drug. The GC—MS analysis of these samples involves the scan-by-scan searching of hundreds of mass spectra, seeking those which show the characteristic pattern of ions.

Recently, we have introduced a computer technique which would be of great assistance in such a search. Isotope cluster chromatography [7] was designed to search GC—MS data sets for mass spectra displaying the isotope cluster patterns resulting from chlorine and/or bromine atoms. These elements have two

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abundant, naturally-occurring stable isotopes; and their mass spectra are very distinctive. We have now expanded that computer program to allow the investigator to specify any patterns of ions for which to search. We report here several potential applications of isotope cluster chromatography to these artificial isotope patterns. In addition to drug metabolism studies, the technique can be used in experiments in which a mixture of stable isotope labeled and non-labeled derivatizing reagents is used. In this case, the computer assists in locating the spectra of compounds which contain a particular functional group which has been derivatized by distinguishing such compounds from others which do not display the isotopic doublet.

MATERIALS AND METHODS

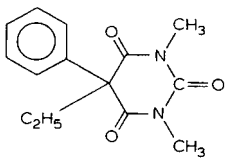
Unless specifically stated, all solvents and reagents were obtained from commercial sources and were used without further purification. [$^2H_4$]Methanol (99.5% isotopically pure) was purchased from Alfa-Ventron (Beverly, MA, U.S.A.).

Methanolic hydrochloric acid was prepared by bubbling dry hydrogen chloride gas through methanol. The molarity was determined by titration against standard sodium hydroxide solution, and was adjusted to approximately 3 *M* hydrochloric acid. The dimethylphenobarbital, stable-isotope labeled dimethylphenobarbital, and spiked serum samples were generous gifts of Dr. Agnes Van Langenhove of M.I.T. (Boston, MA, U.S.A.).

Methyl esters were prepared by dissolving the corresponding carboxylic acid in 200—400 μl of [$^2H_4$]methanol and adding an equivalent amount of 3 *M* methanolic hydrochloric acid. The reaction was conducted at room temperature for 45 min. Excess reagents were removed under a stream of dry nitrogen.

GC—MS analyses were performed on a Hewlett-Packard 5985B system. Chromatography was on a 30 m × 0.32 mm fused silica capillary, wall coated with Durabond DB-1 (J&W Scientific, Orangevale, CA, U.S.A.). The splitless injector and transfer lines to the mass spectrometer were maintained at or above 250°C. Helium at a flow-rate of 1—2 ml/min was used as carrier gas. The column conditions were as indicated in figure legends. The mass spectrometer ion source was at 200°C, and spectra were continuously scanned approximately every 2 sec. The electron energy was 70 eV.

Computer programs were written in BASIC with FORTRAN subroutines; and listings are available from the author. The user enters the relative intensities of the isotope pattern for which he/she is searching. An unknown mass spectrum is broken down into a series of parts, each containing exactly as many masses as are present in the known isotope pattern. Each of these parts is then compared to the known cluster to see if it is similar. A reverse library search-type algorithm is used to determine similarity, as in our previous work [7]. A total score for the unknown mass spectrum is calculated as a weighted sum of all of the similarity indices. When each spectrum in a GC—MS data set has been searched and assigned a score, a plot of score versus scan number produces a chromatogram such as that in Fig. 2a or Fig. 3a.



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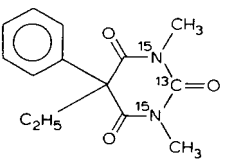

II

RESULTS AND DISCUSSION

The mass spectrum of a mixture of dimethylphenobarbital (I) and dimethyl-[^{13}C,$^{15}N_2$]phenobarbital (II) is shown in Fig. 1. The ion doublets separated by 3 atomic mass units (a.m.u.) at *m/z* 232—235, *m/z* 245—248, and *m/z* 260—263 (shown in boldface type in Fig. 1) are a result of a nearly equimolar mixture of the non-labeled and stable-isotope labeled compounds [8]. This pattern will appear in the mass spectra of the parent drug and any metabolites which retain the stable-isotope label, so it is this pattern which the computer is asked to seek. The user can input any isotope cluster pattern of up to twenty masses at any relative intensities; but in this case, the investigator would specify a doublet of about equal intensities separated by 3 a.m.u. The computer then conducts a scan-by-scan search of any GC—MS data set for the presence of such clusters, just as in the previous description of isotope cluster chromatography [7]. A score is assigned to each mass spectrum reflecting the probability that such a cluster occurs in the spectrum and the abundance of the ions in potential clusters. A plot of these scores versus scan number produces an isotope cluster chromatogram as shown in Fig. 2a. A serum sample spiked with the mixture of labeled and non-labeled dimethylphenobarbitals was chromato-

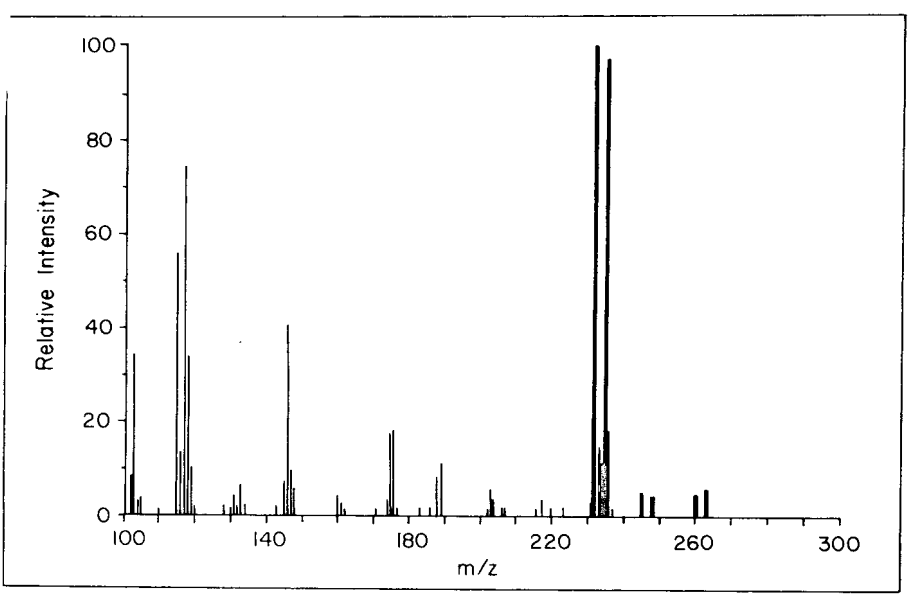

Fig. 1. Mass spectrum of an approximately equimolar mixture of I and II. The ion doublets at *m/z* 232—235, 245—248, and 260—263 are diagnostic (see text) and are highlighted.

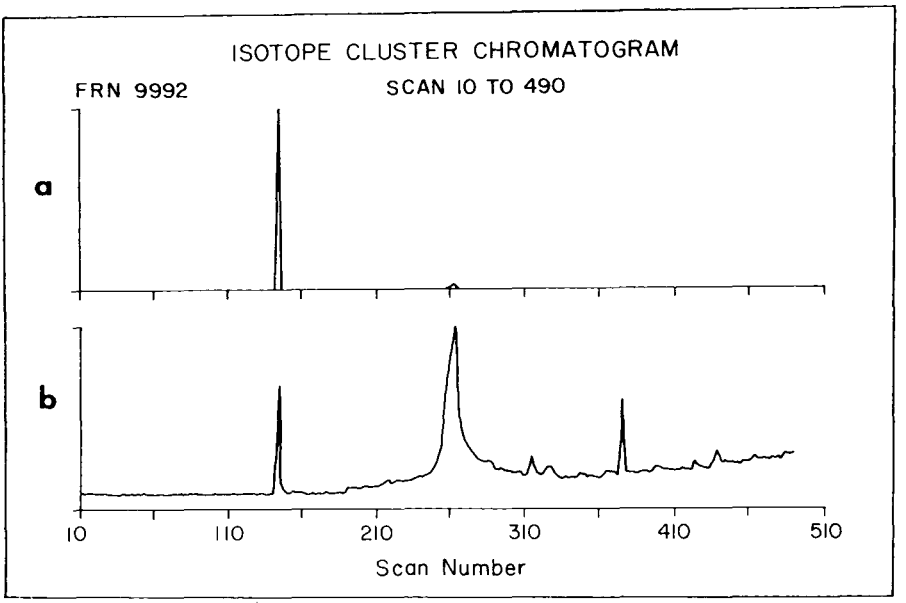

Fig. 2. (a) Isotope cluster chromatogram and (b) total ion profile for the GC—MS analysis of a serum extract spiked with I and II. The GC oven temperature was held at 120°C for 2 min, and then linearly programmed at 10°C/min to 330°C.

graphed and searched. Only one peak, around scan 145, shows a maximum in the isotope cluster chromatogram, indicating an intense doublet of the type sought. The peak corresponds to the expected retention time of dimethyl phenobarbital. If metabolites had been present and had contained the isotope doublet, they would also have been found. Mass chromatograms [9], a useful alternative data reduction method, can only be used in cases where the mass spectrum or structure (and hence, a characteristic ion) is known. The isotope cluster chromatogram locates all metabolites which display the appropriate isotope pattern, regardless of their structure.

Drug metabolism is not the only area of applicability of such a technique. Nau and Riordan [10] have used $H_2{}^{18}O$ to probe the mechanisms and active sites of enzymes. Isotope clusters produced by the incorporation of ^{18}O into peptides could also be located by computer.

There are two phenomena which could possibly distort the isotope patterns observed in the mass spectra of drug metabolites. Mimura and Baba [11] have observed that in the metabolism of the drug, paeonol, an isotope effect causes the non-labeled and stable-isotope labeled drugs to be metabolized at different rates. Thus the isotope cluster of the metabolite is no longer exactly the same as in the original dose mixture. Such an effect is occasionally observed with deuterium labeled compounds, but rarely with ^{13}C, ^{15}N, or ^{18}O.

A second concern is that the stable-isotope labeled and non-labeled compounds may be at least partially separated chromatographically. As a result, in any mass spectrum, the isotope pattern observed would also reflect this separation. Again, the effect is most pronounced with deuterium labeling, particularly in compounds with several deuterium atoms. In our experience,

working with di- and trideutero compounds, we have noticed a slight distortion of the isotope pattern on the leading and trailing edges of a chromatographic peak; but in the peak center, the isotope ratios are as would be predicted. This is consistent with the observations of other workers [11], and has not imposed a serious limitation on the program thus far.

The time required to generate the isotope cluster chromatogram in Fig. 2a was about 5 min to search 480 mass spectra for isotope clusters. During this time, the computer is doing its calculations without user interaction and can be left unattended. Without this data reduction by computer, the analyst would be faced with the task of looking at each of the 480 mass spectra individually, and assessing in each spectrum the likelihood that isotope clusters are present. Clearly a dramatic savings in time and effort can be realized.

Another area of application of this stable-isotope labeling technique is in the formation of chemical derivatives. Methylation [12] and acetylation [13] using mixtures of non-labeled and stable-isotope labeled reagents have been reported. These derivatives produce mass spectra with characteristic isotope patterns which can then be used to identify compounds containing a reactive functional group or to aid in the determination of fragmentation mechanisms. The isotope cluster chromatography program described above can be used to locate these diagnostic isotope clusters in a GC—MS data set as well.

As a simple example, a mixture of compounds including one carboxylic acid was prepared. When the mixture was methylated with methanolic hydrochloric acid including both [$^1H_4$]methanol and [$^2H_4$]methanol, only those compounds capable of being methylated would incorporate the stable isotope label. In this case, the non-labeled and labeled methanol were in a ratio of

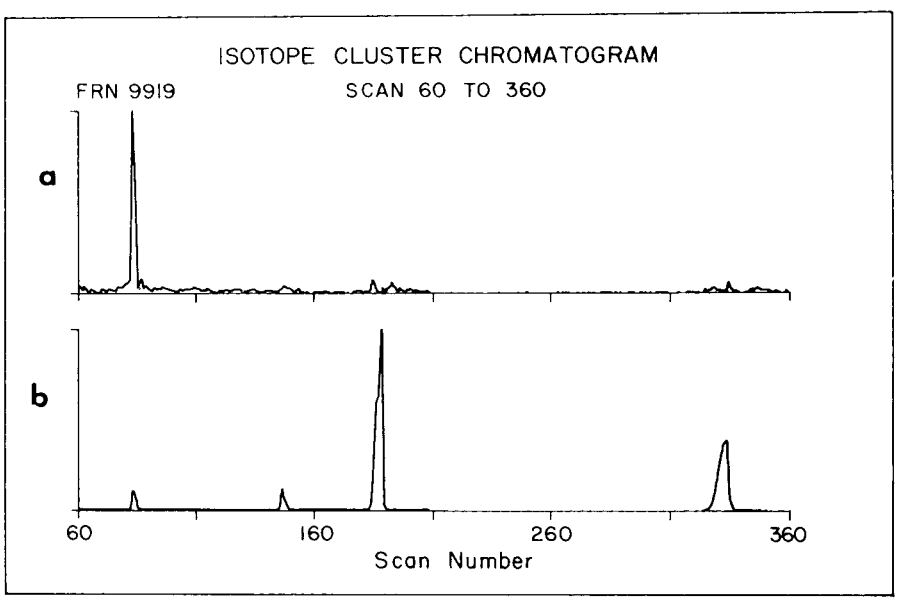

Fig. 3. (a) Isotope cluster chromatogram and (b) total ion profile for the GC—MS analysis of a sample methylated with a mixture of [$^1H_4$]- and [$^2H_4$]methanolic hydrochloric acid. The GC oven temperature was linearly programmed from 100°C to 330°C at 16°C/min.

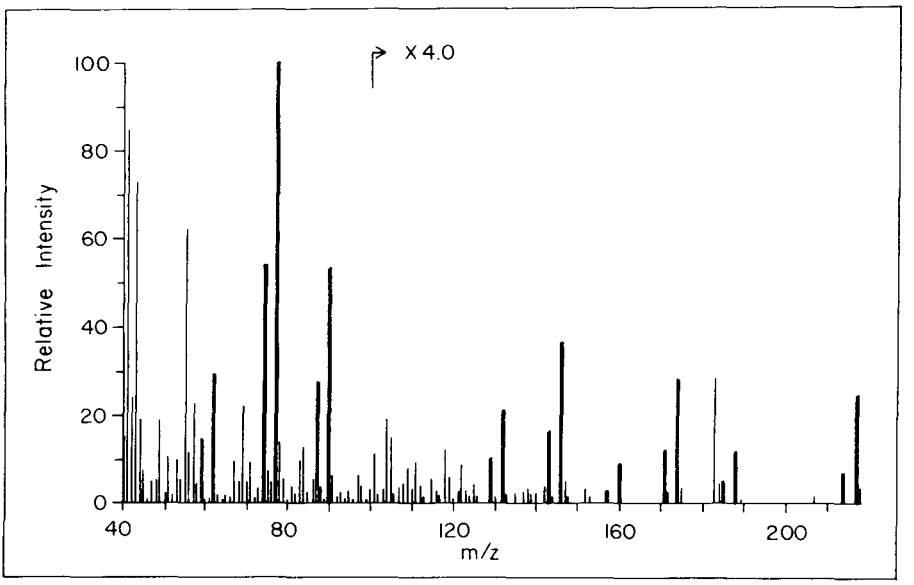

Fig. 4. Mass spectrum of a mixture of [$^1H_3$]- and [$^2H_3$]methyl laurate from scan 83 of Fig. 3. Ion doublets characteristic of the non-label/stable-isotope label (in a ratio of about 1:2) are highlighted.

about 1:2, so compounds which were methylated would show a doublet, separated by 3 a.m.u. in a ratio of about 1:2. This information is given to the computer, which searches the GC—MS data set for the appropriate isotope cluster, and generates the plot shown in Fig. 3a. One peak appears around scan 83 and its mass spectrum is shown in Fig. 4. The isotope doublets are shown in boldface type as before. The spectrum is that of methyl laurate which has been stable-isotope labeled. The mass chromatogram of *m/z* 74 could have been used to locate the methyl laurate as well, since the *m/z* 74 ion is characteristic of saturated fatty acid methyl esters [14]. However, isotope cluster chromatography is a more general technique since it could also locate methyl esters of compounds which do not display a prominent ion at *m/z* 74, such as benzoic acid derivatives.

In summary, the technique of isotope cluster chromatography has been expanded to include a search for any isotope cluster specified by the user. The products of metabolic or chemical reactions can be quickly located by computer, allowing the investigator to concentrate his/her time on those compounds which have incorporated a stable-isotope label. Although the data presented were all collected as positive ions in the electron ionization mode, the technique would work equally well for chemical ionization, and for negative ion mass spectra, provided only that the isotope patterns remain undistorted.

ACKNOWLEDGEMENTS

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Note

Use of O-((—)-menthyl)-N,N′-diisopropylisourea for the preparation of diastereomeric menthyl esters for the chromatographic resolution of enantiomeric carboxylic acids

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One of the techniques commonly employed in the separation of enantiomers involves the preparation of diastereomeric derivatives using an optically pure chiral derivatizing reagent. The diastereomers are then separated by various chromatographic means, and the resulting elution patterns reflect the nature of the underivatized material. The purpose of the study presented here was to examine the usefulness of O-((—)-menthyl)-N,N′-diisopropylisourea as a reagent for preparing (—)-menthyl esters of enantiomeric carboxylic acids.

Previously used methods for the preparation of menthyl esters include acid catalyzed esterification using (—)-menthol [1]. This method requires the use of dry hydrogen chloride gas and a very large excess of (—)-menthol. Menthyl esters have also been prepared by first converting the acid to the acid chloride by refluxing with thionyl chloride [2] or oxalyl chloride [3], followed by treatment of the acid chloride with (—)-menthol. While all of these methods produce the desired end-product, namely diastereomeric (—)-menthyl esters of enantiomeric acids, the derivatization procedures themselves are somewhat involved and require considerable care in the maintenance of anhydrous conditions. Among the proposed advantages of O-((—)-menthyl-N,N′-diisopropylisourea ((—)-MDI) as a chiral derivatizing reagent are the simple techniques involved with its use, the completeness of its reaction with carboxylic acids, and the lack of potential racemization during derivatization.

(—)-MDI is an adduct of (—)-menthol and N,N′-diisopropylcarbodiimide:

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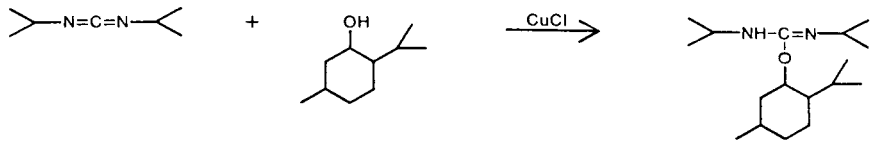
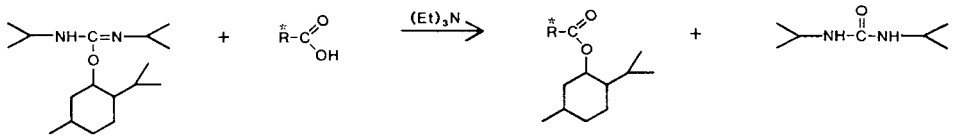


N,N'-diisopropylcarbodiimide (-)-menthol O-((-)-menthyl)-N,N'-diisopropylisourea ((-)-MDI)

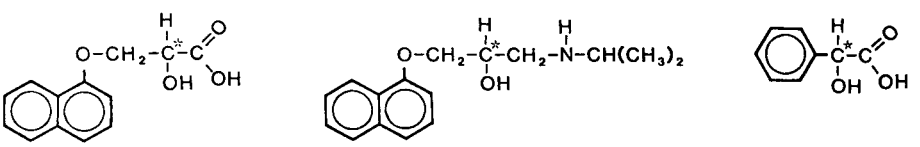
This reaction is catalyzed by monovalent copper. The derivatization reaction produces the two diastereomeric esters and N,N'-diisopropylurea:



(-)-MDI (±) Racemic acid Diastereomeric esters N,N'-diisopropylurea

Tertiary amines catalyze the derivatization; triethylamine was used as the catalyst in these studies.

Naphthoxylactic acid (2-hydroxy-3-(1-naphthoxy)propanoic acid; NLA), a major urinary metabolite of the β-adrenergic antagonist propranolol, and mandelic acid were used as test samples for the (—)-MDI derivatization reagent.



Naphthoxylactic acid (NLA) Propranolol Mandelic acid

Both of these acids have one chiral center. The diastereomeric derivatives were analyzed by capillary gas chromatography (GC) and capillary gas chromatography—mass spectrometry (GC—MS).

MATERIALS AND METHODS

Synthesis of O-((—)-menthyl)-N,N'-diisopropylisourea

(—)-Menthol (7.8 g, 0.050 mol; Mallinckrodt, New York, NY, U.S.A.) and N,N'-diisopropylcarbodiimide (6.3 g, 0.050 mol; Aldrich, Milwaukee, WI, U.S.A.) were dissolved in 25 ml tetrahydrofuran (Aldrich). Copper(I) chloride (10 mg; Fisher, Fair Lawn, NJ, U.S.A.) was added, and the mixture was stirred at room temperature overnight. The mixture was then evaporated in vacuo to 8 ml and chromatographed on a 250-g Al_2O_3 (W200 Basic; Woelm, Eschwege, G.F.R.) column eluting with 250 ml tetrahydrofuran. The tetrahy-

drofuran was removed by evaporation in vacuo, yielding 5.86 g of (−)-MDI (41.6% of theoretical) as a pale yellow liquid. No appreciable decomposition of (−)-MDI was detected four years after synthesis.

Other reagents

(±)-Naphthoxylactic acid was prepared in these laboratories by a procedure analogous to that reported by Nelson and Bartols [4]. Racemic mandelic acid was obtained from Chem Service (Westchester, PA, U.S.A.); (−)-mandelic acid and (+)-mandelic acid were obtained from Aldrich, triethylamine from Eastman Kodak (Rochester, NY, U.S.A.), and ethyl acetate from Fisher. All solvents were ACS reagent grade or better. Ethereal diazomethane was generated from Diazald® (Aldrich).

Equipment

Capillary GC analyses were performed using a Varian 3700 gas chromatograph equipped with a split injector and a flame ionization detector. The column was a Grade A 60 m × 0.25 mm I.D. SP-2100 wall-coated open tubular (WCOT) glass capillary obtained from J. & W. Scientific (Orangeville, CA, U.S.A.). The straightened capillary ends were deactivated with Carbowax 20M.

Capillary GC-MS analyses were performed using the same gas chromatograph and column interfaced through an open-split interface to a Finnigan MAT-212 mass spectrometer equipped with a Spectrosystem SS200 data system.

Derivatization of enantiomeric acids

A 1-mg amount of each sample acid was dissolved in 400 μl of a solution of tetrahydrofuran—triethylamine (9:1, v/v); 10 μl (−)-MDI were added, and the reaction mixture was capped tightly in a vial and heated at 100°C for 16 h. The completeness of reaction was monitored by forming the methyl ester of any unreacted acid by derivatization with diazomethane in diethyl ether followed by capillary GC analysis. No methyl ester peaks were observed after 16 h heating with triethylamine as a catalyst.

Capillary GC—MS resolution of diastereomers

The diastereomeric menthyl naphthoxylactates were resolved under the following conditions: injector temperature 260°C; split ratio 1:350; column temperature programmed from 225°C to 240°C at a rate of 0.2°C/min; average linear velocity 21—22 cm/sec; open-split interface temperature 260°C; line-of-sight temperature 250°C; ion source temperature 240°C; ionization energy 70 eV. The conditions for the resolution of the diastereomeric menthyl mandelates were identical except that the injector temperature was 200°C and the column temperature was programmed from 165°C to 180°C at 0.3°C/min. Both analyses were complete before the final column temperature was reached. During both analyses the mass spectrometer was scanned from 45 to 400 a.m.u. at a rate of 3 sec/decade with an interscan time of 1 sec.

Determination of elution order

Menthyl mandelates. R-(−)-Mandelic acid and *S*-(+)-mandelic acid were

separately derivatized*. Aliquots of the two solutions were mixed in a 4:1 (v/v) ratio (*S*:*R* and *R*:*S*) and analyzed by capillary GC. Elution order was determined based upon the retention times of the separately derivatized *R* and *S* standards and the relative peak intensities of the two mixtures.

Menthyl naphthoxylactates. Because of the unavailability of optically pure *R*- and *S*-naphthoxylactic acid, and because NLA is a major urinary metabolite of propranolol, *R*-naphthoxylactic acid (R-NLA) was extracted from the urine of a rat dosed with S-(—)-propranolol. The sample was a 24-h urine collection from a male Sprague—Dawley rat injected intraperitoneally with 10 mg/kg of *S*-(—)-propranolol·HCl (Ayerst Labs., New York, NY, U.S.A.). The total urine volume was 14 ml. A 2-ml aliquot was adjusted to pH 1.5 with 6 *N* hydrochloric acid and extracted with 2 × 5 ml ethyl acetate. The combined organic phases were evaporated to dryness under a stream of nitrogen; the residue was derivatized using 90 μl tetrahydrofuran—triethylamine (9:1) and 10 μl (—)-MDI. A 6-μl aliquot of this solution was mixed with 6 μl of previously derivatized racemic NLA of comparable concentration. The resulting mixture was analyzed by selected ion monitoring (*m/z* 370, the molecular ion) capillary GC—MS. The elution order was determined by comparing the relative peak intensities of derivatized racemic NLA with and without the added derivatized urinary R-NLA.

RESULTS

(—)-MDI was found to react to completion with carboxylic acids to form menthyl esters after 16 h heating in tetrahydrofuran with triethylamine as a catalyst.

The retention behavior of the diastereomeric menthyl mandelates and menthyl naphthoxylactates from capillary GC—MS analysis is presented in

TABLE I

RETENTION BEHAVIOR OF THE DIASTEREOMERIC MENTHYL NAPHTHOXYLACTATES AND MENTHYL MANDELATES ON AN SP-2100 WCOT CAPILLARY COLUMN

Analysis	Column temperature	Adjusted retention time*, t'_R (min, ± 0.1)	Carboxylic acid enantiomer
Menthyl naphthoxylactates	225—240°C at 0.2°C/min	51.2	*R*-(—)
		53.7	*S*-(+)**
Menthyl mandelates	165—180°C at 0.3°C/min	40.5	*R*-(—)
		42.6	*S*-(+)***

*The elution time of a non-retained compound (butane) was subtracted from the absolute retention times to obtain the adjusted retention times.
**Determined from the data presented in Table II.
***Determined from chromatograms of derivatized optically pure mandelic acid standards.

*In the Cahn—Ingold—Prelog system of chiral notation, used here for the purposes of consistency, D-(—)-mandelic acid has the *R* configuration.

Table I. The retention times presented are adjusted for the void volume of the system. Exemplary chromatograms from the two analyses are presented in Figs. 1 and 2. Fig. 1 is a retrospective single ion plot of the molecular ion (*m/z* 370) of menthyl naphthoxylactate. No molecular ion was observed for the menthyl mandelates; the plot presented in Fig. 2 is a retrospective plot of *m/z* 107, corresponding to the $[(C_6H_5)C(OH)H]+$ fragment of menthyl mandelate. Both sets of diastereomers were resolvable on an SP-2100 WCOT capillary column with slight peak overlap. Baseline separation was obtainable under isothermal column conditions (225°C for the menthyl naphthoxylactates and 170°C for the menthyl mandelates) with some sacrifice in analysis time and peak broadening.

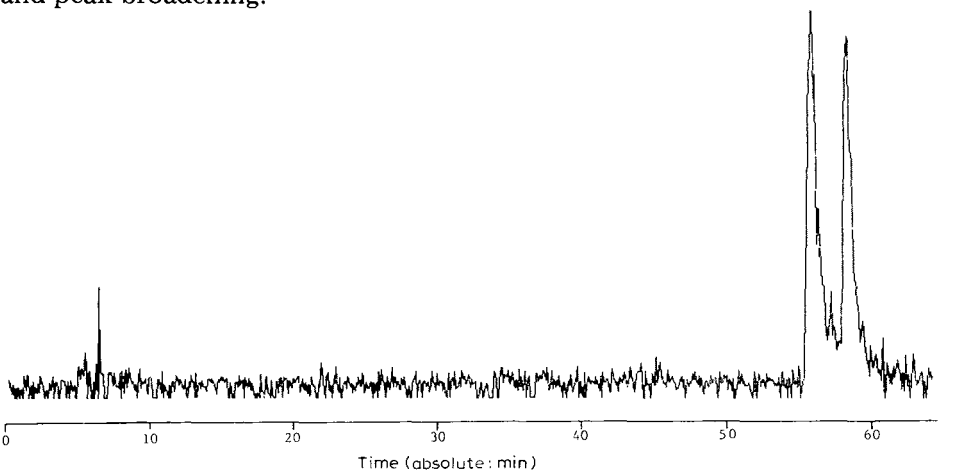


Fig. 1. Capillary GC—MS chromatogram of the diastereomeric menthyl naphthoxylactates. A 3-μl sample was injected (7—8 μg). The chromatogram is a restrospective single-ion plot of the molecular ion (*m/z* 370). See text for GC—MS parameters.

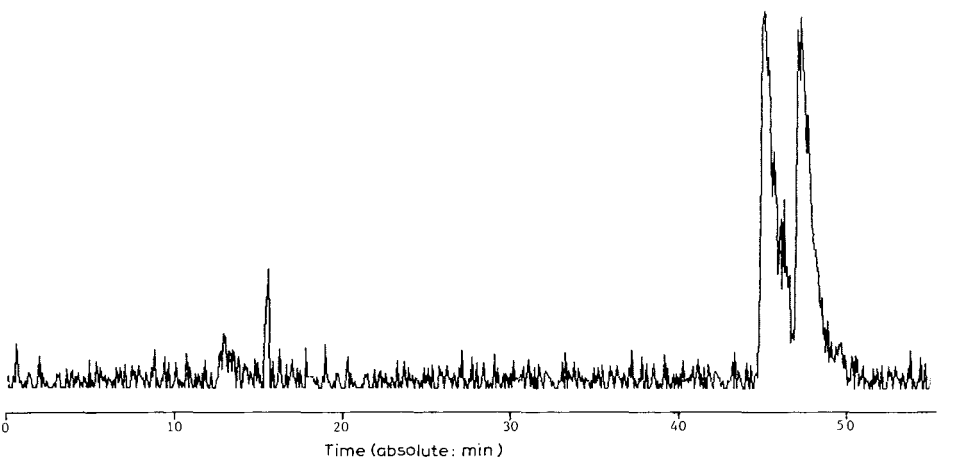


Fig. 2. Capillary GC—MS chromatogram of the diastereomeric menthyl mandelates, as a retrospective single-ion plot of the $[(C_6H_5)C(OH)H]^+$ fragment (*m/z* 107) of menthyl mandelate. The sample size was 3 μl (7—8 μg). For GC—MS conditions, see text.

The elution order of the menthyl mandelates was determined by comparing the retention times of the separately derivatized optically pure mandelic acid enantiomers, and by comparing the relative peak intensities of 4:1 (v/v) mixtures of the separately derivatized enantiomers. Under the conditions of the analysis, (—)-menthyl-*R*-(—)-mandelate eluted with a peak maximum at 40.5 min (adjusted retention time), and (—)-menthyl-*S*-(+)-mandelate eluted at 42.6 min. These retention times were reproducible to within ± 0.1 min. The relative peak intensities of the two 4:1 mixtures were consistent with the above elution order.

In order to determine the order of elution of the menthyl naphthoxylactates, *R*-naphthoxylactic acid (R-NLA) was extracted from the urine of a rat dosed with *S*-(—)-propranolol. The shift in the notation of the absolute configuration derives from the shift in priorities in the Cahn—Ingold—Prelog system of notation. It is assumed that the chiral center of the β-blocking side chain of propranolol remains unaltered during metabolism to NLA. The extracted R-NLA was derivatized and mixed with derivatized racemic NLA. Table II presents the relative peak intensities of this mixture and those of derivatized racemic NLA alone [from selected ion monitoring (*m/z* 370) capillary GC—MS]. From the results of the work of Nelson and Bartols [4], R-NLA is the negative rotating isomer. (—)-Menthyl-*R*-(—)-naphthoxylactate was found to elute before (—)-menthyl-*S*-(+)-naphthoxylactate on an SP-2100 column.

TABLE II

DETERMINATION OF THE ORDER OF ELUTION OF THE DIASTEREOMERIC MENTHYL NAPHTHOXYLACTATES FROM SELECTED-ION MONITORING (*m/z* 370) CAPILLARY GC—MS

Derivatized sample	Adjusted retention time, t'_R (min, ± 0.1)	Relative peak intensity
Racemic NLA	51.2	0.95
	53.7	1.00
Urinary R-NLA and racemic NLA	51.2	1.50
	53.7	1.00

DISCUSSION

O-((—)-Menthyl)-N,N′-diisopropylisourea has been synthesized and found to be useful for the preparation of diastereomeric (—)-menthyl esters of enantiomeric acids. (—)-Menthyl esters (from (—)-menthol) have been used in the past in the diastereomeric separation of lactic and glyceric acids [1], acyclic isoprenoid acids [2], phytanic [5] and pristanic [6] acids, and chrysanthemic acids [7]. The techniques involved with the use of (—)-MDI are relatively simple, with the minor disadvantage of the requirement of overnight heating in order to effect completeness of reaction. While this study has

demonstrated the applicability of (−)-MDI to analytical samples, it could be useful on a preparative scale as well. For instance, a large quantity of a racemic acid could be treated with (−)-MDI, and the resulting diastereomers resolved by preparative high-performance liquid chromatography. The separate esters could then be hydrolyzed and the optically pure enantiomers isolated. Such a procedure may offer advantages over repeated fractional crystallization in terms of yield, purity, or both.

The determination of the order of elution of the menthyl mandelates was straightforward due to the availability of optically pure standards and the extremely reproducible retention times obtainable from capillary GC techniques. While the determination of the elution order of the menthyl naphthoxylactates was indirect, it afforded a further demonstration of the usefulness of (−)-MDI in its application to the concentration levels of samples obtained from biological fluids. (−)-MDI thus has a wide range of potential applications as an analytical tool, and could be useful for preparative work as well.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatographic procedure for the analysis of urinary 3-methoxy-4-hydroxymandelic acid

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(First received February 10th, 1982; revised manuscript received February 2nd, 1983)

Measurements of 3-methoxy-4-hydroxymandelic acid (VMA) in urine are a small but important portion of the workload of many clinical chemistry laboratories. VMA is the main metabolite of dopamine and norepinephrine or epinephrine, and determination of VMA in urine has some importance in the diagnosis of neuroblastoma and in treatment of this disease and Parkinson's disease.

Although many techniques have been applied to the assay of VMA, their complexity, inadequate sensitivity, poor reproducibility or non-specificity have seriously compromised their value in the diagnosis of neural crest tumors.

Among methods for separating and identifying VMA in urine are paper chromatography [1, 2], thin-layer chromatography (TLC) [3, 4], spectrophotometry [6—8], electrophoresis [9, 10], gas chromatography [11—13], gas chromatography—mass spectrometry [14—16] and, more recently, high-performance liquid chromatography (HPLC) [17—25].

Paper chromatography and TLC procedures lack precision and require too much time for a routine procedure. Although gas chromatographic methods offer specificity and sensitivity, the procedures are too complex for routine use in a clinical laboratory. The other methods are less specific.

HPLC would appear to be adaptable to VMA assay. Direct HPLC analysis for VMA in urine, after its separation by column chromatography, is complicated because many other urinary compounds absorb at wavelengths of 254 nm or less, where VMA absorbs strongly. Thus, if one uses a highly sensitive UV detector, extensive clean-up of the urine before chromatography is required.

Therefore, VMA determination in urine samples is carried out in two steps: (1) extraction of VMA from the urine matrix by column chromatography with graphitized carbon black (GCB), and (2) quantitative determination of

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the eluate by HPLC with reversed-phase column and UV detection.

The present procedure is compared with that developed by Pisano et al. [8].

EXPERIMENTAL

Materials and methods

HPLC apparatus. We used a high-performance liquid chromatograph, series 3, with a Model LC 75 detector (both from Perkin-Elmer, Norwalk, CN, U.S.A.) and a Rheodyne Model 7125 syringe loading sample injector (Berkeley, CA, U.S.A.).

The instrument was operated in the isocratic mode and the absorbance of the eluent was monitored at 280 nm, with the aid of a Perkin-Elmer Model 56 strip-chart recorder. The reversed-phase column of 10 ↘

strip-chart recorder. The reversed-phase column of 10 μm average particle size used was a Perkin-Elmer C18/10 (25 × 0.46 cm, No. 258-0184). All the experiments were carried out at room temperature.

Reagents and adsorbent. All the reagents used were of highest purity (A.C.S. certified grade). Methanol (special grade for liquid chromatography), phosphoric acid, potassium dihydrogen phosphate were from Carlo Erba (Milan, Italy). For the VMA standard, a stock solution of VMA (1 g/l, purchased from Sigma, St. Louis, MO, U.S.A.) was prepared in 10 mmol/l hydrochloric acid. This solution is stable for many months at 4°C.

The GCB (Carbopack B, Supelco, Bellefonte, PA, U.S.A.) is characterized by a surface area of 100 $m^2\ g^{-1}$ and 80—100 mesh size.

Chromatographic conditions. The low-strength eluent (0.01 *M* KH_2PO_4, pH 3.0) was filtered through a 0.22-μm filter (No. GSWPO 4700; Millipore Corp., Bedford, MA, U.S.A.). The high-strength eluent was methanol. The ratio between low- and high-strength eluent was 99:1. The flow-rate was 1.2 ml/min. Chromatographic peaks were quantitated by measuring absorbance at 280 nm.

Evaluation of the chromatograms. Initial peak identification of VMA was performed on the basis of retention times and comparison with the reference compound. Peak identity was also confirmed using stopped-flow UV spectra. The UV spectrum was then scanned in the region between 220 and 320 nm.

Procedure

Urine samples. Urines (24-h) used for the correlation and normal value study were collected in glass bottles containing 10 ml of 6 *M* hydrochloric acid.

Extraction procedure using GCB column. A glass column (I.D. 0.4 cm) with PTFE cock was filled with 250 mg of GCB (80—100 mesh) to a height of 5 cm. The GCB was retained by two small plugs of silanized glass wool. The column was packed by passing through 5 ml of methanol and 5 ml of distilled water. Then 1 ml of acified urine (pH 2.0 with HCl) was passed through the column by gravitational flow.

After the urine had passed through the column, the column was washed with 0.5 ml of 0.01 *M* hydrochloric acid. Then VMA was eluted from the

GCB by methanol at a rate of 0.5 ml/min. The first fraction of 0.5 ml was discarded. The methanol fraction between 0.5 and 1.9 ml was collected and dried at 40°C by a gentle stream of nitrogen.

The residue was dissolved in 100 μl of methanol and 10 μl of this solution were analyzed by HPLC.

RESULTS AND DISCUSSION

The majority of clinical laboratories are still utilizing non-specific colorimetric VMA assays. Many of these procedures have too long analysis times, and frequently lack the sensitivity and specificity required. HPLC is potentially a powerful technique for the separation and quantitation of compounds in complex samples.

The use of a purification procedure with the GCB column and subsequent analysis by HPLC shows the following advantages: short analysis time, selectivity, sensitivity and the possibility to be automated.

Experiments were conducted in order to obtain the best analytical conditions for the separation of VMA from other UV-absorbing urine constituents. The possibility to operate in the isocratic mode and the use of a UV detector was obtained by the clean-up step for urine using GCB as adsorbent. This material allows the isolation of VMA in 1 ml of acidified urine and, in the subsequent elution, a marked selectivity with respect to the other UV-absorbing constituents.

Elution and recovery curves

It is possible to determine the minimum amount of solvent necessary for an efficient recovery from the elution and recovery curves.

In Fig. 1 the elution curve of VMA from urine passed through the GCB column is shown. On the ordinate, q_i is the amount recovered within the portion of solvent indicated; on the abscissa, q_t is the total amount of VMA.

The elution curve is only slightly tailing in the right part. This allows the total recovery of VMA simply by using small amounts of methanol (fraction between 0.5 and 1.9 ml).

Choice of mobile phase

Use of highly polar mobile phase is required to elute VMA as a sharp, symmetrical band, when chromatographed on a C18 reversed-phase column. The retention time of VMA depends on the pH of the mobile phase. We chose a pH of 3.0 because it results in a favorable retention time for VMA and is well within the buffer range for orthophosphate.

Chromatographic peaks were identified by retention time with the reference compounds and stopped-flow UV scanning. The use of stopped-flow scanning coupled with the identification retention time is very helpful in a complex biological matrix such as urine.

Recovery

Recovery studies were performed by adding specified quantities of VMA to aqueous solutions of urines. Then 1-ml aliquots were submitted to the

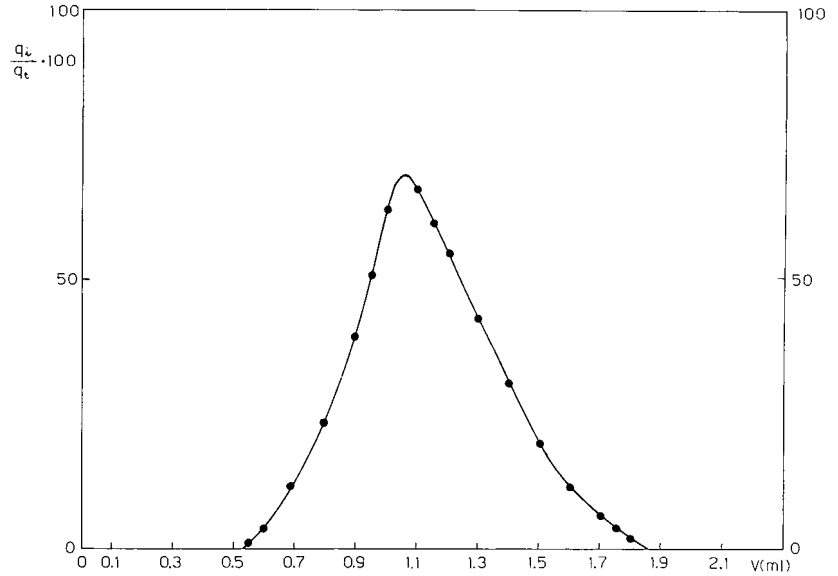

Fig. 1. Elution curve of VMA from urine.

purification procedure. We performed analytical recovery studies using aqueous standard to determine the absolute recovery of VMA. Recoveries of VMA from aqueous solution using the adsorption column method varied between 97% and 102% with a mean of 98 ± 2.4% (n = 6) for VMA concentrations in the range 1—50 μg/ml.

Table I shows the analytical recovery of VMA added to urine. Recoveries are always more than 97%. The recoveries shown in Table I indicate that VMA can be measured in aqueous solution or urine, with equivalent results. The analytical recovery studies indicate the excellent efficiency and reproducibility of this procedure.

Linearity of detector response and detection limit

Linearity of detector response was determined by plotting peak heights or areas versus the amount of injected VMA. The response was found to be linear over the entire working range (1—50 μg/ml). The detection limit per 1 ml of urine is 0.5 μg.

TABLE I

ANALYTICAL RECOVERY OF VMA ADDED TO URINE

VMA in urine (mean ± S.D., μg/ml)	VMA added (μg/ml)	VMA found (mean ± S.D., μg/ml)	Recovery (mean ± S.D., %)	n
1.15 ± 0.06	2.00	2.05 ± 0.08	97 ± 1.3	6
3.20 ± 0.07	2.00	5.11 ± 0.08	98 ± 2.5	6
2.10 ± 0.06	2.00	4.15 ± 0.09	101 ± 3.3	6
2.71 ± 0.08	5.00	7.60 ± 0.13	98 ± 1.9	6
1.90 ± 0.05	10.00	11.59 ± 0.20	97 ± 2.3	6
2.90 ± 0.09	20.00	22.60 ± 0.38	99 ± 2.1	6

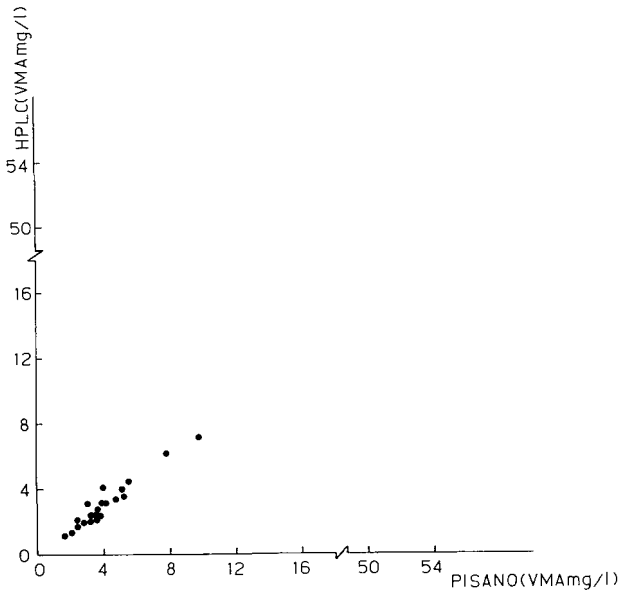

Fig. 2. Correlation between the present method (Y) and the method of Pisano (X).

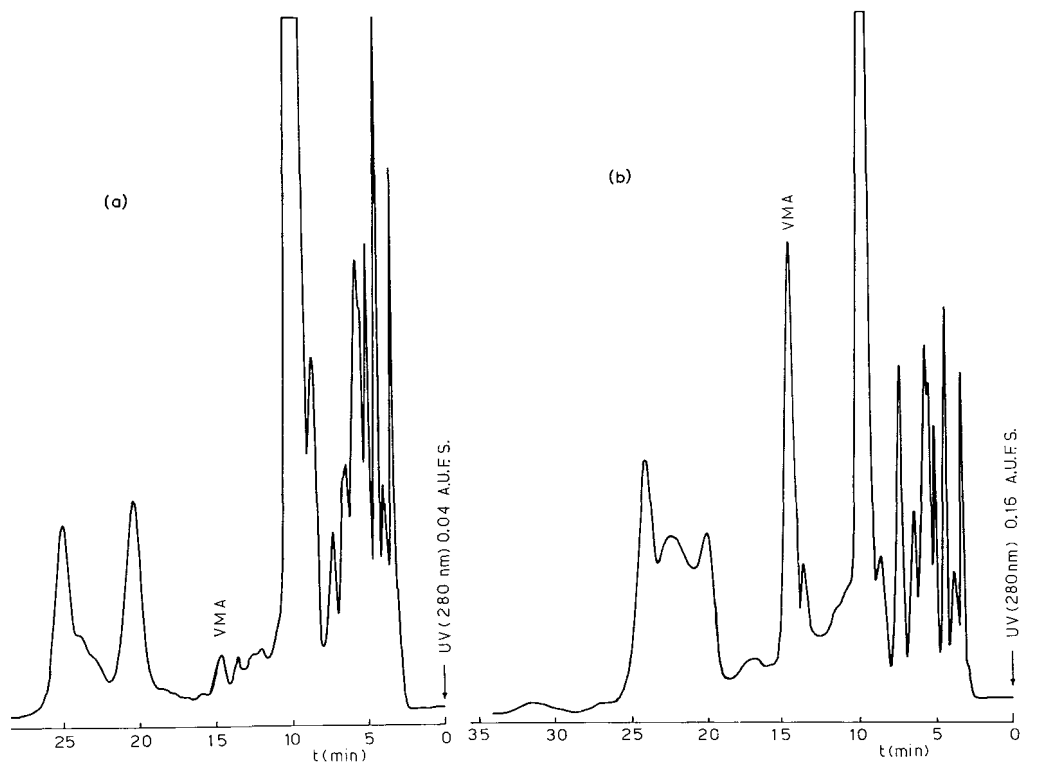

Fig. 3. Chromatograms obtained for 24-h urine samples (a) from a normal person (1.15 μg/ml VMA) and (b) from a patient with neuroblastoma (50.8 μg/ml VMA).

Correlation with comparison method

Twenty-two urine samples were assayed by the method of Pisano et al. [8] and by the present procedure. The two methods were run in parallel; the results are plotted in Fig. 2. The data fit the regression equation $Y = 1.15X - 1.22$, where X is the Pisano method, with a correlation coefficient of 0.987. With few exceptions, this procedure gave lower VMA values than did the classical Pisano method, as shown in Fig. 2. This bias may be attributed to increased specificity in the chromatographic method.

CONCLUSION

The procedure described here shows the advantages of short time, minimal sample, high sensitivity, good accuracy and reproducibility for analyzing VMA concentration in urine. The method is applied to the assay of urine from patients with neuroblastoma, pheochromocytoma and Parkinson's disease. Human urinary VMA values obtained by this method are in agreement with those of other workers [19, 23].

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Note

Modified sample preparation for high-performance liquid chromatographic—electrochemical assay of urinary catecholamines

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(First received November 17th, 1982; revised manuscript received January 21st, 1983)

High-performance liquid chromatography with electrochemical detection (HPLC—ElCD) has gained wide acceptance as a method for measuring catecholamine concentrations in physiological fluids. Advantages of the HPLC—ElCD method include its ease, rapidity of sample preparation, and low cost. A one-step alumina batch extraction has been used successfully for sample preparation of plasma [1, 2], but in applying the technique for urinary catecholamine measurements, a one-step alumina extraction has proven inadequate, because confounding peaks of unknown identity can interfere with the catecholamine peaks [3, 4]. As a result, current HPLC—ElCD methods include additional sample preparation steps [3—5]. The relatively simple, rapid, modified sample preparation described here has dramatically improved the quality of chromatographic recordings for urine catecholamines in this laboratory. The method allows urine-derived eluates to be injected into the same HPLC—ElCD apparatus used for plasma prepared with the alumina extraction, with simultaneous determination of free norepinephrine (NE), epinephrine (E), and dopamine (DA).

EXPERIMENTAL

Sample collection and handling

Urine samples assayed using the HPLC—ElCD technique were obtained from healthy female inpatient volunteers who were not allowed to smoke cigarettes, ingest caffeine-containing or catecholamine-rich foodstuffs, or to take any medications except occasional acetaminophen while on study.

Twenty-four hour total urine collections in 20 ml 6 *N* hydrochloric acid

were refrigerated until the next day. The urine specimens were stored in 20-ml aliquots at —70°C in plastic scintillation vials without other additives.

Reagents

Reagents for the HPLC—ElCD technique included Woelm Super 1 alumina, acid-washed according to the method of Anton and Sayre [6], glacial acetic acid, hydrochloric acid, disodium EDTA, sodium bicarbonate, sodium hydroxide, and sodium acetate (Fisher Scientific, Pittsburgh, PA, U.S.A.); acetonitrile and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.); Tris (Bethesda Research Labs., Bethesda, MD, U.S.A.); heptanesulfonic acid (Fisher or Aldrich); norepinephrine, epinephrine, dopamine, and N-methyldopamine (Sigma, St. Louis, MO, U.S.A.); and distilled, deionized water (Milli-Q, Millipore, Bedford, MA, U.S.A.).

Equipment

The HPLC—ElCD apparatus consisted of a Waters 6000A solvent delivery system, U6K injector, μBondapak C_{18} 30 cm × 3.9 mm reversed-phase stainless-steel column containing 10-μm irregular particulate packing, solvent clarification kit for degassing the mobile phase, and guard column packed with C_{18} Porasil; a Bioanalytical Systems LC4 or LC4A amperometric detector with TL5 glassy carbon electrode; and an LKB or Fisher Recordall strip-chart recorder or Waters Data Module. Waters C_{18} columns and silica Sep-Paks were used for the modified sample preparation as described below.

Mobile phase

The chromatographic mobile phase was prepared as follows. To 1 l water were added 6.8 g sodium acetate, 100 mg EDTA, and 1 g heptanesulfonic acid. The pH was adjusted to 4.8 with 2 *N* hydrochloric acid and the liquid filtered using a vacuum pump and 0.45-μm aqueous filter. Then, 70 ml were discarded and replaced with acetonitrile. The mobile phase was degassed as necessary, usually daily, by stirring under vacuum. The pump was set at 1.0 ml/min, the detector at 10 nA/V, 0.50 V applied potential, and the recorder at 1 V full scale.

Assay steps

A C_{18} Sep-Pak was washed with 10 ml methanol and a silica Sep-Pak with 6 ml of a solution containing 5 ml 1.0 *M* sodium bicarbonate at pH 8.5 and 1 ml 50 g/l EDTA. The C_{18} Sep-Pak was washed with 10 ml water. The C_{18} and silica Sep-Paks were stored in this state overnight in a referigerator prior to urine assay the next day.

The acidified urine was thawed at room temperature. Five ml were passed through the C_{18} Sep-Pak, followed by 1 ml water, and collected in a sample tube. A 2-ml aliquot was mixed with 6 ml of the above described EDTA—bicarbonate solution and the mixture passed through the silica Sep-Pak. The silica Sep-Pak was washed once with 5 ml water. The catecholamines were eluted from the silica by 4 ml of 1% acetic acid. Of this 4 ml, 1 ml was assayed through the batch alumina extraction step. This 1 ml was placed in a 1.5 ml plastic sample tube containing about 10 mg alumina; 400 μl of 1 *M* Tris -20 g/l

EDTA, which had been adjusted to pH 8.6 with hydrochloric acid, were added. The tube was shaken vigorously for 20 min, centrifuged, and the supernatant discarded. The alumina was washed once with 1 ml water and the catecholamines were then desorbed with 100 μl of 0.2 *M* acetic acid. A 50-μl aliquot of the eluate was injected into the HPLC column.

By comparison with a mixture of 100 ng/ml norepinephrine, epinephrine, and dopamine external standards, where 50 μl (5 ng) had been directly injected and where 1 ml (100 ng) was assayed in parallel with the urine, urinary catecholamine excretion was calculated according to the following equation:

Catecholamine excretion (ng per 24 h) = peak height of catecholamine in urine eluate ÷ peak height of external standard × 20 (ng/ml) × volume excreted (ml per 24 h).

Overall recovery through the sample preparation steps was about 40–50% for each of the catecholamines — about 90% through the C_{18} step, 70–75% through the silica step, and 70—75% through the alumina step. No difference in recoveries was obtained among the three catecholamines at any step in the sample preparation.

RESULTS

Fig. 1 shows representative chromatograms of directly injected NE, E, DA, and N-methyldopamine (NMDA) standards and of urine-derived eluates at various stages in the sample preparation. Fig. 1 demonstrates that NE, E, and DA were clearly resolved from each other and that no interfering peaks occurred with the modified sample preparation. Across a total of 181 different

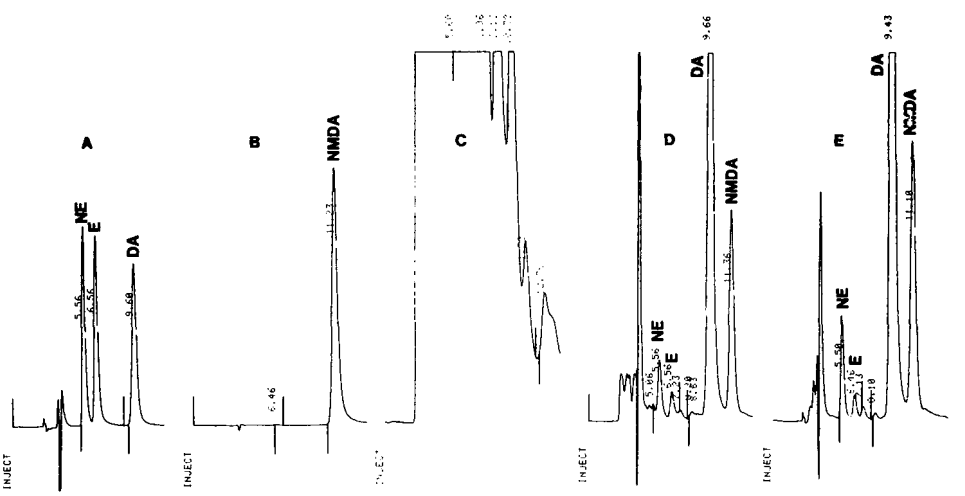

Fig. 1. Chromatographic recordings at various stages of the sample preparation. (A) Injection of 50 μl (5 ng) norepinephrine (NE), epinephrine (E), and dopamine (DA) standards; (B) 1 μl (5 ng) N-methyldopamine (NMDA) standard; (C) 200 μl of partially purified urine sample after C_{18} Sep-Pak; (D) 200 μl of same sample after C_{18} and silica Sep-Paks; (E) 50 μl of same sample after C_{18}, silica, and then alumina batch extraction. Displayed retention times are in hundredths of a minute.

urine specimens, the average norepinephrine excretion per 24 h (± 1 S.D.) was 37 ± 25 μg, epinephrine 7 ± 5 μg, and dopamine 278 ± 187 μg. These results agree well with those obtained by other, older techniques [7]. Adequate chromatography was obtained for all specimens, although occasionally a small peak occurred just after norepinephrine, just after epinephrine, or just before dopamine. After only the C_{18} step, the solvent front invariably was very wide and completely obscured any catecholamine peaks.

DISCUSSION

In this report a modified sample preparation is presented for analysis of urine catecholamines using HPLC—ElCD. Addition of sample purification steps with commercially available C_{18} and silica pre-packed columns prior to alumina batch extraction resulted in excellent chromatographic records, whereas the assay of urine after only an alumina extraction has yielded unreliable results [3, 4]. The modified technique allows injection of urine-derived eluates into the same chromatographic—electrochemical equipment as for plasma-derived eluates, but because of the relatively small recoveries the sample preparation described here for urine can not be used for plasma. Addition of the Sep-Pak columns to the procedure increases the cost of the catecholamine assay by about US$ 2 per sample. Although they can be reused, only new columns were involved in the present study.

Because of the dietary and other restrictions imposed on the healthy women whose urine was assayed, it is possible that some foodstuffs, medications, or disease states may not yield as satisfactory results. The rather large standard deviations as fractions of the mean probably represent real variability in catecholamine excretion rates both within and across individuals, since assays conducted on samples collected for different days in the same individuals resulted in standard deviations of similar magnitude, whereas intra-assay standard deviations among replicates of the same sample were much smaller.

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Note

Determination of adenine, adenosine and related nucleotides at the low picomole level by reversed-phase high-performance liquid chromatography with fluorescence detection

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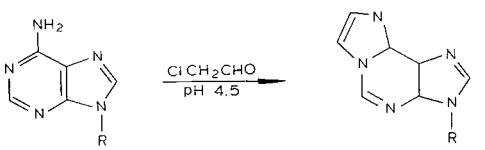
The determination of individual nucleotides and nucleotide phosphates at the low picomole level is not particularly straightforward. Conventional chromatographic techniques such as ion-exchange chromatography coupled with UV detection [1—4] are not always sufficiently sensitive, whilst enzymatic techniques, such as the bioluminescent lucerferin—luciferase reaction with adenosine 5′-triphosphate (ATP), are highly sensitive and specific but require specialist equipment and expertise and may not always be suitable for routine use.

The reaction of adenine and its analogues with chloroacetaldehyde to form the highly fluorescent 1,N^6-etheno derivatives has been described [5—8] and utilised in a number of analytical procedures [9, 10], however it has not previously been possible to separate the more polar components (i.e. the nucleotide phosphates) without resorting to ion-exchange chromatography [10]. Recently, it has been shown that reversed-phase high-performance liquid chromatography (HPLC) can be utilised to separate nucleotide phosphates [11, 12] and it was therefore decided to determine whether the 1,N^6-etheno derivatives of the adenine nucleotides could be similarly separated.



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EXPERIMENTAL

Materials

ATP, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), cyclic AMP (cAMP), adenosine (ADO) and adenine (ADE) were purchased from Sigma (London, Great Britain). Standard solutions were made up in borate buffer (0.05 *M*, pH 10.0) and were stable at room temperature.

Chloroacetaldehyde (chloroethanol) was prepared by distillation from a chloroacetaldehyde dimethyl acetal (Aldrich, Gillingham, Great Britain)—5% sulphuric acid (1:5, v/v) mixture. The distillate (b.p. 85—86°C) was diluted with doubly distilled water to give a 4 *M* solution which was stable at room temperature.

Phosphate (0.05 *M*, pH 7.0) and acetate (1.0 *M*, pH 4.5) buffers were prepared from analytical-grade reagents. The phosphate buffer was made up in HPLC-grade water (Rathburn Chemicals, Walkerburn, Great Britain) and was subsequently filtered (0.5 μm) and further purified before use by passage through a pellicular reversed-phase column (Whatman Co:Pell ODS).

Methods

Aliquots of the nucleotide solution, acetate buffer and chloroacetaldehyde (50:10:1, v/v/v) were mixed together in glass stoppered test tubes and then incubated for a minimum of 30 min in a boiling water bath. The resultant solutions were then injected directly into the chromatograph.

Chromatography

A high-performance liquid chromatograph consisting of two Waters Assoc. 6000A pumps, a Model 660 solvent programmer and a U6K injector coupled to a LDC Fluoromonitor III fluorescence detector (mercury lamp, 254-nm interference exciting filter and 410—700 nm emission filter) were used throughout.

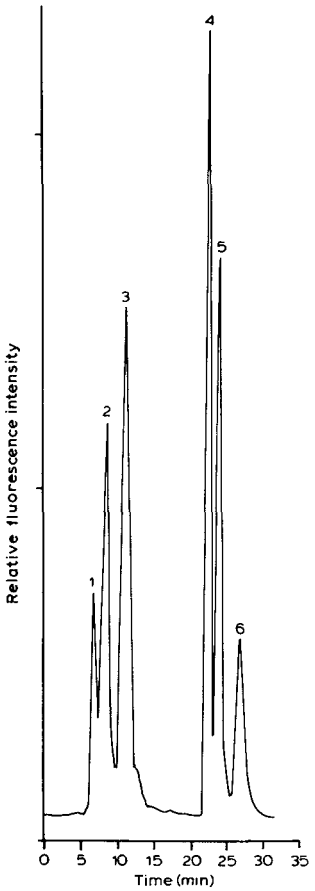
Elution was performed by programming (Curve 8, 15 min) from 97% phosphate buffer (0.05 *M*, pH 7.0), 3% methanol solution (Solution A) to a 75% phosphate buffer, 25% methanol solution (Solution B).

The chromatographic separation was performed on a 30-cm column packed with Whatman ODS-2 (10 μm) reversed-phase packing. The analytical column was protected by a 5-cm guard column packed with Whatman Co:Pell ODS medium. A flow-rate of 1 ml min^{-1} was used throughout.

RESULTS

A typical chromatogram of a six-component nucleotide mixture (derivatised as described under *Methods*) is shown in Fig. 1. Improved separation of the ATP/ADP pair can be obtained by reducing the amount of organic modifier in Solution A, but only at the expense of some peak broadening which may detract slightly from the overall sensitivity.

The effects of incubation time on the formation of the various derivatives are shown in Fig. 2. It is clear from this that reaction is essentially complete after 30 min in all cases and this time was therefore used in all subsequent work.



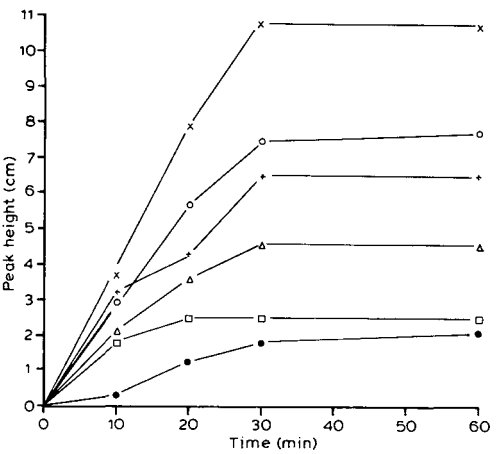


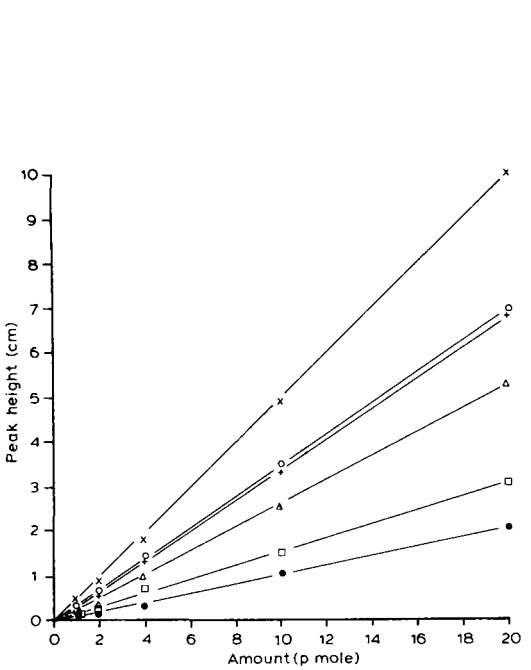
Fig. 1. A typical chromatogram of the derivatives of a six-component mixture prepared as described in the text. Peaks: 1 = ATP; 2 = ADP; 3 = AMP; 4 = cAMP; 5 = ADO; 6 = ADE.

Fig. 2. The effect of incubation time on the derivative formation. Mixtures prepared as described in the text were incubated for various periods of time, cooled and subsequently injected into the chromatograph. □ = ATP; △ = ADP; + = AMP; × = cAMP; ○ = ADO; • = ADE.

Calibration curves for each of the components are shown in Fig. 3. Linearity is preserved up to some hundreds of picomoles and the curves are independent of injection volume of at least 500 μl. With a detection limit of 0.5—1 pmol for each compound, solutions with concentrations as low as 2 pmol ml^{-1} may be satisfactorily analysed.

The reagent blank is very low and is negligible over the portions of the chromatogram of interest. No significant interferences with the overall procedure have been detected. Cytidine, which does form a weakly fluorescent derivative, has its emission maximum at 347 nm compared with 410 nm for the adenine derivatives and its contribution to fluorescence is removed by the emission filter. The technique is therefore specific to adenine and related compounds.

The reproducibility of the technique was assessed by derivatising five identical aliquots of a standard mixture of the six compounds (1 nmol ml^{-1} of



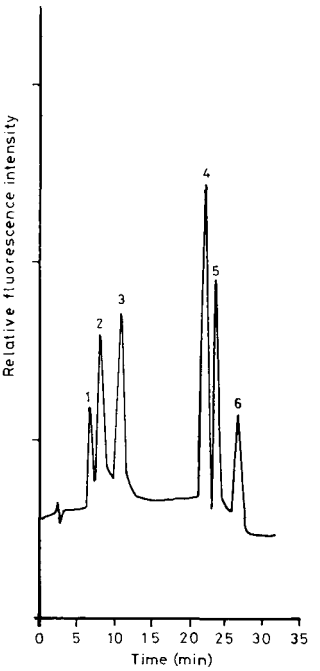


Fig. 3. Calibration curves for each of the six adenine compounds examined. □ = ATP; △ = ADP; + = AMP; × = cAMP; ○ = ADO; • = ADE.

Fig. 4. A typical chromatogram of the chloroacetaldehyde derivatised extract of phytoplankton collected from Irish Sea water (10.0 μl injection). Peaks as in Fig. 1.

each component) and injecting 20 μl (20 pmol). Coefficients of variation of 3.75, 1.78, 2.99, 1.83, 1.69 and 3.18% were found for ATP, ADP, AMP, cAMP, ADO and ADE, respectively. Once prepared the stability of the derivatives is excellent and they may be safely stored at room temperature for at least a week without measurable change.

A further interesting feature of this technique is that if it is only required to analyse for the nucleotide phosphates, then this may be achieved by isocratic elution with solution A in approximately 10 min. The derivatives of the less polar components are retained on a guard column which may be cleared periodically by elution with solution B.

The method has been successfully utilised by the author for the analysis of adenine related compounds in marine phytoplankton. The plankton cells were concentrated from sea water by filtration and the adenine compounds were extracted with boiling borate buffer and subsequently derivatised in the manner described previously. An example of the type of chromatogram obtained is shown in Fig. 4 from which it can be seen that there is no evidence of interference from other co-extracted compounds. This technique is at present under investigation with a view to using it to obtain improved standing crop estimates.

The high specificity of the chloroacetaldehyde reaction with adenine related compounds to form a fluorescent derivative has made it a useful tool in the

study of enzyme systems, RNA/DNA and biological fluids in general (for review see ref. 6). The reaction combined with the simple HPLC technique described in this paper is likely to find a considerable number of applications in future biological studies.

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Note

Improved determination of betaxolol in biological samples by capillary column gas chromatography

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(Received November 11th, 1982)

Betaxolol (SL 75 212) is a new cardioselective β-adrenoceptor blocking agent with a long half-life of about 16 h and high bioavailability of about 80% [1—4].

During the early pharmacokinetic studies, the determination of the drug in blood and urine was carried out by a gas chromatographic method with a classical packed column using propranolol as the internal standard [5]. This method was quite satisfactory for studies in volunteers and in patients on monotherapy [1, 2, 4, 6, 7].

However, during long-term studies, when occasional polytherapy occurred, we could observe, in a few cases, interferences with both the betaxolol and its internal standard. It was then necessary to improve the efficiency of the original chromatographic method by the use of capillary columns. In addition, we thought it preferable to replace propranolol as internal standard by an analogue of betaxolol with a longer retention time, allowing higher analysis temperature and less risk of interference.

This paper describes the improved method for the accurate quantitation of betaxolol in blood, urine and tissues. The method has proved useful both for pharmacokinetic studies and for therapeutic drug monitoring.

EXPERIMENTAL

Standard and reagents

Betaxolol [4-(2-cyclopropylmethoxyethyl)-1-phenoxy-3-isopropylamino-propan-2-ol] and SL 76 020 [4-(2-cyclobutylmethoxyethyl)-1-phenoxy-3-iso-propylaminopropan-2-ol] were synthesized by Dr. Manoury, Chemistry Depart-

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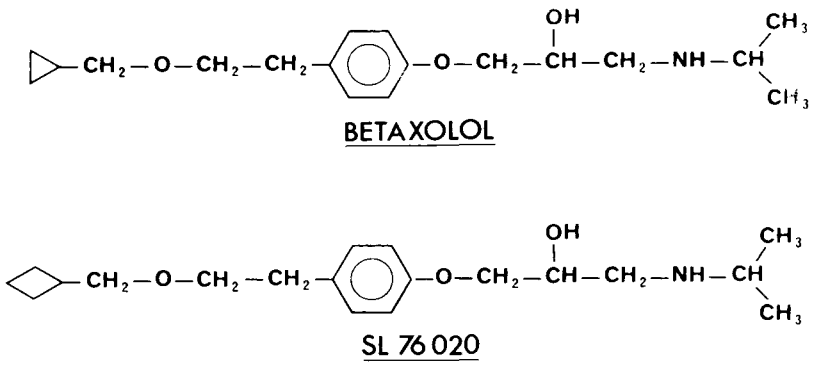

Fig. 1. Structural formulae of betaxolol and its internal standard, SL 76020.

ment LERS-Synthélabo (France). Their structural formulae are given in Fig. 1.

Analytical grade diethyl ether, ethyl acetate, sodium hydroxide, hydrochloric acid and *n*-hexane were obtained from Merck (Darmstadt, G.F.R.) and heptafluorobutyric anhydride (HFBA) was from Pierce (Rotterdam, The Netherlands).

Gas—liquid chromatographic conditions

Analyses were performed under isothermal conditions on a Model 5880 A Hewlett-Packard or a Model Sigma 4 Perkin-Elmer gas chromatograph, both equipped with a ^{63}Ni linear electron-capture detector.

A 0.02-mm OV-101 phase thickness quartz capillary column (25 m × 0.2 mm I.D.) was pretreated and tested by the manufacturer (Spiral, Dijon, France)

The operating conditions were: column temperature 225°C; injector temperature 300°C; split ratio 1:10; carrier gas argon—methane (95:5, v/v) 1 ml/min, make-up gas (argon—methane) 40 ml/min; detector temperature 300°C.

Samples of 1 µl were injected with the automatic injector HP 7672 A coupled with the HP 5880 gas chromatograph.

Calibration graph and quantitation

Standard solutions of betaxolol (1 µg/ml) and SL 76 020 (1 µg/ml), prepared in methanol, were stable for at least two months when kept at −20°C in the dark.

The calibration curves were prepared by adding 1, 5, 15, 30, and 50 ng betaxolol and 50 ng of SL 76020 to 1 ml of blank blood. The samples were extracted according to the method described below, and the extract was derivatized by heating with 200 µl of a solution of HFBA (10%, v/v) in ethyl acetate at 50°C for 10 min. In order to remove the excess reagent, this solution was then evaporated to dryness under a gentle stream of nitrogen at 60°C. To the dry residue 100 µl of hexane were added and 1 µl of this solution was injected onto the column. The curves were prepared by plotting the ratios of the peak height of betaxolol to the internal standard, against the known amounts of betaxolol. This curve was used to calculate the amount of betaxolol in unknown samples.

Extraction procedure for blood, urine and tissues

SL 76020 (50 ng) as internal standard, 100 μl of 2 *N* sodium hydroxide, and 7 ml of freshly distilled diethyl ether were added to 0.5–2 ml of blood in a 10-ml glass stoppered test-tube. The tubes were gently shaken on a rotating mixer for 15 min and then centrifuged at 4°C for 5 min at 800 *g*. The ether phase was transferred to another series of test-tubes containing 2.5 ml of 0.2 *N* hydrochloric acid, mixed on a Vortex mixer for 15 sec and then centrifuged for 2 min at 800 *g*. The upper organic phase was discarded. A further 5 ml of diethyl ether were added to the aqueous phase and the agitation and centrifugation repeated. After discarding the ether, 300 μl of 2 *N* sodium hydroxide solution were added to the aqueous phase together with 7 ml of diethyl ether. After extraction on a Vortex for 15 sec and centrifugation, 6.5 ml of the ether phase were transferred to another series of test-tubes evaporated to dryness under nitrogen in a water bath at 40°C, and then derivatized with HFBA as described above. An internal calibration curve with various amounts of betaxolol added to blank blood was carried through the procedure with the unknown samples.

An identical procedure was used for the analysis of urine and tissues.

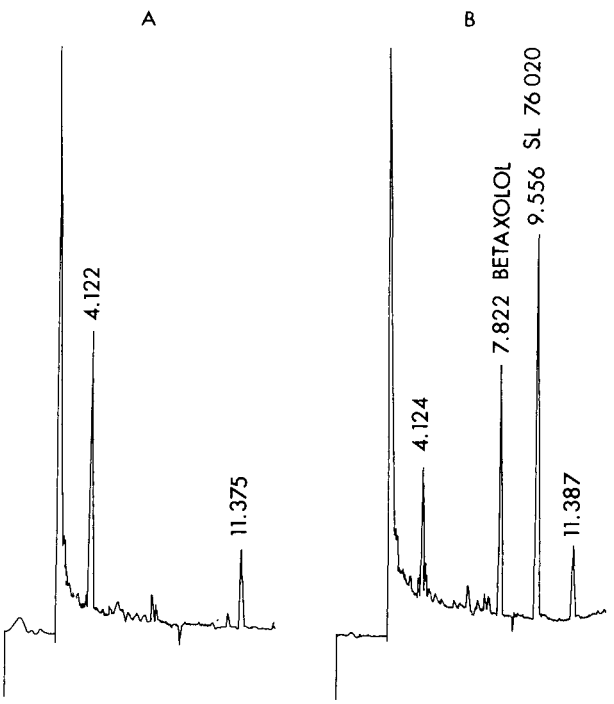

Fig. 2. Gas chromatograms, obtained with the capillary column, of a patient blood extract. (A) Blood collected before the beginning of the administration of betaxolol (t = 0); (B) blood collected 12 h after the oral administration of a 20-mg dose. No interfering peaks are present in the blood collected at t = 0.

RESULTS AND DISCUSSION

The chromatograms of a blood sample collected from a patient before the administration of betaxolol (time = 0) and a blood sample drawn 12 h after dosing with 20 mg of betaxolol are shown in Fig. 2. The retention times of betaxolol and SL 76 020 were 7.8 and 9.5 min, respectively. The peaks of betaxolol and its internal standard are well separated and no interfering peaks from endogenous substances or xenobiotics are present.

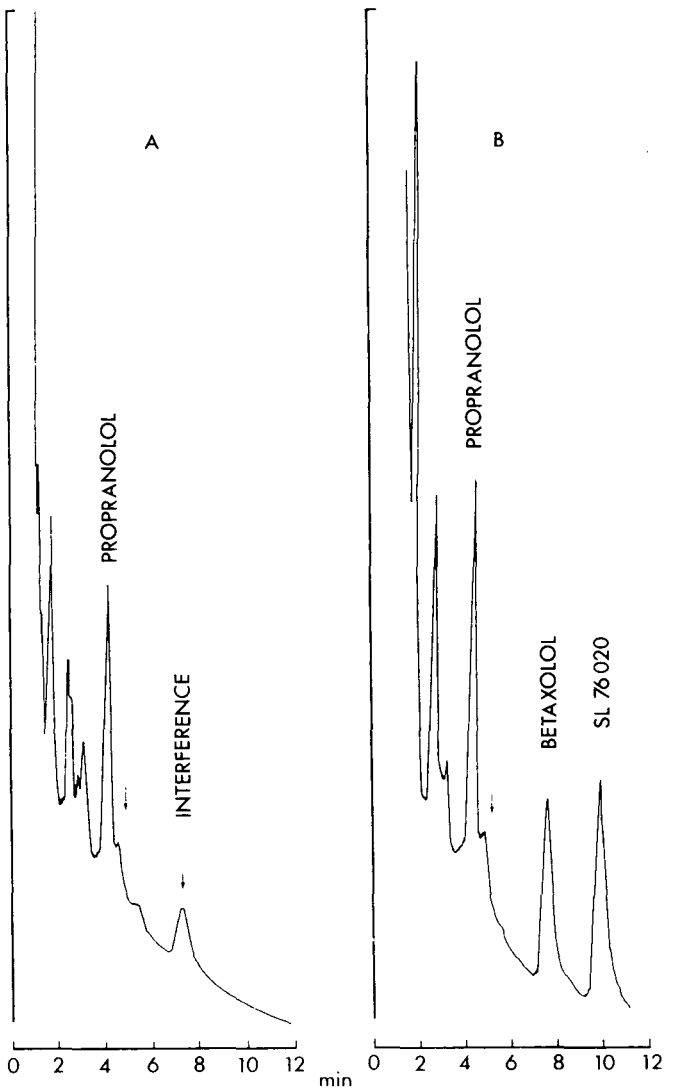

Fig. 3. Gas chromatograms, obtained with the packed column, of the same blood extract shown in Fig. 2. Propranolol was also added in this experiment. (A) Blood collected at $t = 0$: arrows indicate two peaks interfering with propranolol and betaxolol. (B) Blood collected 12 h after betaxolol dosing: the peak interfering with propranolol is still evident, whilst the other one is completely masked by the peak of betaxolol, which is thus overestimated.

The same samples, spiked also with propranolol, were analysed by the previously published method in which the separation is carried out on a packed column. As can be seen in Fig. 3, two interferences were present in the chromatogram. Fig. 3A shows the chromatogram obtained at time 0; an interfering peak at the end of the peak of propranolol and a second one with a retention time almost identical to that of betaxolol are present. Fig. 3B shows a chromatogram obtained from the sample collected 12 h after dosing and in which one can observe again the interference with propranolol, but not the interference with betaxolol, probably because betaxolol masks completely the interfering peak. Substituting propranolol by SL 76 020 should eliminate the interference with the internal marker, as shown in Fig. 3A, but would not avoid the possible interfering peak with betaxolol. The higher resolution of the capillary column overcomes this problem, as shown in Fig. 2.

The possible interference between betaxolol, SL 76 020 and a certain number of cardiovascular and diuretic drugs which could be associated with beta-blocker therapy, has been investigated. Guanethidine, clonidine, chlortalidone, furosemide, lidocaine, quinidine, diazepam, diltiazem, amiodarone and nitroglycerine have been added to samples containing betaxolol and SL 76 020, at concentrations known to be present during chronic treatment. No interfering peaks any of these compounds were observed. Moreover, many other beta-blockers such as alprenolol, oxprenolol, atenolol, acebutolol and metoprolol have a retention time shorter than that of betaxolol in our chromatographic conditions and cannot interfere with betaxolol.

The reproducibility of the method is shown in Table I. The coefficient of variation ranges from about 10% at lower concentration to 4% at higher concentration. For 1 ng, the coefficient of variation is 8.6%. It must be emphasized that these values were not obtained during a single experiment, but from 31 calibration curves prepared for routine analysis over a period of two months. Compared with the previously published procedure, the limit of sensitivity was lowered from 1 to 0.5 ng/ml of blood or urine with the present capillary column method. As already observed for betaxolol and propranolol,

TABLE I

CONCENTRATION OF BETAXOLOL FOUND IN PLASMA FOR KNOWN AMOUNTS OF THE DRUG ADDED

Amount added to plasma (ng)	No. of determinations	Amount recovered (ng/ml, mean ± S.D.)	Coefficient of variation* (%)
1	13	1.04 ± 0.09	8.6
5	10	4.6 ± 0.4	8.8
15	41	15.3 ± 1.6	10.4
30	31	30.1 ± 1.7	5.6
50	31	48.3 ± 2.4	4.9

*Coefficient of variation = $\frac{\text{S.D.} \times 100}{\text{mean}}$.

the ratio of the peak heights of betaxolol and its new internal standard (SL 76 020) is not modified over a one-week period, confirming the excellent stability of the HFBA derivatives. The absolute recovery of the method calculated independently for betaxolol and SL 76 020, of about 90%, confirmed the values previously obtained [5].

More than 1500 samples have been analysed with the capillary column method and no interferences have been detected or observed up to now.

CONCLUSIONS

The use of a quartz capillary column increased the sensitivity and especially the specificity of the previous packed column method. This two-fold increase in sensitivity obtained with the capillary column cannot be very relevant for clinical drug monitoring in chronically treated patients whose blood level at steady-state is about 40 ng/ml, but may improve the accuracy of the determination in the terminal phase of pharmacokinetic studies. The main advantage of the described method is the increase of selectivity due to the improved separation power of the capillary column. The possibility of interferences due to the concomitant intake of other xenobiotics cannot be excluded but it remarkably reduced, and samples which could not be analysed with the previous method give well-separated peaks necessary for quantitation. This method must be preferred to the previous one for routine monitoring of betaxolol in patients undergoing polytherapy.

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CHROMBIO. 1636

Note

Determination of oxcarbazepine in human plasma by high-performance liquid chromatography

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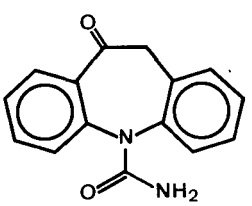
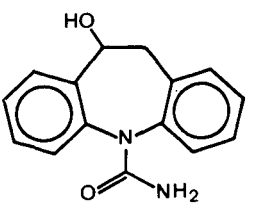
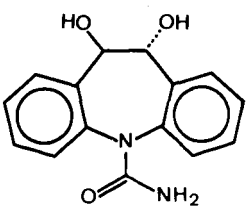
Oxcarbazepine (I), a potent anticonvulsant [1], is the keto-homologue of carbamazepine, a well-known antiepileptic drug (Fig. 1). After the administration of single oral doses of oxcarbazepine to volunteers, only low plasma concentrations of the unchanged compound have been observed [2]. Therefore, in pharmacokinetic studies the concentrations of both the parent compound



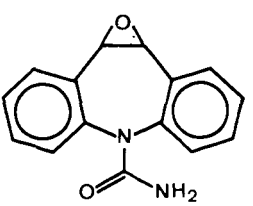
I Oxcarbazepine



II GP 47 779



III CGP 10 000



IV GP 49 023

Fig. 1. Structures of: I, oxcarbazepine (10,11-dihydro-10-oxo-carbamazepine); II, GP 47 779 (10,11-dihydro-10-hydroxy-carbamazepine); III (10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine); and IV (10,11-epoxy-carbamazepine).

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and the main metabolite 10,11-dihydro-10-hydroxy-carbamazepine, GP 47 779 (II), as well as of a minor metabolite 10,11-dihydro-10,11-*trans*-dihydroxy-carbamazepine, CGP 10 000 (III), need to be measured. For the simultaneous determination of these three compounds a sensitive high-performance liquid chromatographic (HPLC) assay has been developed using 10,11-epoxy-carbamazepine (IV) as the internal standard.

EXPERIMENTAL

Chemicals

All solvents and reagents were of analytical grade (Fluka, Buchs, Switzerland, and Merck, Darmstadt, G.F.R.) and were used without further purification. Oxcarbazepine, GP 47 779 (II), CGP 10 000 (III) and the internal standard originated from Ciba-Geigy Ltd., Basle, Switzerland. Water was deionised, distilled in a glass apparatus and was filtered through a 0.45-μm Millipore® filter before use.

Apparatus

A Hewlett-Packard isocratic liquid chromatograph (Model 1082 B) equipped with an automatic sampling system (Model 79842 A) was used. The variable-wavelength detector (Model 79875 A) was operated at 210 nm and an LC terminal (Model 79850 B) was used for programming and peak area integration.

The column (25 cm × 3.2 mm I.D.) was packed with LiChrosorb RP-18, 10 μm (Merck). The mobile phase (acetonitrile—water, 20:80) was used at a flow-rate of 1 ml/min and at a column temperature of 30°C.

Preparation of standard solutions

Stock solutions of oxcarbazepine and the metabolites were prepared by dissolving 10 mg of the compound in 100 g of water containing 20% of ethanol (10% of ethanol for the metabolites).

Aliquots of these stock solutions were combined and diluted with water to yield a concentration of 2 μg/g for each of the three compounds. This solution served to prepare spiked plasma samples for calibration curves and recovery analyses. Weighing of aliquots was preferred because of higher precision and better documentation.

Preparation of the internal standard solution

A stock solution was prepared by dissolving 10 mg of 10,11-epoxy-carbamazepine in 100 ml of water containing 10% of ethanol. An aliquot was diluted with water to yield a concentration of 2 μg/ml. To each analytical plasma sample 1 μg of 10,11-epoxy-carbamazepine was added using a Repipette® sampler. The working solutions, when kept at 5°C, were found to be stable for at least four weeks.

Procedure

Weigh (Mettler AC 100 balance) 0.5 g of plasma into a 16 × 70 mm disposable glass ampoule and dilute with 1 g of water. (For calibration curves and recovery analyses add known amounts of the three compounds instead of 1 g of

water.) Add 0.5 ml (Repipette® sampler) of an aqueous solution of the internal standard and shake for 5 min (Heidolph DSG 304 vertical mixer). Then add 7 ml of extraction solvent (diethyl ether—dichloromethane, 2:1) and 1.0 g of solid sodium carbonate-10-hydrate to saturate the aqueous phase. Seal the ampoule with a polyethylene cap and shake for 30 min (Infors TR 1 horizontal shaker at 200 rpm). Centrifuge for 5 min (MSE Multex at 940 *g*) and freeze the aqueous phase in a dry ice—ethanol mixture. Transfer the organic layer into a 16 × 40 mm disposable glass ampoule and evaporate the solvent by gently blowing nitrogen into the ampoule at 40°C. Place the ampoule in a desiccator and evaporate for 10 min to eliminate completely the dichloromethane. Reconstitute the extraction residue in 0.2 ml of the mobile phase. Transfer the solution into a micro injection vial. Seal with a septum cap and centrifuge for 1 min to ensure no air bubbles or particulate matter will be injected into the chromatograph.

Calibration

To establish calibration curves, plasma samples with known concentrations were prepared by adding oxcarbazepine and the two metabolites to 0.5 g of blank human plasma. After addition of 1 μg of the internal standard, the samples were processed as described above.

A 20-μl volume of the reconstituted extract of each sample was injected and peak area ratios of the compound to the internal standard plotted against the

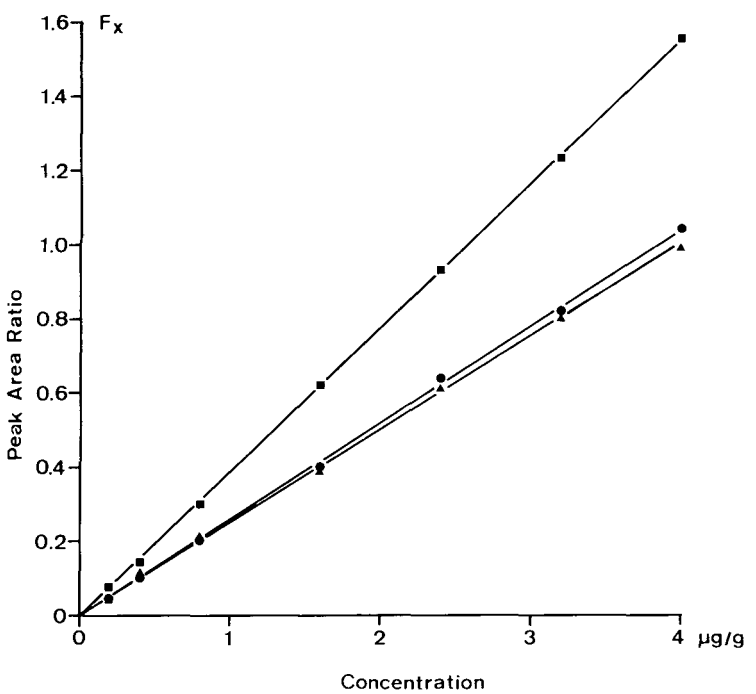

Fig. 2. Peak area ratios of test compounds to internal standard versus concentration. Calibration curves for oxcarbazepine (● —●), II (■ —■) and III (▲ —▲).

compounds concentrations given. Calibration curves for all three compounds were linear (r=0.999) in the range 0.2—4.0 μg/g (Fig. 2).

In the routine analysis of large series of plasma samples single-point calibration was used to calculate the concentrations of oxcarbazepine and the metabolites.

RESULTS

Extractability

The extractability of the three compounds and of the internal standard from plasma was determined by comparison of the peak area after direct injection of known amounts to that resulting after injection of the reconstituted extracts of spiked samples that underwent the whole work-up procedure. Covering the concentration range 0.2—2.0 μg/g the extractabilities (mean ± S.D.) were 80 ± 4% for oxcarbazepine, 90 ± 4% for II, 67 ± 4% for III and 94 ± 6% for the internal standard.

Precision and accuracy

Recovery analyses of spiked plasma samples were analysed together with each series of analytical samples. The results of recovery analyses from six independent series and the respective coefficients of variation (CV%, between-day) are given in Table I. Typical chromatograms are shown in Fig. 3.

TABLE I

MEAN VALUES OF RECOVERY ANALYSES PERFORMED ON SIX DIFFERENT DAYS USING SINGLE-POINT CALIBRATION

	Given (μg/g)	Found (μg/g) (mean ± S.D.)	Precision between-day (CV%)	Accuracy (%)
Oxcarbazepine	0.200	0.220 ± 0.016	7.3	110.0
	0.400	0.401 ± 0.035	8.7	100.3
	0.800	0.790 ± 0.049	6.2	98.8
	1.600	1.589 ± 0.037	2.3	99.3
	2.400	2.374 ± 0.077	3.2	98.9
	3.200	3.237 ± 0.080	2.5	101.2
II	0.200	0.218 ± 0.031	14.2	109.0
	0.400	0.419 ± 0.045	10.7	104.8
	0.800	0.803 ± 0.044	5.5	100.4
	1.600	1.612 ± 0.043	2.7	100.8
	2.400	2.384 ± 0.048	2.0	99.3
	3.200	3.188 ± 0.057	1.8	99.6
	4.000	3.935 ± 0.020	0.5	98.4
III	0.400	0.469 ± 0.034	7.2	117.3
	0.800	0.822 ± 0.056	6.8	102.8
	1.600	1.691 ± 0.143	8.5	105.7
	2.400	2.403 ± 0.109	4.5	100.1
	3.200	3.135 ± 0.233	7.4	98.0
	4.000	3.828 ± 0.043	1.1	95.7

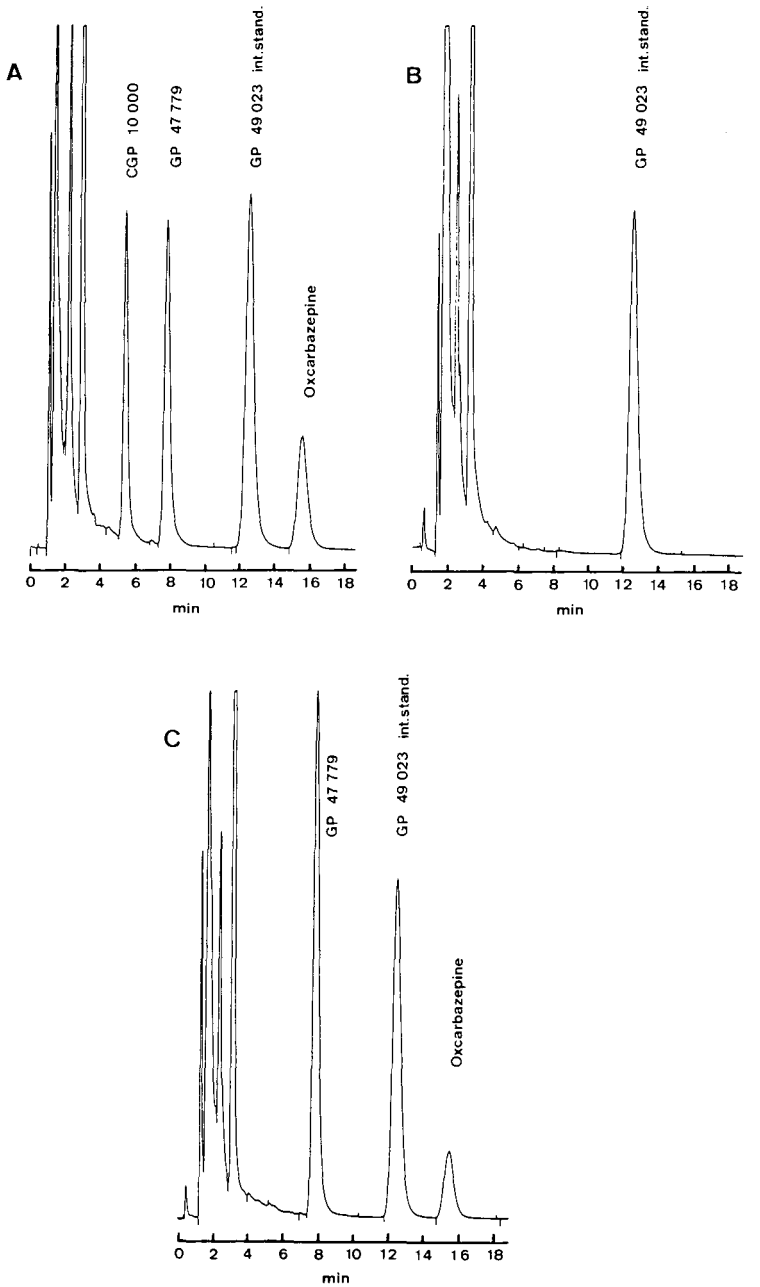

Fig. 3. (A) Chromatogram obtained from analysis of a spiked human plasma sample containing 0.8 μg/g each of oxcarbazepine, II and III. (B) Chromatogram obtained from analysis of a blank plasma sample. (C) Chromatogram obtained from analysis of a plasma sample withdrawn from a healthy volunteer after administration of one 300-mg tablet of oxcarbazepine.

Plasma concentrations of oxcarbazepine and its metabolites

After administration of one 300-mg tablet of oxcarbazepine to a healthy volunteer, the plasma concentrations of unchanged oxcarbazepine reached a maximum level of 0.4 μg/g 1 h after intake of the dose, and dropped below the limit of quantitation (0.1 μg/g) within 3 h.

The main metabolite II reached a maximum plasma concentration of 2.5 μg/g 4 h after administration.

The plasma concentrations of the minor metabolite III never exceeded the limit of quantitation (0.1 μg/g) after the 300-mg dose (Fig. 4).

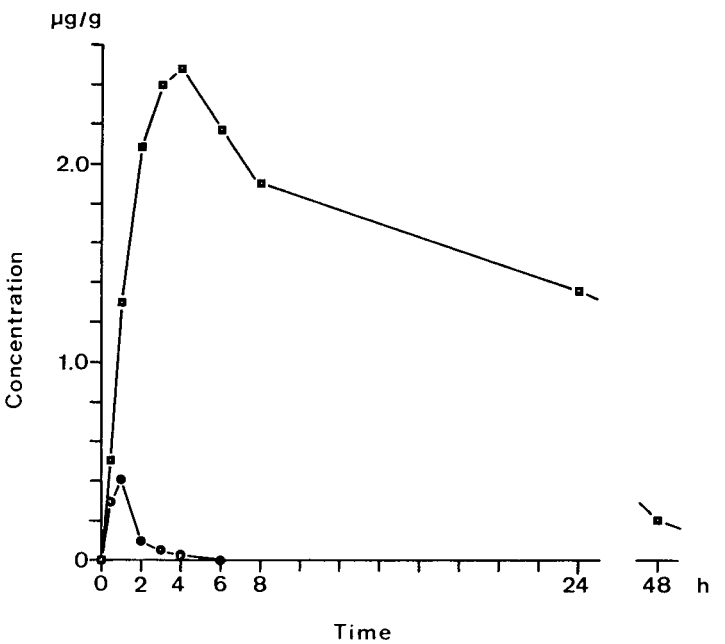

Fig. 4. Plasma concentrations of oxcarbazepine (•——•) and of the main metabolite II (▪——▪) after administration of 300 mg of oxcarbazepine to a healthy volunteer.

CONCLUSIONS

The HPLC assay described for routine analysis of oxcarbazepine and two of its metabolites using 10,11-epoxy-carbamazepine as internal standard is highly sensitive due to the good extractabilities and the use of optimum wavelength for the detection of all compounds. The procedure is able to quantitate plasma levels as they emerge after single oral doses of oxcarbazepine.

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CHROMBIO. 1641

Note

Ion-exchange high-performance liquid chromatography in drug assay in biological fluids

II. Verapamil

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In continuation of our study of the potentialities of ion-exchange sorbents in high-performance liquid chromatography (HPLC) of drugs in biological fluids [1], we applied such sorbents for the determination of verapamil in serum, saliva and urine. Verapamil hydrochloride [2,8-bis(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile hydrochloride] is a potent cardiovascular drug with antianginal and antiarrhythmic activity [2].

Current methods for analysing verapamil in biological fluids are based on gas—liquid chromatography [3—6] and HPLC with reversed-phase columns [7—9]. Two of them [3,9] are able to determine also the N-desmethylated metabolite of verapamil (norverapamil) which is pharmacologically active [2]. The method presented here allows good separation of unchanged verapamil and its three main metabolites: norverapamil, 2-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaheptanitrile (D 617) and 2-(3,4-dimethoxyphenyl)-2-propylamino-3-methylbutyronitrile (D 620).

While this work was in progress two papers appeared concerning HPLC determination of verapamil. One of them [10] described ion-paired reversed-phase separation of verapamil and its seven metabolites, but did not allow norverapamil determination. Cole et al. [11] separated the same compounds as we did using adsorption column, but their method was suitable only for plasma or serum. It is necessary also to note that potassium bromide, which was used by Cole et al. as a mobile phase component, is not recommended along with other halide salts by manufacturers of commonly used liquid chromatographic systems.

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EXPERIMENTAL

Apparatus and columns

The chromatographic system consisted of a Model 110 pump (Altex, Berkeley, CA, U.S.A.), a Model 7120 injection valve (Rheodyne, Berkeley, CA, U.S.A.) and a Model FS-970 fluorescence detector (Schoeffel, Westwood, NY, U.S.A.). The separation was carried out on a 25 cm × 4.6 mm I.D. Partisil 10 SCX column (Altex), with a precolumn of 4 cm × 3.2 mm I.D. packed by us with the same sorbent. Recorder Model 355 (Linear Instruments, Irvine, CA, U.S.A.) was used.

Reagents and standards

Pentane and amyl alcohol were of pure grade and were distilled in glass prior to use. Diethylamine was of pure grade and was distilled over sodium hydroxide. Acetonitrile (LiChrosolv®, E. Merck, Darmstadt, G.F.R.) was used as received. Other reagents were of analytical grade or of chemically pure grade. The water used for preparation of solutions and the mobile phase was double distilled in glass.

Verapamil hydrochloride (LEK, Ljubljana, Yugoslavia) and glaucin hydrochloride (Tatchimpharmpreparaty, Kasan, U.S.S.R.) were used for standard solution preparations. Three concentrations were prepared: 100, 10 and 1 μg/ml.

Extraction procedure

Samples of serum (1 ml), saliva (1 ml) or urine (0.1—1.0 ml aliquot) were pipetted into 15-ml glass-stoppered Pyrex glass tubes (silanised before assay). In the case of urine the volume was adjusted to 1 ml with distilled water. Then 50—100 μl of the internal standard solution (glaucin) of the appropriate concentration were added followed by 100 μl of 0.1 *N* potassium hydroxide solution. Samples were extracted for 30 sec with 5 ml of pentane—amyl alcohol (20:1) using a Vortex mixer. Layers were separated by centrifugation for 5 min at 500 *g*; the organic layer was transferred into the conical tube and extraction was repeated with 2 ml of the same mixture for 15 sec. The combined extract was vortexed with 100 μl of 0.1 *N* sulphuric acid. After a short centrifugation the acidic extract (50—90 μl) was injected into the column.

Chromatographic conditions

The mobile phase was prepared by mixing acetonitrile (200 ml), diethylamine (0.86 ml), glacial acetic acid (0.57 ml) and water (up to 1000 ml). The mixture was degassed under vacuum. The flow-rate was 2 ml/min, the column temperature was ambient.

The excitation wavelength was 206 nm, the emission filter was not used. Sensitivity was set at 3.5; time constant was 0.5 sec.

Quantitation

The internal standard method was used for verapamil determination using glaucin (4,5,7,8-tetramethylaporphine hydrochloride) as the internal standard. Calibration curves were obtained by assaying the samples spiked with verapamil

in the concentration range 10—500 ng/ml (10—1000 ng/ml for urine). Peak height ratios were plotted against verapamil concentrations. Recovery was assessed by comparing peak heights after analysis of extracted drugs and standard solutions.

Mass spectrometry

The peaks were collected in conical tubes and the mobile phase was evaporated by rotary evaporator at 60°C. Residue was reconstituted in methanol. The mass spectra were obtained on a Varian MAT-112 mass spectrometer at 50 eV with direct injection of samples.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of extracts of blank serum (a) and serum spiked with verapamil and internal standard (glaucin), 20 and 10 ng/ml, respectively (b). In Fig. 2a the chromatogram of urine free of drugs is presented. As one can see, there are no peaks on the chromatograms which interfere with verapamil and glaucin. The same results were obtained with saliva. Fig. 1c and Fig. 2b show, respectively, chromatograms of extracts of serum and urine taken from a patient who received verapamil orally. Peak 5 represents glaucin, and peak 4 is intact verapamil; this was confirmed by the mass spectrum, which fully coincides with one published [6]: *m/e* 454 (M^+, relative intensity 0.5%), 305 (3%), 304 (29%), 303 (100%), 261 (1%), 260 (5%). Peak 3 was identified as norverapamil: *m/e* 440 (M^+, 0.5%), 439 (0.7%), 290 (24%), 289 (100%), 260 (10%); this also corresponded to published data [6]. Peak 2 represents D 617, the product of N-dealkylation of verapamil: *m/e* 291 (1%), 290 (M^+, 3%), 259 (1%), 247 (3%), 219 (1%), 216 (2%). The peak of maximum intensity had *m/e* 44, which coincided with previous data [6]. The mass spectrum of peak 1 is presented in Fig. 3. The *m/e* values of peaks in it correspond to the fragmentation scheme of D 620, but relative intensities differ from those presented previously [6]. We suppose, however, that peak 1 corresponded exactly to the product of verapamil N-dealkylation because the rest of the verapamil metabolites had phenolic hydroxy groups and could not be extracted from alkaline medium.

Thus, our method gives the possibility to determine simultaneously intact verapamil and its three main metabolites. As we did not have the metabolite standards at our disposal we only quantitated intact verapamil.

In Table I regression equations of the calibration curves for the determination of verapamil in serum, saliva and urine are presented. Because the therapeutic levels of verapamil do not exceed 500 ng/ml [2], the calibration curves for serum and saliva were constructed in the range 10—500 ng/ml. For urine the range 10—1000 ng/ml was chosen. The correlation coefficients for the regression lines given in Table I represent the excellent linearity of the calibration curves.

The recovery was determined at two levels of verapamil and glaucin in serum, saliva and urine, and the results are given in Table II. As one can see, the recovery of verapamil exceeds 65% and that of glaucin 70%.

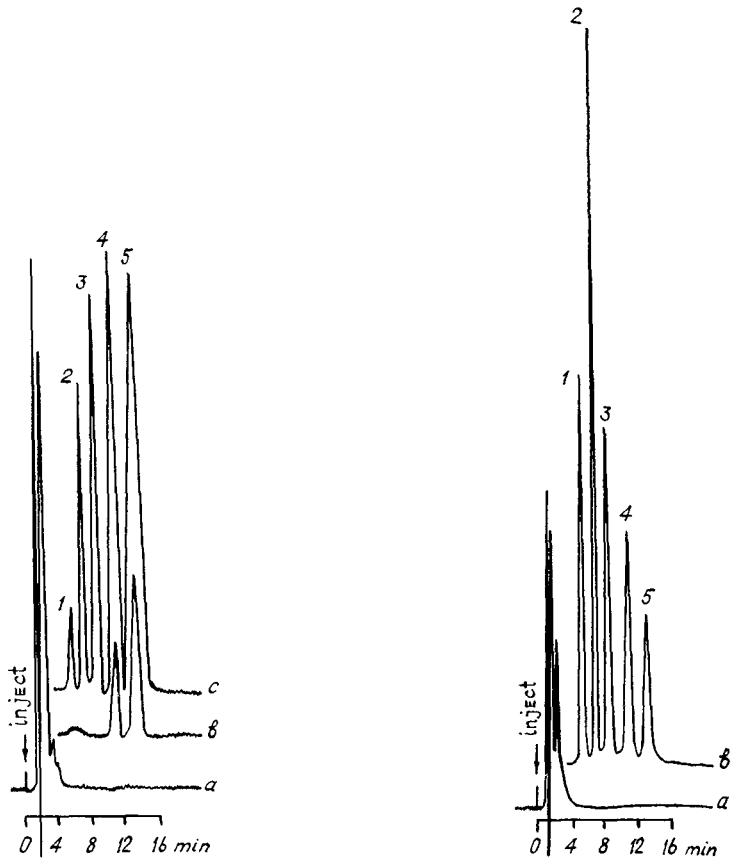

Fig. 1. (a) Chromatogram of the extract of a blank serum. (b) Chromatogram of the extract of the same serum spiked with 20 ng of verapamil and 10 ng of glaucin (the scale range is 0.02 μA). (c) Chromatogram of the serum extract of a patient who received verapamil orally. Peaks: 1 = D 620, 2 = D 617, 3 = norverapamil, 4 = verapamil, 5 = internal standard (glaucin).

Fig. 2. (a) Chromatogram of the extract of a blank urine. (b) Chromatogram of the urine extract of a patient who received verapamil orally. Peak identification as in Fig. 1.

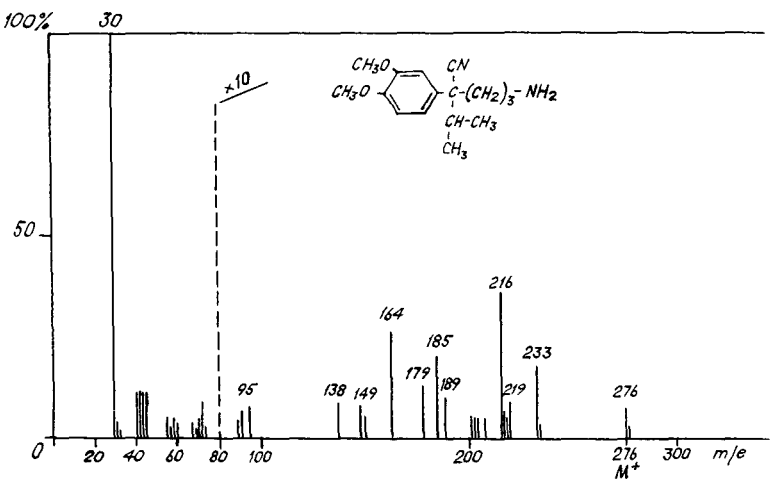

Fig. 3. Mass spectrum of metabolite 1. For mass spectrometric conditions see text.

TABLE I

REGRESSION EQUATIONS OF THE CALIBRATION CURVES FOR VERAPAMIL ASSAY IN SERUM, SALIVA AND URINE

Biological fluid	Regression equation*	Correlation coefficient
Serum	$y = 0.0030x + 0.0102$	0.995
Saliva	$y = 0.0031x + 0.0165$	0.995
Urine	$y = 0.0038x + 0.0313$	0.990

*x = verapamil concentration; y = verapamil to glaucin peak height ratio.

TABLE II

EXTRACTION YIELD OF VERAPAMIL AND GLAUCIN FROM SERUM, SALIVA AND URINE

Concentration added (ng/ml)		n	Recovery (%)	Coefficient of variation (%)
Serum				
Verapamil	20	10	67.8 ± 1.9	9.1
	100	5	71.7 ± 2.8	8.6
Glaucin	50	5	78.2 ± 2.7	7.7
	100	5	80.1 ± 2.4	7.5
Saliva				
Verapamil	20	5	66.5 ± 3.8	11.9
	100	7	66.7 ± 1.5	5.8
Glaucin	50	7	80.4 ± 2.3	7.7
	100	5	80.7 ± 2.2	6.5
Urine				
Verapamil	50	5	80.5 ± 4.2	11.5
	500	6	85.4 ± 1.4	4.2
Glaucin	100	5	69.5 ± 1.3	4.2
	500	5	72.2 ± 1.2	4.0

TABLE III

ACCURACY OF THE DETERMINATION (day-to-day) OF VERAPAMIL IN SERUM, SALIVA AND URINE

$n = 5$.

Concentration of verapamil (ng/ml)		Coefficient of variation (%)
Serum	20	5.5
	100	4.9
Saliva	20	7.7
	100	6.2
Urine	50	10.3
	500	9.0

TABLE IV

DRUGS WHICH DO NOT INTERFERE IN VERAPAMIL DETERMINATION

Amitriptyline	Isosorbide dinitrate
Amphetamine*	Lidocaine
Amylobarbital	Mexiletine
Aspirin	Nadolol*
Barbital	Nitroglycerin
Butabarbital	Oxazepam
Diazepam	Papaverine
Digoxin	Phenobarbital
Dilantin	Prazosin*
Ephedrine	Propranolol*
Etmosin	Reserpine
Furosemide	Secobarbital
Hypothiazide	Trimecaine

*These drugs interfered with metabolite peaks.

In Table III the data on the accuracy of the varapamil determination are presented. The detection limit of the method is about 5 ng/ml (peak-to-noise ratio 5:1) which is sufficient for the pharmacokinetic study of verapamil.

In Table IV the drugs which do not interfere in the determination of intact verapamil are listed. Certain drugs interfered with the metabolite peaks.

In conclusion, a specific and sensitive method has been developed for verapamil assay in serum, saliva and urine using a cation-exchange Partisil 10 SCX column and fluorescence detection, which has some advantages over published methods. The extraction procedure is not time-consuming because the stage of solvent evaporation is excluded. During a day 30 samples may be worked-up. The acid reextract can be stored at 4°C for several days without any change. The chromatographic analysis of one sample takes no more than 16 min.

ACKNOWLEDGEMENTS

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CHROMBIO. 1650

Note

Determination of oxyphenbutazone in human plasma by high-performance liquid chromatography

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Oxyphenbutazone is not only a metabolite of phenylbutazone but it is also extensively used as a drug. Several methods have been described for its quantitative determination in biological fluids, including two laborious gas chromatographic methods [1, 2]. High-performance liquid chromatographic (HPLC) methods have been published recently. Most of them allow the simultaneous determination of phenylbutazone and its main metabolite oxyphenbutazone. Chromatography is performed either on an adsorption column [3, 4] with a sensitivity of 0.77—1.54 μmol/l or on a reversed-phase column [5—11], with the disadvantages of a poor limit of quantitation when the method is performed without extraction [5—7], a lack of accuracy when there is no internal standard [8—10], and a more laborious procedure when two extraction steps are required [11].

This paper describes a simple and rapid procedure which permits the determination of oxyphenbutazone down to 0.154 μmol/l in human plasma by HPLC with a one-step extraction and with phenylbutazone as internal standard.

EXPERIMENTAL

Chemicals and reagents

Oxyphenbutazone and phenylbutazone were supplied by Ciba-Geigy (Basle, Switzerland). Potassium dihydrogen phosphate, phosphoric acid 85% and pH 5 Titrisol buffer were purchased from Merck (Darmstadt, G.F.R.). Toluene (Pestipur, SDS, Peypin, France) and methanol were of analytical grade.

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Chromatography equipment and conditions

Chromatography is performed on a Hewlett-Packard 1081 B high-performance liquid chromatograph equipped with a fixed-wavelength (254 nm) UV detector. The peak areas are obtained with a Perkin-Elmer data system (Sigma 10) connected to the chromatograph. The chromatographic column (stainless-steel tube, 25 cm × 4.7 mm I.D.) is filled with LiChrosorb RP-18, 10 μm particle size (Merck 9334), using the slurry packing technique. Chromatography is performed at room temperature using the degassed mobile phase methanol—phosphate buffer pH 4 (63:37, v/v) at a constant flow-rate of 1.5 ml/min. The top pressure is about 90 bars.

Extraction

Five-hundred microliters (3.2 nmol) of a phenylbutazone methanolic solution are introduced into a glass tube and evaporated under nitrogen at 37°C. Then 1 ml of plasma, 1 ml of buffer pH 5 and 5 ml of toluene are added. The tube is stoppered and shaken mechanically for 15 min at 300 rpm (Infors shaker), then centrifuged for 10 min at 2500 *g*. A maximum volume of organic phase is transferred into another tube and taken to dryness under a nitrogen stream at 45°C. Two hundred and fifty microliters of the mobile phase are added to the dry residue, the tube is shaken on a Vortex mixer and 40 μl are injected onto the chromatographic column.

Calibration

The calibration samples are prepared by introducing 500 μl of the internal standard solution and 50 μl of suitable methanolic solutions containing oxyphenbutazone into glass tubes. Methanol is evaporated under nitrogen, the compounds are redissolved in 1 ml of plasma. The added amounts of oxyphenbutazone correspond to plasma concentrations ranging from 0.154 to 154 μmol/l. A calibration curve is generated on every analysis day.

RESULTS AND DISCUSSION

Recovery and precision

Various spiked plasma samples were prepared and analysed several times. Table I shows the within-day reproducibility over the concentration range 0.154—154 μmol/l. The six replicate samples of each concentration were analysed on the same day.

Table II shows the day-to-day reproducibility of standard curves. A least-squares ln—ln regression line was generated from the 29 data points of six standard curves and each concentration added to plasma was calculated from this regression line.

Reproducibility of calibration curves

Day-to-day reproducibility was tested by expressing each data point as a percentage of the value read off the ln—ln line for the corresponding concentration (Table II). The distribution of these normalized (concentration independent) data had an overall average (± C.V.) of 101.0 ± 12.9%. This result shows a variability especially for the lower concentrations, and it is necessary to establish a calibration curve daily.

TABLE I

PRECISION AND RECOVERY IN THE WITHIN-DAY DETERMINATION OF OXYPHENBUTAZONE APPLIED TO SPIKED HUMAN PLASMA SAMPLES

Concentration added (μmol/l)	Mean concentration found (n = 6) (μmol/l)	Coefficient of variation (%)	Mean recovery (%)
0.154	0.142	6.7	92
0.617	0.601	6.9	97
1.54	1.50	3.6	97
3.09	3.18	2.6	103
15.4	16.70	4.5	108.5
30.9	30.15	5.5	98
154	144	6.2	93

Mean recovery ± C.V. (%) 98.4 ± 7.1

TABLE II

DAY-TO-DAY REPRODUCIBILITY OF CALIBRATION CURVES USED TO DETERMINE OXYPHENBUTAZONE IN PLASMA

Concentration added to plasma (μmol/l)	Peak area ratios							$100 \times \frac{E}{C}$
	Experimental (E)						Calculated from ln–ln regression line (C)	
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6		
0.154	0.017						0.0273	64
		0.033						120
			0.029					106
				0.031				113
					0.019			70
						0.032		117
0.617	0.111						0.114	97
		0.140						123
			0.115					101
				0.108				95
					0.105			92
						0.110		96
3.09	0.625						0.603	104
		0.651						108
			0.651					108
				0.693				115
					0.631			105
						0.701		116
30.9	—						6.49	—
		6.54						101
			6.24					96
				5.93				91
					7.13			110
						7.08		109
154	32.83						34.21	96
		33.19						97
			32.56					95
				31.22				91
					32.63			95
						33.85		99

Average ± C.V. (%): 101.0 ± 12.9

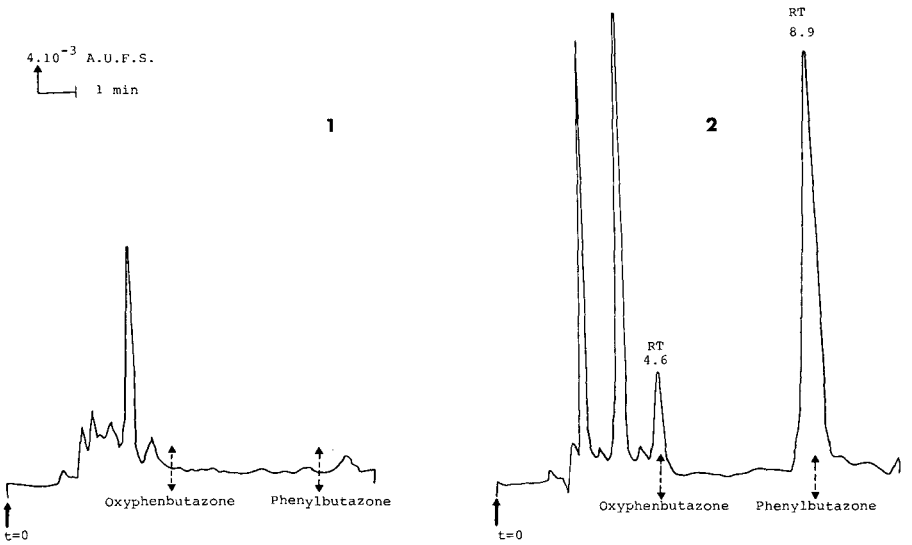

Fig. 1. Examples of chromatograms: (1) human plasma blank (1 ml of plasma); (2) human plasma containing 0.617 μmol/l oxyphenbutazone and 3.2 nmol of phenylbutazone (internal standard). RT = retention time (min).

Plasma interference and selectivity

Fig. 1 shows the chromatograms corresponding to extracts of 1 ml of human plasma and of the same plasma spiked with 617 nmol/l oxyphenbutazone and 3.2 nmol of internal standard. These chromatograms demonstrate that there is no interference from the normal plasma components.

Oxyphenbutazone in urine

The HPLC method described above was not applied to determine oxyphenbutazone in human urine. A very low percentage of the dose (2.3%) has been found as the unchanged drug in human urine [12]. Moreover, the metabolism of oxyphenbutazone is not well known: it was only reported that about 25% of the dose is found in urine as the oxyphenbutazone O-glucuronide [13, 14].

Stability

Oxyphenbutazone was found to be stable in plasma for at least three months when kept at −20°C.

Application

This method was applied to study the oxyphenbutazone plasma concentrations in six subjects after a single administration of one 250-mg oxyphenbutazone suppository (Fig. 2).

CONCLUSIONS

With the described method, plasma concentrations down to 154 nmol/l (50 ng/ml) of oxyphenbutazone can be determined using a 1-ml sample. This limit of quantitation is convenient for pharmacokinetic and bioavailability

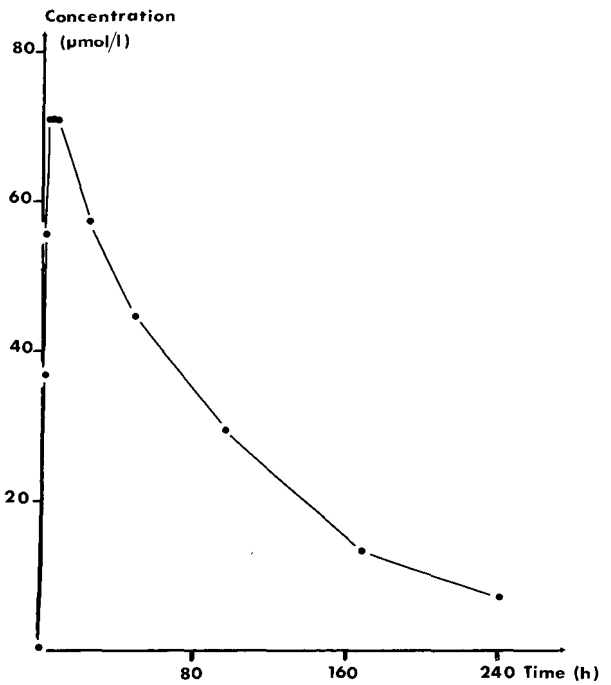

Fig. 2. Oxyphenbutazone mean concentrations in plasma after administration of one 250-mg oxyphenbutazone suppository to six subjects.

studies. It also permits oxyphenbutazone determinations in small volumes of plasma (100 μl), which could be useful when the drug is administered to children.

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Note

Analysis of D-penicillamine by high-performance liquid chromatography with glassy carbon electrochemical detection

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(First received September 10th, 1982; revised manuscript received January 28th, 1983)

Electrochemical detection in conjunction with high-performance liquid chromatography (HPLC) has mostly been applied to the analysis of catecholamines and related compounds. The use of glassy carbon electrodes in electrochemical detection for the analysis of thiol compounds is a new application of the technique and underlines the importance of electrochemical detection in fields other than those related to catechols.

Penicillamine (α-amino-β-methyl-β-mercaptobutanoic acid) is a compound of importance in the therapy of several diseases, including rheumatoid arthritis [1], Wilson's disease [2] and in the treatment of heavy metal poisoning [3].

Measurement of low concentrations of penicillamine in biological fluids presents certain difficulties, in that amino acids and naturally occurring thiol compounds interfere with most assays, and the highly polar nature of both penicillamine and the interfering substances makes separation difficult. Several HPLC techniques for the analysis of penicillamine have been reported recently, they incorporate various detection techniques, including fluorescence derivatisation [4, 5], post-column reaction [6] with Ellman's reagent, performic acid oxidation followed by amino acid analysis procedures [7] and electrochemical detection utilising a mercury-based electrode [8].

Of these, the optical methods require derivatisation before or after chromatography; some are time consuming. The Ellman post-column technique [6] requires a post-column reactor which utilises an extra HPLC pump, and suffers from limited sensitivity which makes it useful for urine analysis, but not for plasma.

Electrochemical detection employing a mercury-based electrochemical detector has been applied to penicillamine assay [7]; however the mercury-

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based electrode employed is difficult to maintain and prone to sudden sensitivity changes. The glassy carbon electrode on the other hand is simple to maintain and has a long life during which its detection sensitivity changes little. The glassy carbon electrode has the added advantage that when operated at a potential of +800 mV non-thiol endogenous compounds present in plasma samples appear not to interfere with analysis of blood samples.

MATERIALS AND METHODS

Chemicals

D-Penicillamine, L-cysteine, DL-homocysteine and glutathione were purchased from Sigma (Poole, Great Britain). 1-Heptanesulphonic acid sodium salt (HSA) was purchased from Magnus Scientific (Sandbach, Great Britain). All other chemicals were of general laboratory reagent grade.

Collection and preparation of blood samples for HPLC

Blood samples (5 ml) were collected from three patients receiving penicillamine (125 mg daily) for the therapy of rheumatoid arthritis, and from volunteers not receiving the drug. The samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) (G.D. Searle & Co., High Wycombe, Great Britain; blood tubes KE/5). The presence of EDTA was necessary to inhibit the blood clotting process and to prevent formation of penicillamine disulphide on storage. As soon after collection as possible the plasma was separated by centrifugation ($2 \cdot 10^3 g$, 10 min) and the plasma proteins precipitated by the addition of trichloroacetic acid (TCA) (200 μl plasma plus 200 μl 18% w/v TCA), followed by standing at 0°C for 10 min. The precipitated protein was removed by centrifugation, and the clear supernatant analysed by HPLC with electrochemical detection, as described below.

It was necessary to precipitate plasma proteins and maintain acid conditions to permit storage (i.e. for several hours) of the penicillamine-containing solution without considerable losses of penicillamine [9].

HPLC equipment

The HPLC system comprised a Constametric III solvent pump (Laboratory Data Control, Stone, Great Britain), a Rheodyne injection valve (Rheodyne, Berkeley, CA, U.S.A.), a Hypersil ODS 25 cm × 4 mm I.D.; particle size 5 μm analytical column (Magnus Scientific) equilibrated with heptanesulphonic acid [6], detection was electrochemical at a potential of +800 mV. The electrochemical detector (LCA 15) and glassy carbon electrode were purchased from EDT Research (London, Great Britain).

Elution solvent

The mobile phase, previously described by Beales et al. [6], consisted of aqueous phosphate buffer (0.25 *M*; pH 7.4) containing EDTA ($4 \cdot 10^{-4}$ *M*) and HSA ($3.5 \cdot 10^{-4}$ *M*). It was used at a flow-rate of 1 ml min^{-1}.

Investigation of endogenous thiols and penicillamine

Aliquots (10 μl) of solutions (10 μg ml^{-1} in 100 mg% aqueous EDTA) of

authentic penicillamine, cysteine, homocysteine and glutathione (solutions prepared immediately before use) were injected onto the analytical column in order to determine the retention volumes of these compounds. Samples (10 μl) of diluted and deproteinised plasma were injected for plasma level determinations.

Calibration graph

A calibration graph was prepared for absolute penicillamine levels between 5 and 20 ng injected onto the column.

Assessment of accuracy

Samples (n = 9) containing 10 ng of D-penicillamine in 10 μl of 100 mg% EDTA were analysed by the above technique. Quantitative variation in the analytical procedure was calculated from these data.

RESULTS AND DISCUSSION

Penicillamine was readily detectable at a glassy carbon electrode at a potential of +800 mV (Fig. 1B). The penicillamine eluted with k' = 1.5 (V_0 = 1.6 ml), gave a linear calibration between 5 and 20 ng in a 10-μl sample, above this value the graph formed a plateau. The detection limit for the technique was 2 ng (i.e. 0.4 μg cm^{-3} plasma) in comparison with 10 ng for the Ellman post-column colorimetric detection system [6].

Comparison of deproteinised plasma samples from volunteers not given penicillamine with patients receiving penicillamine for the treatment of rheumatoid arthritis, demonstrated a peak with a k' corresponding to penicillamine in the latter group (Fig. 1A). Interfering compounds which co-chromatograph with penicillamine were not present in the control plasma. Plasma levels of free penicillamine in three patients receiving a daily dose of 125 mg of penicillamine were 2.5 ± 0.5 μg ml^{-1} (mean ± S.D.), approximately 8—9 h after the penicillamine dose.

Interference by naturally occurring thiols was found not to be a problem since cysteine (k' = 0), homocysteine (k' = 0.3) and glutathione (k' = 0.1) all eluted at a very short retention volume, and thus were well separated, chromatographically, from penicillamine (k' = 1.5).

Since electrochemical detection of penicillamine relies upon oxidation of the thiol group, its known metabolites, namely penicillamine disulphide and S-methylpenicillamine [10] were not detectable, this technique is therefore specific for penicillamine per se.

Quantitative variation in the analytical procedure was determined for nine samples of 10 ng penicillamine in 10 μl total injection volume. The meter deflection for these samples was 10.8 ± 0.73 nA, therefore the intra-sample variation was 13.5%. Penicillamine is readily oxidised in aqueous solution to form penicillamine disulphide. This reaction is inhibited by acid conditions in the presence of EDTA [9]; however, in order to minimise inaccuracies in quantification it is essential to prepare standard solutions of, and a standard curve for, penicillamine daily and to analyse plasma samples within 1 h of their collection and deproteination.

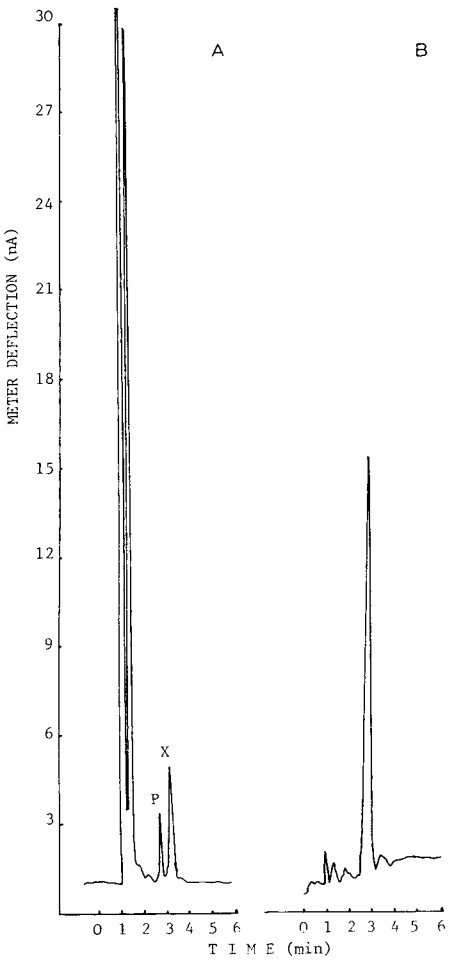

Fig. 1. (A) Analysis of a plasma sample from a patient receiving D-penicillamine using a reversed-phase HPLC column eluted with phosphate buffer (0.25 *M*; pH 7.4) containing EDTA and HSA (flow-rate = 1 ml min^{-1}) followed by electrochemical detection. Peaks: P = penicillamine; X = unidentified endogenous compound also present in control plasma samples. (B) Analysis of 15 ng of authentic D-penicillamine by the same technique as described above.

While ion pairing with HSA seems improbable as the mechanism of penicillamine retention at the pH used in the analytical procedure, good resolution and peak shape for penicillamine in biological samples were not found unless HSA was present. It seems possible that the HSA—octadecyl combination effectively resembles an ion-exchange column and that penicillamine retention was by an ion-exchange mechanism.

HPLC with glassy carbon electrochemical detection is therefore a technique which may be applied to the analysis of penicillamine. The procedure is of high sensitivity and thus may be used for the analysis of plasma samples obtained from patients receiving penicillamine for the treatment of rheumatoid

arthritis. The technique therefore has applications in the routine clinical laboratory and in studies on pharmacokinetics in the research laboratory.

The observations that cysteine, homocysteine and glutathione are electrochemically active underlines the potential usefulness of electrochemical detection at a glassy carbon electrode for the detection of thiol compounds, generally, an application which has received little attention to date.

CONCLUSIONS

A method for the determination of penicillamine in plasma is described. The method utilises reversed-phase HPLC with electrochemical detection at a glassy carbon electrode (+800 mV). The HPLC system used comprised a C_{18} reversed-phase column (Hypersil ODS) and used phosphate buffer (pH 7.4) containing heptanesulphonic acid ($3.5 \cdot 10^{-4}\,M$) and EDTA ($4 \cdot 10^{-4}\,M$) as the mobile phase. A high degree of linearity was found within the on-column injection range 5—20 ng. For 10-ng samples the electrochemical detection response was 10.8 ± 0.73 nA ($n = 9$).

The technique provides a rapid, sensitive method for determining penicillamine in the plasma of patients being treated with the drug.

ACKNOWLEDGEMENTS

The authors are grateful to Dr M. Snaith of University College Hospital for providing the blood samples used in this study and to the Wellcome Trust for financial support.

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Note

Simultaneous determination of nafimidone [1-(2-naphthoylmethyl)imidazole], a new anticonvulsant agent, and a major metabolite in plasma by high-performance liquid chromatography

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(First received November 8th, 1982; revised manuscript received January 24th, 1983)

Nafimidone hydrochloride [1-(2-naphthoylmethyl)imidazole] (I) is a new anticonvulsant agent under investigation for use in the treatment of generalised tonic-clonic and partial seizures [1, 2]. Nafimidone has been shown to be rapidly metabolised in rat, dog, monkey and man with the formation of a major non-conjugated metabolite 1-[2-hydroxy-2-(2-naphthyl)ethyl]imidazole (II) [3]. This metabolite has been shown to possess similar anticonvulsant properties to the parent compound [4].

This paper describes two simple high-performance liquid chromatographic (HPLC) methods for the quantitation of nafimidone and its major metabolite in plasma. The methods employ UV and fluorescence detection, the latter involving wavelength switching to optimise sensitivity. The methods have been used to analyse plasma obtained following the administration of nafimidone hydrochloride to dog and monkey.

EXPERIMENTAL

Materials

All reagents were of at least analytical grade except in the case of diethyl ether which was redistilled in glass before use. HPLC grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, Great Britain). Heptanesulphonic acid, used as an ion-pair reagent, was of HPLC grade (Fisons Scientific Apparatus, Loughborough, Great Britain).

Nafimidone, [^{14}C]nafimidone, metabolite II and internal standards, 1-[2-(2-naphthoyl)ethyl]imidazole (III) and 1-[(6-methyl-2-naphthoyl)methyl]-

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imidazole (IV) were supplied as hydrochloride salts by Syntex Research (Palo Alto, CA, U.S.A.).

Preparation of standard solutions

Standard solutions of nafimidone, metabolite II and internal standards III and IV were prepared in 0.02 *M* citrate buffer (pH 2.75). These solutions (5—15 $\mu g\ ml^{-1}$) stored at 4°C were stable for at least three months.

Extraction procedure

Duplicate aliquots of plasma (0.05—0.5 ml) were spiked with internal standard [III (UV) or IV (fluorescence), 10—200 ng] buffered by the addition of McIlvaine buffer (0.19 *M* Na_2HPO_4—0.0064 *M* citric acid, pH 7.6, 1 ml) [5] and extracted once with diethyl ether (6 ml). This pH was employed as nafimidone is unstable at high pH. Extraction consisted of mechanised rotation in stoppered tubes followed by centrifugation. The ether fraction was then extracted with 0.1 *M* hydrochloric acid (1 ml). Following removal of the ether fraction the aqueous fraction was again buffered with McIlvaine buffer (pH 7.6, 2 ml) and extracted with diethyl ether (4 ml). The organic layer was then transferred to conical tubes and evaporated to dryness under oxygen-free nitrogen (45°C). The residue was re-dissolved in 40—100 μl of HPLC mobile phase prior to analysis.

A calibration line was prepared by extraction of aliquots of control plasma (0.5 ml) spiked with nafimidone and metabolite II over the expected concentration range.

Instrumentation

Analyses were performed on a Spectra-Physics Model SP8000A liquid chromatograph (Spectra-Physics, St. Albans, Great Britain) equipped with a Waters Assoc. (Cheshire, Great Britain) Model 710A Intelligent Sample Processor (WISP).

A Model CE2012 variable-wavelength UV detector (Cecil Instruments, Cambridge, Great Britain), or a Model PE3000 fluorescence detector (Perkin-Elmer, Beaconsfield, Great Britain) was used as flow monitor.

Chromatography

Two HPLC systems were used for quantitation of nafimidone and metabolite. Both systems utilised stainless-steel columns 150 mm × 4.5 mm I.D. When UV detection was used columns were packed with ODS Hypersil (5 μm) (Shandon Southern Products, Runcorn, Great Britain) and operating conditions were: mobile phase, methanol—acetonitrile—0.2 *M* ammonium carbonate (40:8:52. v/v), flow-rate, 1 ml min^{-1}; detector, 248 nm (for simultaneous detection of nafimidone and metabolite II) or 225 nm (optimum for detection of metabolite II). Use of fluorescence detection required a different system: column, Partisil 10 ODS-3 (silylated) (Whatman, Maidstone, Great Britain), (10 μm); mobile phase, methanol—acetonitrile—water (all containing 0.005 *M* heptanesulphonic acid and 0.087 *M* acetic acid (44:4:41, v/v), flow-rate 1 ml min^{-1}; detector, for nafimidone and internal standard IV λ_{ex} 245 nm, λ_{em} 456 nm, for metabolite II λ_{ex} 255 nm, λ_{em} 333 nm.

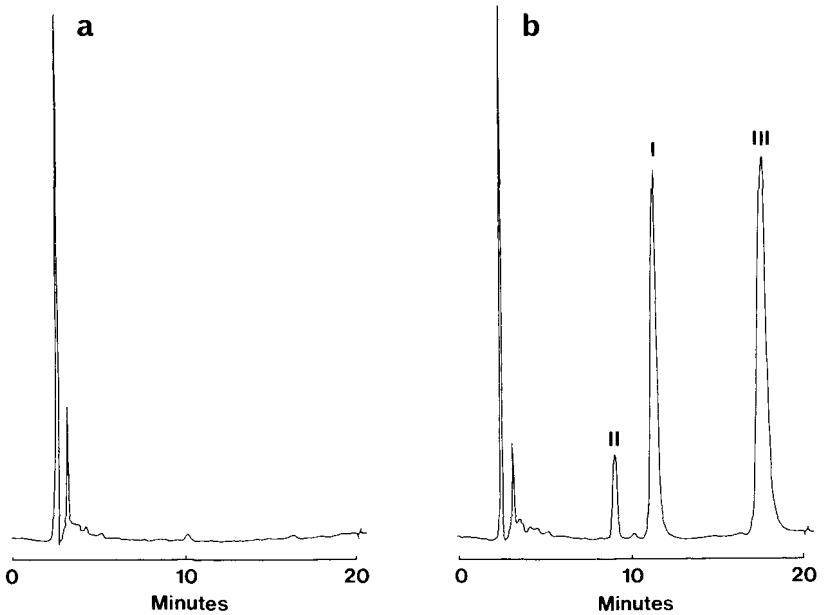

Fig. 1. Chromatograms of extracts of control plasma (a) and of plasma from a male volunteer following administration of nafimidone hydrochloride showing nafimidone (I), metabolite II, and internal standard (III) using UV detection (248 nm) (b).

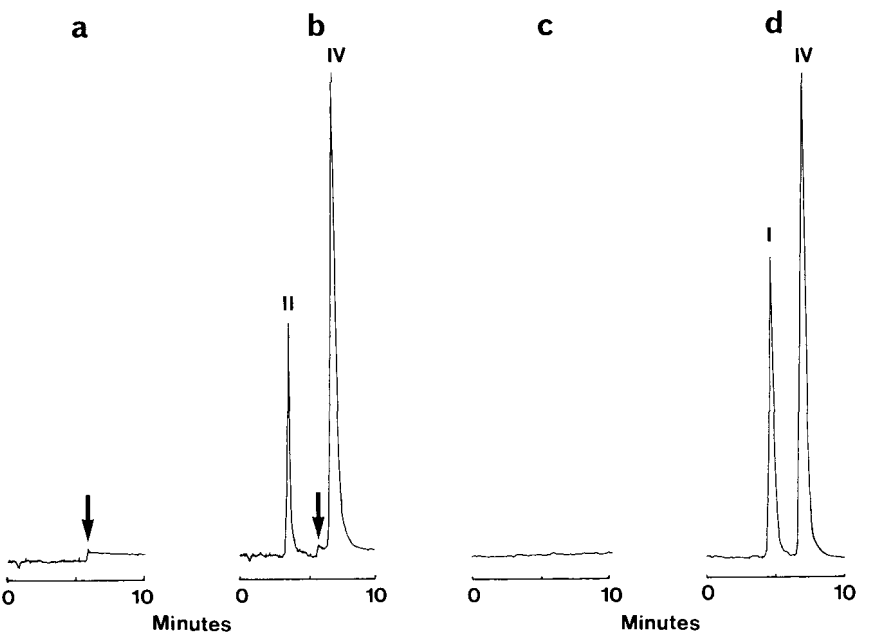

Fig. 2. Chromatograms of extracts of control plasma (a, c) and of plasma from a male volunteer following administration of nafimidone hydrochloride (b, d) showing nafimidone (I), metabolite II and internal standard (IV). Arrows indicate wavelength change from λ_{ex} 255 nm to 245 nm, λ_{em} from 333 nm to 456 nm (a, b). For c and d, λ_{ex} = 245 nm; λ_{em} = 456 nm.

Using the ion-pair HPLC system resolution of nafimidone and metabolite II was incomplete and two injections were made of each extract with the fluorimeter set up for each compound in turn. Nafimidone is not detectable at the optimum excitation/emission wavelengths for metabolite II and the converse is also true. Chromatograms are shown in Figs. 1 and 2.

RESULTS AND DISCUSSION

The precision and accuracy of the method using both detectors was determined by preparing pools of plasma containing nafimidone and metabolite II at several concentrations. Multiple analyses were performed using this spiked plasma and the accuracy and assay variance computed from the results. The results using each detection method are given in Table I.

TABLE I

PRECISION AND ACCURACY FOR THE DETERMINATION OF NAFIMIDONE (I) AND METABOLITE (II) IN PLASMA USING HPLC WITH UV OR FLUORESCENCE DETECTION

UV				Fluorescence			
Actual concn. (ng ml^{-1})	Observed (mean ± S.D., $n = 6$)	Variance	% of actual	Actual concn. (ng ml^{-1})	Observed (mean ± S.D., $n = 6$)	Variance	% of actual
Nafimidone							
26	25.5 ± 0.43	1.7	98	43.6	41.7 ± 0.69	1.6	96
127	135 ± 5.1	3.8	106	209	207 ± 7.9	3.8	99
362	378 ± 15.2	4.0	104	396	388 ± 26.0	6.7	98
Metabolite II							
204	209 ± 11.2	5.4	102	511	519 ± 23.7	4.6	102
798	811 ± 38.6	4.8	102	2440	2530 ± 138	5.5	104
1900	1940 ± 108	5.6	102	4640	4710 ± 354	7.5	102

The recovery of nafimidone from plasma was checked by spiking aliquots of control plasma with [^{14}C]nafimidone, extracting as described and determining the amount of radioactivity in the final extract by liquid scintillation counting. Plasma was spiked at 100 ng ml^{-1} and 1 μg ml^{-1} and the percentage recoveries in the final ether extract were 65 ± 7% (S.D.) and 74 ± 5% (S.D.), respectively.

The detection limits for each compound vary with the method used. The limits for each compound for each method are given in Table II. These are based on the extraction of 0.5 ml plasma. The signal-to-noise ratios for the peaks at the limits range from 3 to 6.

Using the extraction methods described no plasma components interfere with the assay. Metabolite II is the only non-conjugated metabolite to have

TABLE II

DETECTION LIMITS FOR THE QUANTITATION OF NAFIMIDONE (I) AND METABOLITE II IN PLASMA USING HPLC WITH UV OR FLUORESCENCE DETECTION

Limits are based on extraction of 0.5 ml plasma. Wavelengths are expressed as nm, detection limits are expressed as ng ml^{-1}.

Detector	Compound	λ_{abs}	λ_{ex}	λ_{em}	Detection limit
UV	I	248	—	—	10
UV	II	248	—	—	100
UV	II	225	—	—	10
Fluorescence	I	—	245	456	3
Fluorescence	II	—	255	333	5

been isolated and identified so it cannot be said with certainty that the assay is specific. However radiochemical studies have shown that II is the main, if not only, non-conjugated metabolite found in plasma of rat, dog and monkey.

Analysis of control plasma spiked with nafimidone or metabolite II has shown that there is no detectable interconversion of the two compounds in vitro.

The above methods of analysis were applied to plasma samples from dog and monkey following single oral doses (10 mg kg^{-1}) of nafimidone hydrochloride administered in solution (0.2% w/w in 0.02 *M* citrate buffer, pH 2.75). Plasma profiles of nafimidone and metabolite II are shown in Fig. 3.

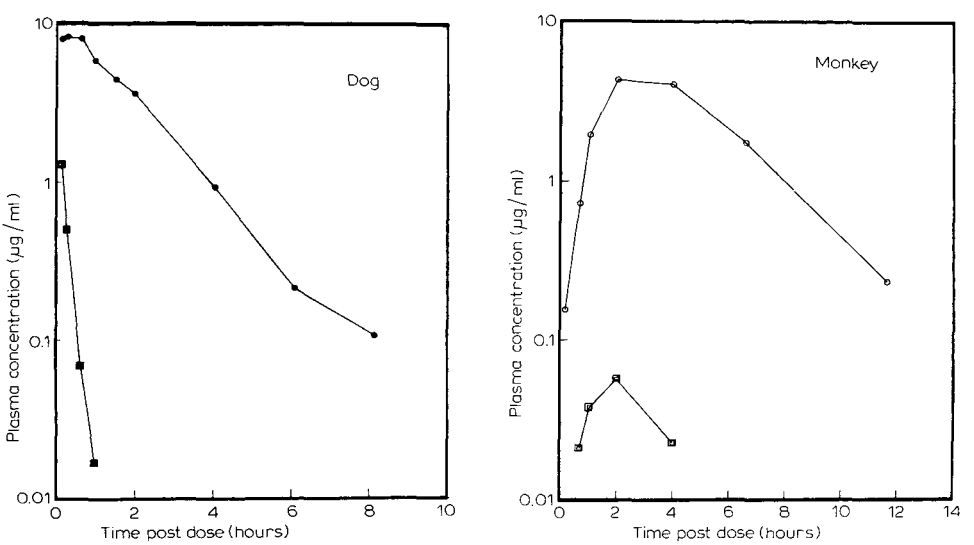

Fig. 3. Plasma profiles of nafimidone (I) (▪, □) and metabolite II (•, ○) in dog and monkey following single oral doses of nafimidone hydrochloride at a dose level of 10 mg kg^{-1}.

The interaction of the commonly administered anticonvulsants, phenytoin, carbamazepine, phenobarbitone and sodium valproate with the assay of nafimidone and metabolite II has been studied. None of the anticonvulsants fluoresce at the excitation—emission wavelengths used so do not interfere with the fluorescence assay. However, using the UV assay modification of the mobile phase is necessary to resolve carbamazepine from the compounds of interest. None of the other drugs interfere with the UV assay.

Both of the above methods afford similar limits of detection with regard to the metabolite II. As this is the more significant species biologically either method will be suitable for the pharmacokinetic and clinical evaluation of nafimidone. The methods are also suitable for the quantitation of the metabolite II in saliva and thus will allow the validation of salivary estimation as a means of therapeutic monitoring.

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Note

High-performance liquid chromatographic assay for zomepirac and its main metabolite in urine

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(First received November 30th, 1982; revised manuscript received February 4th, 1983)

Zomepirac (Z) [5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid] is an orally active, non-narcotic analgesic agent [1, 2].

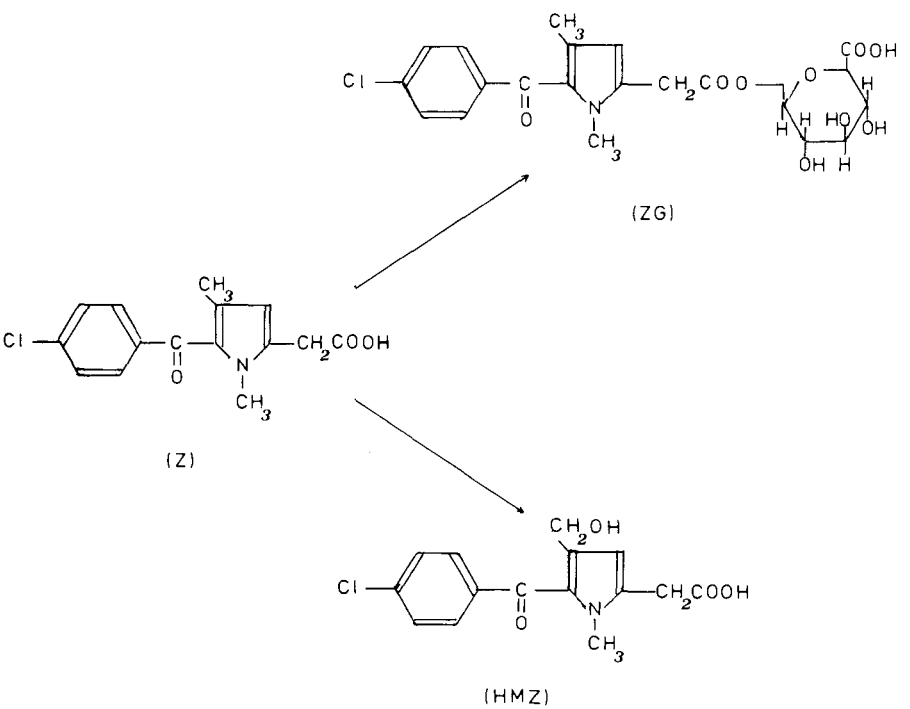

Fig. 1. Metabolic pathway for zomepirac in man.

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The metabolism of zomepirac was studied using [^{14}C]zomepirac and the major urinary metabolites in man were identified by chromatographic and spectroscopic data [3]. Zomepirac is primarily metabolized by conjugation with glucuronic acid to give zomepirac glucuronide (ZG), while hydroxylation yielding hydroxymethylzomepirac (HMZ) is a minor pathway (Fig. 1).

Zomepirac was determined in plasma by normal- [4] and reversed-phase [5] high-performance liquid chromatography (HPLC). Recently gas chromatography—mass spectrometry for determining zomepirac in urine was reported [6]. However, this method required hydrolysis of the conjugated drug followed by extraction and derivatization, and allows the detection only of free zomepirac. In continuing our work on the application of HPLC in biomedical analysis [7, 8], we developed a simple and sensitive method for the quantitative determination of zomepirac and zomepirac glucuronide in human urine. The procedure described involves direct reversed-phase HPLC, using propyl 4-hydroxybenzoate or dantrolene sodium as internal standard.

The peaks were detected at 254 or 317 nm and quantities of each assay component as low as 100 ng/ml could be measured.

EXPERIMENTAL

Materials

Zomepirac sodium was provided by SIT (Mede, Italy).

Zomepirac glucuronide was extracted from urine samples and separated by chromatography on a Sephadex LH-20 column [3]; its structure was confirmed by mass spectra.

Propyl 4-hydroxybenzoate (Fluka, Buchs, Switzerland) and dantrolene sodium (SIT) have been used as internal standards for the assay at 254 and 317 nm, respectively.

All the solvents were of HPLC grade (LiChrosolv; Merck, Darmstadt, G.F.R.). Water was deionized, distilled from alkaline permanganate and filtered through a 0.45-μm membrane filter (Type HA, Millipore, Bedford, MA, U.S.A.).

Chromatography

Chromatographic analyses were performed on a component system consisting of a Model 6000A pump, equipped with a Model U6K universal injector, a Lambda-Max Model 480 ultraviolet (UV) detector, and a Model 730 data module (Waters Assoc., Milford, MA, U.S.A.).

Samples were chromatographed at room temperature on a μBondapak C_{18} column (30 cm × 4 mm I.D., 10 μm) (Waters Assoc.). A pre-column (2.5 cm × 4 mm I.D.) packed with μBondapak C_{18} Corasil was used to prevent deterioration of the main column.

The separations were obtained using acetonitrile—water brought to pH 2.8 with phosphoric acid (50:75) as a mobile phase with the flow-rate of 2 ml/min at 140 bars.

Peaks were detected by 254 or 317 nm absorbance measurements (a.u.f.s. 0.02) with the linearity verified for assay components quantities up to 125 μg/ml.

Solutions

Stock solutions of zomepirac sodium and zomepirac glucuronide were prepared with a concentration of 0.5 mg/ml in the mobile phase. Propyl-4-hydroxybenzoate and dantrolene sodium were dissolved in the mobile phase to give a concentration of 0.1 mg/ml.

Calibration curves

Into individual 10 ml volumetric flasks were placed 1 ml of urine, 1 ml of internal standard (I.S.) solution and accurately pipetted volumes (in the range of 0.005—0.25 ml) of zomepirac sodium and zomepirac glucuronide standard solutions. After dilution to 10 ml using the mobile phase, replicate injections of 10 μl were made for each sample.

Assay method

To 1.0 ml of urine was added 1.0 ml of internal standard solution and diluted to 10 ml with the mobile phase.

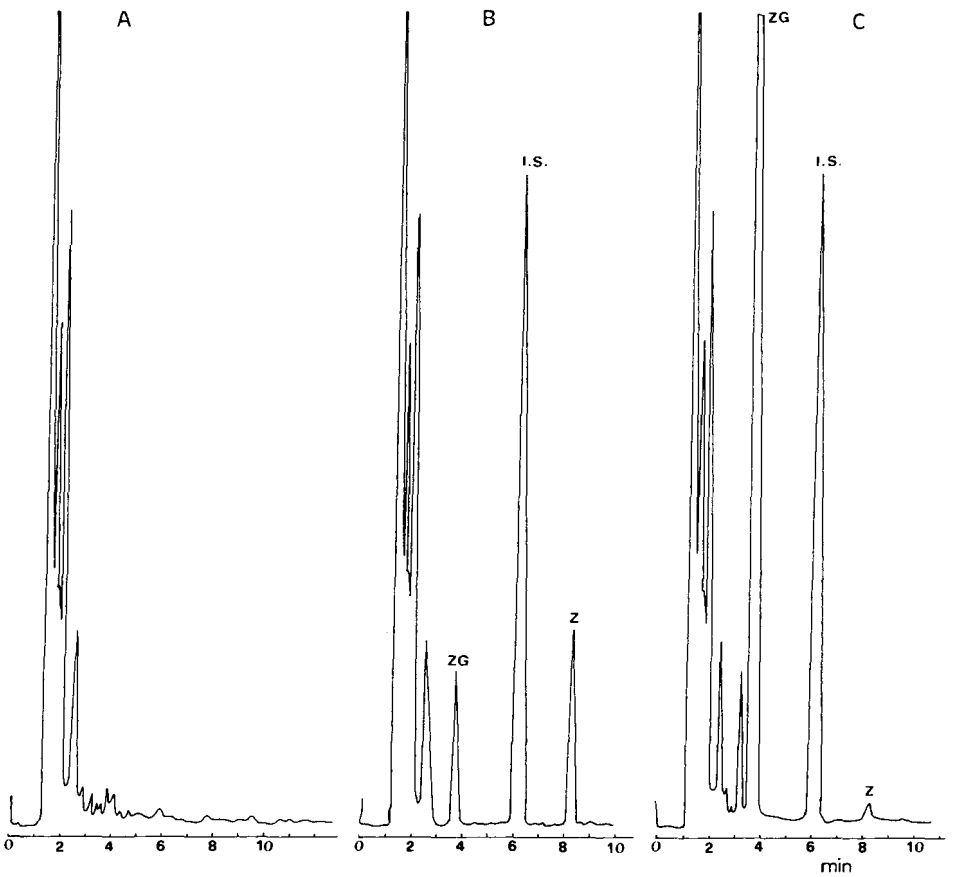

Fig. 2. Chromatograms of (A) blank urine, (B) standard urine containing zomepirac (Z), zomepirac glucuronide (ZG), and propyl-4-hydroxybenzoate (I.S.), 100 ng of each, and (C) urine sample from a volunteer 2 h after the ingestion of a 100-mg tablet of sodium zomepirac. UV detection: 254 nm.

After vortex mixing, replicate injections of 10 μl were made. To assess specificity, a control urine sample (1 ml) was fortified at 100 μg/ml with zomepirac sodium and zomepirac glucuronide and processed through the assay as specified above.

RESULTS AND DISCUSSION

Fig. 2A and B show chromatograms obtained from blank urine (A) and from standard urine (B) containing zomepirac, zomepirac glucuronide, and propyl-4-hydroxybenzoate (internal standard), 100 ng of each. Fig. 2C is a chromatogram of a representative sample from a volunteer following the oral ingestion of a 100-mg zomepirac sodium tablet (Zomax: Cilag-Chemie). These chromatograms demonstrate the lack of interference and the specificity of the assay procedure for the measurement of zomepirac and its main metab-

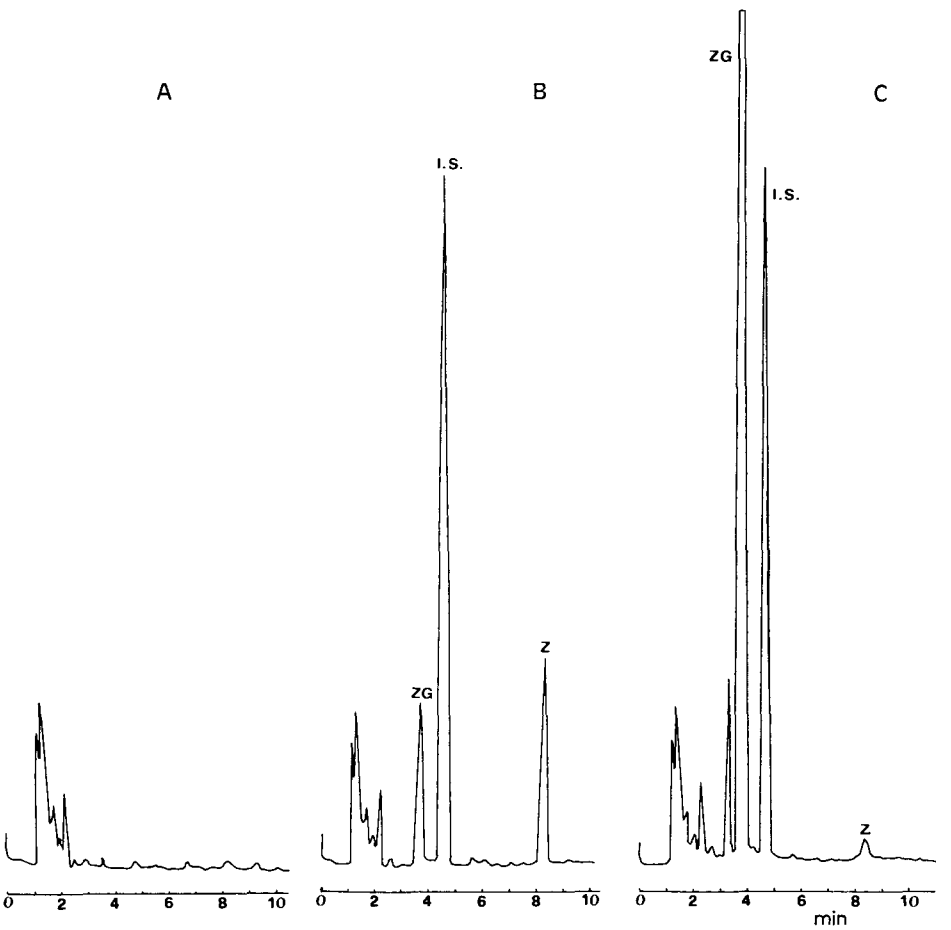

Fig. 3. Chromatograms of (A) blank urine, (B) standard urine containing zomepirac (Z), zomepirac glucuronide (ZG) and sodium dantrolene (I.S.), 100 ng of each, and (C) urine sample from a volunteer 2 h after the ingestion of a 100-mg tablet of sodium zomepirac. UV detection: 317 nm.

olite in urine. The peak eluted with a retention time of 3.2 min and detected at both 254 and 317 nm is probably due to hydroxymethylzomepirac. However, owing to the difficulty in isolating this minor metabolite, no direct evidence can be produced. Interfering peaks from endogenous substances were diminished by monitoring the eluate at 317 nm, where zomepirac and zomepirac glucuronide absorb strongly. In these conditions, sodium dantrolene has been used successfully as the internal standard (Fig. 3A—C).

The method was evaluated over a concentration range of 2.5—125 μg of zomepirac sodium and zomepirac glucuronide per ml of urine. The spiked samples were taken through the analytical procedure. Linear relationships between the ratios of peak areas (zomepirac sodium and zomepirac glucuronide to internal standards) and concentrations (μg/ml) of zomepirac sodium and

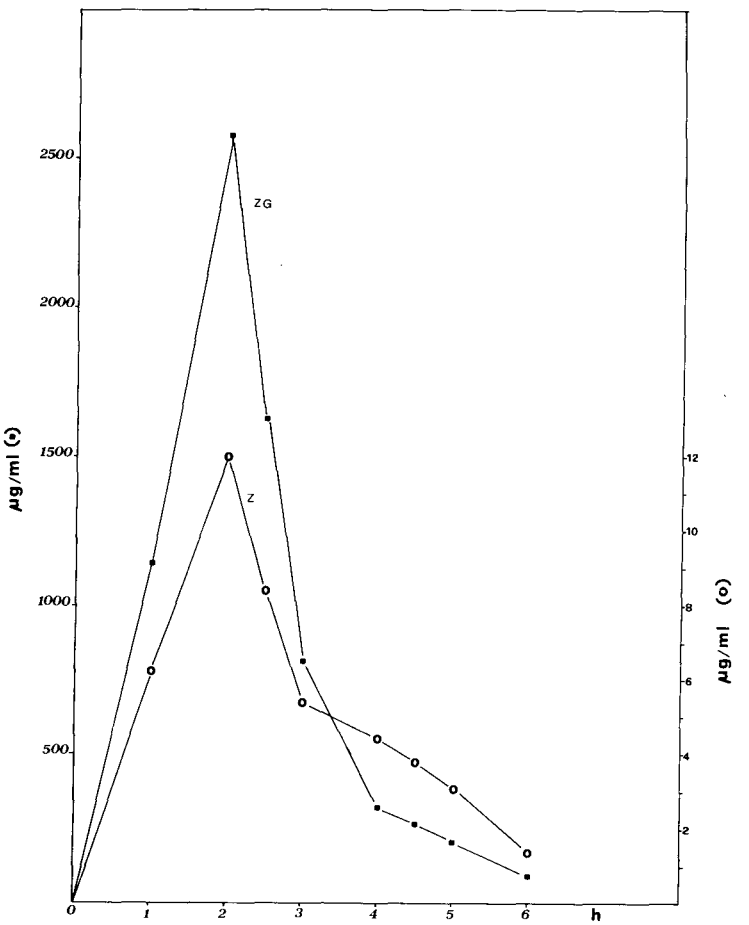

Fig. 4. Urine zomepirac glucuronide (▪) and zomepirac (○) concentration—time curves obtained in a volunteer following oral administration of a 100-mg tablet of zomepirac sodium.

zomepirac glucuronide were found and can be expressed by the following equations

zomepirac sodium

$Y = 0.0043X - 0.0162$ $r = 0.996$ (I.S. = propyl-4-hydroxybenzoate)

$Y = 0.0049X - 0.0165$ $r = 0.982$ (I.S. = sodium dantrolene)

zomepirac glucuronide

$Y = 0.0028X - 0.0181$ $r = 0.986$ (I.S. = propyl-4-hydroxybenzoate)

$Y = 0.0032X - 0.0149$ $r = 0.989$ (I.S. = sodium dantrolene)

where Y represents the peak area ratio and X the concentration (μg/ml).

Intra-day assay variations (C.V. %) were determined by assaying the spiked samples four times on the same day and were 4.2% and 3.2% for zomepirac sodium and zomepirac glucuronide, respectively. Inter-day assay variations were obtained by analyzing the samples daily for two weeks and resulted in 3.8% and 4.5% for zomepirac sodium and zomepirac glucuronide, respectively.

The lowest limit of detection has been found to be approximately 100 ng/ml. This is adequate since the peak urine concentrations of zomepirac and zomepirac glucuronide following a therapeutic dose (100 mg) are much higher.

A representative plot of urine zomepirac and zomepirac glucuronide concentrations vs. time for a volunteer subject following a 100-mg oral dose is shown in Fig. 4. The results indicate that in man the drug is cleared almost entirely by conjugation.

In conclusion, this HPLC method is a simple, reproducible and sensitive procedure.

ACKNOWLEDGEMENT

The authors greatly appreciate the technical expertise of Mr. De Alberti Secondo.

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CHROMBIO. 1661

Note

Simultaneous determination of some radiosensitizing and chemotherapeutic drugs in plasma by thin-layer chromatography

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Hypoxic cell radiosensitizers, particularly misonidazole, are extensively used in radiotherapy. Since it has been demonstrated that misonidazole can enhance the cytotoxicity of some chemotherapeutic agents [1], recent attention has been directed to the possible therapeutic use of the radiosensitizing drugs in combination chemotherapy [2]. In order to assess the clinical potential of combining radiosensitizers with cytotoxic drugs, the simultaneous study of their pharmacokinetics could be of interest. Consequently, we have developed a thin-layer chromatographic (TLC) method for assaying at the same time some chemotherapeutic agents and radiosensitizers. The drugs that have been investigated are misonidazole (MIS), its metabolite desmethylmisonidazole (DEMIS), 1-methyl-2-nitro-5-vinylimidazole (L 8580), cyclophosphamide (CYP) and 5-fluorouracil (f-FU).

EXPERIMENTAL

Reagents

All chemicals and solvents were of analytical grade. CYP was obtained from Schering (Milan, Italy); MIS, DEMIS, ipronidazole (IPR), ornidazole (ORN), and 5-FU were from Hoffmann-La Roche (Basel, Switzerland). 2-Methyl-5-nitroimidazole (MNI) was supplied by Aldrich Europe (Beerse, Belgium), and L 8580 was from Lepetit (Milan, Italy). All drugs were used without further purification.

Stock standard solutions of the compounds to be assayed were prepared in methanol. All solutions were kept refrigerated in tin-foil-wrapped flasks and prepared fresh every two weeks. Working standard solutions were made by dilution to appropriate concentrations with methanol, and discarded after a single use.

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Extraction procedure

Sep-Pak C_{18} extraction. Plasma samples of 1 ml volume containing known amounts of CYP, MIS, and L 8580 or IPR [as internal standard (I.S.)] or alternatively known amounts of L 8580, CYP, and MNI (as I.S.) were treated with 1 ml of borate buffer (pH 10). The mixture was passed through a Sep-Pak C_{18} cartridge pre-wetted with 3 ml of methanol and 5 ml of buffer solution. The cartridge was washed with 1.5 ml of borate buffer, 1.5 ml of water, and 1.5 ml of methanol. This last fraction, which completely eluted the drugs from the cartridge, was vacuum evaporated to dryness keeping the temperature below 40°C. The residue was redissolved in 100 μl of methanol and 1-μl aliquots were chromatographed with the eluents indicated in Table III.

Solvent extraction. To 1 ml of plasma spiked with known amounts of 5-FU, L 8580 and ORN (as I.S.) or alternatively with MIS, DEMIS, 5-FU and L 8580 (as I.S.), CYP, L 8580 and MNI (as I.S.), and MIS, DEMIS, CYP and L 8580 (as I.S.), was added an equal volume of a saturated ammonium sulphate solution. The mixture was extracted twice with 8 ml of isopropanol—ethyl acetate (1:4, v/v). The extraction was performed by a mechanical shaker for 5 min. After centrifugation the organic phase was vacuum-evaporated to dryness and processed as described above.

Chromatographic procedure

A 1-μl sample was spotted on pre-coated layers of silica gel G-60 F_{254} (aluminium plates 0.25 mm thick, Merck, Darmstadt, G.F.R.) with a Camag (Muttenz, Switzerland) micro-applicator. The layers were pre-washed with the eluent mixture to be used. The TLC plates were developed in saturated (paper-lined) tanks at room temperature. After development, the layers were air-dried and the spots were quantitated by scanning densitometry by means of a Camag TLC/HPTLC 76500 scanner. The readings were performed at 320 nm for MIS, DEMIS, MNI, ORN, and IPR, and at 254 nm for 5-FU and L 8580. After this scanning, the plates were heated for 10 min at 260°C for detecting CYP as previously described [3], and then scanned at 254 nm. The standard curves were prepared by spotting 1 μl of working standard solutions and plotting peak areas against ng applied.

RESULTS AND DISCUSSION

Sep-Pak C_{18} extraction

On the basis of the good results obtained for the extraction of CYP from plasma buffered at pH 10 [3], the same procedure was utilized for extracting the other drugs. The results obtained showed a complete retention of CYP, MIS, L 8580, IPR and MNI by the cartridge. On the other hand, DEMIS and 5-FU were not retained. These two drugs were not retained even if the plasma was buffered at pH 4 or pH 6. As a consequence, we resorted to a solvent extraction from plasma for 5-FU and DEMIS. The recovery and reproducibility of the Sep-Pak C_{18} extraction method are presented in Table I.

Solvent extraction

5-FU was extracted from plasma as described by Min and Garland [4]

TABLE I

RECOVERY OF DRUGS FROM PLASMA AND REPRODUCIBILITY OF THE SEP-PAK C_{18} EXTRACTION

Drug	Amount added (μg/ml)	Recovery (%)	Reproducibility (6 samples)		
			Mean (μg/ml)	± S.D.	C.V. (%)
CYP	31.8	94.6	30.1	1.5	5.1
MIS	52.9	90.2	47.7	2.2	4.6
L 8580	36.1	98.7	35.6	1.1	3.2
IPR	55.7	83.1	46.3	2.2	4.8
MNI	20.2	97.7	19.7	0.8	4.0

TABLE II

RECOVERY OF DRUGS FROM PLASMA AND REPRODUCIBILITY OF THE SOLVENT EXTRACTION

Drug	Amount added (μg/ml)	Recovery (%)	Reproducibility (6 samples)		
			Mean (μg/ml)	± S.D.	C.V. (%)
5-FU	26.7	97.0	25.9	1.1	4.3
L-8580	32.5	101.3	32.1	1.6	4.9
ORN	41.1	101.6	40.5	1.5	3.8
MIS	54.4	94.2	51.3	2.3	4.4
DEMIS	32.9	86.4	31.7	1.6	5.0
CYP	37.9	93.8	35.6	1.1	3.2
MNI	27.9	99.8	27.8	1.1	3.9
IPR	43.0	45.4	—	—	—

utilizing, however, isopropanol—ethyl acetate (1:4, v/v) as the organic phase. In such a way the mean recovery obtained was very good, 97%. We did not perform the benzene wash because we found no effect on the baseline of the chromatogram of the blank plasma. The solvent extraction procedure was found to be suitable also for MIS, its metabolite DEMIS, L 8580, CYP, and ORN, but not for IPR. As a consequence, IPR cannot be used as internal standard when performing solvent extraction. The recovery and reproducibility of the solvent extraction method are presented in Table II.

Chromatographic procedure

The $R_F \times 100$ values given in Table III each represents the average of a minimum of three separate chromatographic runs. Under the conditions described under Experimental all R_F values were reproducible (C.V. = 4%). Fig. 1 shows typical chromatograms obtained from plasma samples spiked with different mixtures of the drugs under investigation. Also shown in the figure are the chromatograms of plasma blanks processed by both the extraction procedures. As can be seen, the drugs are well separated and free from interferences from biological components. The calibration graphs calculated

TABLE III

$R_F \times 100$ VALUES OF CHEMOTHERAPEUTIC AND RADIOSENSITIZING DRUGS IN VARIOUS SOLVENT SYSTEMS

Compound	Eluting solvent*			
	I	II	III	IV**
CYP	33	11	52	
MIS	41			36
DEMIS	23			13
L 8580	60	59	71	74
5-FU		19	18	26
MNI		25	33	
ORN		28		
IPR	60			

*Solvent systems used: I = ethyl acetate—dichloromethane—methanol (5:3:1, v/v); II = ethyl acetate—dichloromethane—methanol (15:10:1, v/v); III = dichloromethane—methanol (7.5:1, v/v); IV = chloroform—ethyl acetate—ethanol (7.5:7.5:1, v/v). Solvent run = 8 cm.
** Double development.

as a function of the amount applied to the layer were linear up to 2 μg of CYP, 1 μg of MIS, DEMIS, 5-FU and L 8580 at least (corresponding to 200 μg/ml for CYP, and 100 μg/ml for the other drugs). The detection limits were about 50 ng applied to the layer of CYP, and 10 ng of the other compounds; i.e. the sensitivity of the assay, using 1 ml of plasma, was 5 μg/ml for CYP, and 1 μg/ml for MIS, DEMIS, 5-FU and L 8580. These concentration ranges can obviously change depending on the volume of plasma assayed and/or the volume of solvent used to redissolve the residue after extraction.

CONCLUSIONS

The TLC method described for the simultaneous determination of CYP and 5-FU with some radiosensitizing drugs in plasma samples is simple, rapid and reliable. The usual levels of radiosensitizers in plasma to be analyzed are within the upper and the lower concentration limits reported above [5]. The proposed method is less sensitive for assaying CYP and 5-FU than the gas chromatographic methods commonly used, but equivalent to the electrochemical and high-performance liquid chromatographic (HPLC) techniques for assaying the radiosensitizers [5]. Nevertheless, the levels of CYP and 5-FU detectable by the TLC method cover the major part of the levels usually found in plasma [6—8]. The method allows the simultaneous determination of radiosensitizers and chemotherapeutic agents by two separate scans, yet showing an equivalent sensitivity for all the tested drugs. Analysis of the same preparation could be also performed by HPLC but in this case the sensitivity for CYP would be worse because of the low molar extinction coefficient of CYP even if the detection is performed at 200 nm [9]. Therefore, the method described here proves suitable for the study of the pharmacokinetics of radiosensitizers when given in combination with chemotherapeutic

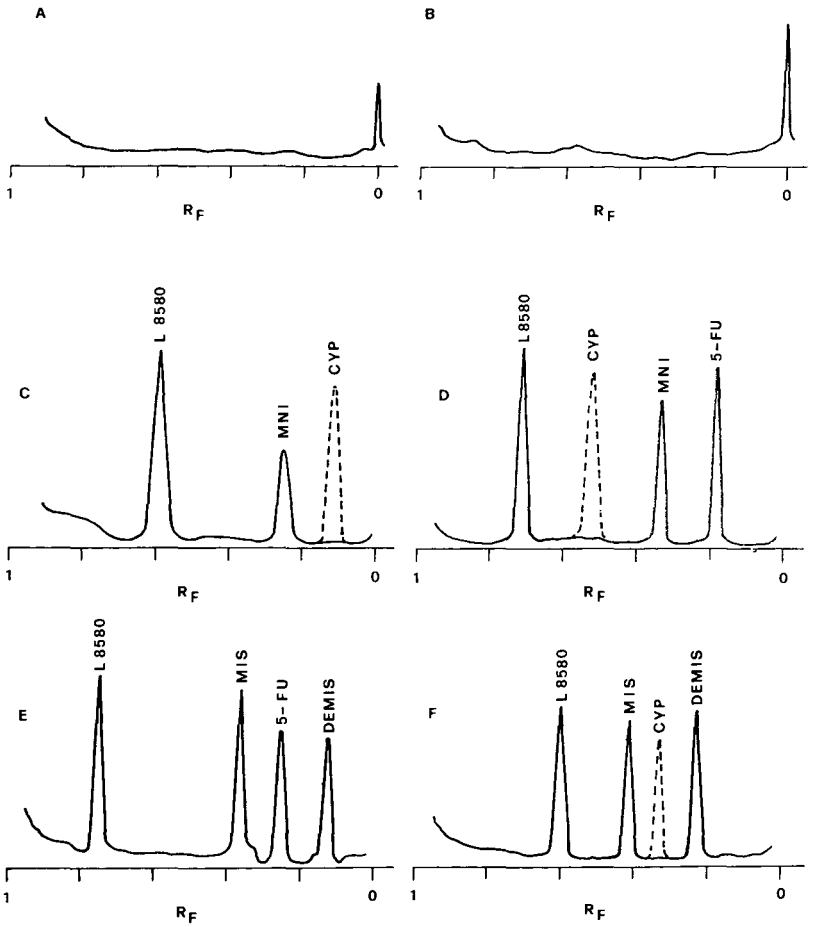

Fig. 1. Chromatograms of: (A) plasma blank from Sep-Pak C_{18} extraction; (B) plasma blank from solvent extraction; (C—F) plasma samples spiked with chemotherapeutic and radiosensitizing drugs. (- - - -), reading performed after heating at 260°C. Eluents used: I for A and F, II for B and C, III for D, IV for E. Abbreviations as in the text.

agents, or for studies which need short analysis times, and simple manipulations, especially when radiolabelled drugs are used or qualitative tests are required.

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CHROMBIO. 1644

Letter to the Editor

Cross-channel contamination of prostaglandins during separation by thin-layer chromatography

Sir,

We wish to report a problem arising when samples containing substantially different amounts of prostaglandins (PGs) are run on adjacent channels on thin-layer chromatography (TLC) plates.

Whatman LK-5D TLC plates with 19 channels are often used to separate prostaglandins prior to radioimmunoassay [1, 2]. In our laboratory, urine after the addition of acetone, adjustment of pH to a value between 4.0 and 4.5 and extraction with chloroform, is spotted onto TLC plates. The chloroform—acetone extract is taken to dryness and reconstituted in methanol (100 μl) and applied to the TLC plate in 50-μl aliquots. Plates are developed in an organic phase of ethyl acetate—isooctane—acetic acid—water (80:50:16:90). Duplicates of each sample are assayed, and separated from each other on the plate by other samples, or by water blanks and a quality control of pooled urine. Blank values are in the range 10—20 pg/ml for thromboxane B_2 and PGE_2 and 10—50 pg/ml for 6-keto-$PGF_{1\alpha}$.

When measuring levels of 6-keto-$PGF_{1\alpha}$ of several ng/ml in rabbit urine we sometimes observed an increase in blank values from 50 to 600 pg/ml, and a corresponding increase in quality control urines from 374 pg to 800 pg. This only occurred when urines containing relatively large amounts of 6-keto-$PGF_{1\alpha}$ (of the order of 16 ng/ml) were run in channels adjacent to the blank or quality control. As this suggested that cross-channel contamination might be occurring, the following investigation was carried out.

Blanks and quality controls were either set up alongside channels that were unoccupied (A) or spotted with samples containing ng/ml quantities of PGE_2 and 6-keto-$PGF_{1\alpha}$ (B). Care was taken to apply the sample to the middle of the preadsorbent area no lower than 5 mm from the bottom of the plate to avoid flooding adjacent channels or contaminating the developing solvent. Our results show that there was definite contamination between adjacent channels resulting in high values for both blanks and quality control samples (Fig. 1). When the experiment was repeated using water blanks spiked with 10 ng of 6-keto-$PGF_{1\alpha}$ or PGE_2, the existence of cross-channel contamination was confirmed, indicating that the phenomenon was not limited only to those samples

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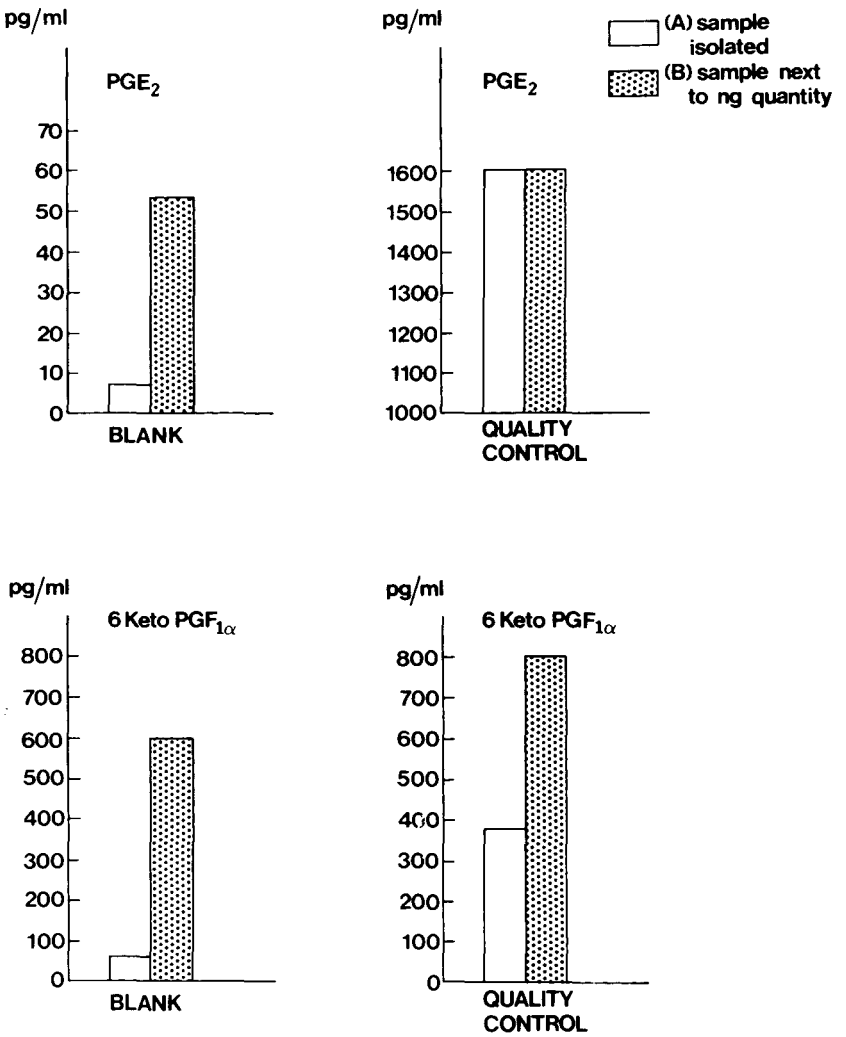

Fig. 1. Blank and quality control values for PGE_2 and 6-keto-$PGF_{1\alpha}$ when run in isolated channels or alongside channels containing ng/ml quantities of PGE_2 and 6-keto-$PGF_{1\alpha}$.

containing urinary extracts which may contain impurities affecting leaching from one channel to the next.

The problem was overcome by running blanks, quality controls and samples in duplicate in adjacent channels, leaving an unspotted channel between each pair of samples. The samples will then only cross contaminate their duplicates and this will be corrected by the estimation of recovery of the ^{3}H-labelled material added initially. This technique successfully eliminates the cross-channel contamination which previously gave rise to errors when samples in adjacent channels varied markedly in PG concentration. The only disadvantage of this procedure is the additional costs arising from the use of 11 rather than

the full 19 channels on the TLC plates. The phenomenon described may arise with substances other than prostaglandins for which these plates are used.

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CHROMBIO. 1648

Letter to the Editor

Determination of ketanserin in plasma by reversed-phase high-performance liquid chromatography

Sir,

Ketanserin (3-{2-[4-(4-fluorobenzoyl)-1-piperidinyl] ethyl}-2,4-[1H,3H]-quinazolinedione) is a potent new serotinergic receptor antagonist completely devoid of agonist activity. It has been shown to possess anti-hypertensive properties in experimental animals and man [1—4] and is currently being evaluated as a hypotensive drug. It appears to be free of serious side effects or systemic toxicity in man.

Recently, in this Journal, Kacprowicz et al. [5] have described a high-performance liquid chromatographic (HPLC) method for the determination of ketanserin in human plasma. Independently, in this laboratory, a conceptually similar, though procedurally different, method for the determination of this drug has been developed. The methodology is described briefly below.

A 1-ml sample of human plasma, spiked with 75 ng of the internal standard (R 46594), was treated with 1 ml of borate buffer, pH 10, and extracted with 5 ml of diethyl ether for 15 min. Following centrifugation at 500 *g*, the ether phase was removed, treated with 1 ml of 0.1 *M* sulphuric acid and extracted for a similar period. The ether phase was discarded (following centrifugation) and to the residual aqueous phase were added 100 μl of 4 *M* sodium hydroxide. This aqueous phase was finally extracted with 5 ml of diethyl ether for 15 min; the organic phase was removed following centrifugation, reduced to dryness under nitrogen and reconstituted in 40 μl of mobile phase. A 20-μl aliquot of this solution was injected into the chromatograph.

The liquid chromatograph comprised an Altex Model 100A pump, a 15 cm × 4.6 mm I.D. analytical column packed with 5-μm Ultrasphere ODS particles (both Altex Scientific, Berkeley, CA, U.S.A.) and a Pye-Unicam Model LC-UV ultraviolet detector (Pye-Unicam, Cambridge, Great Britain). The analytical column was fitted with a 5 cm × 4.6 mm I.D. pre-column packed with 30—38 μm Co-Pell ODS (Whatman, Maidstone, Great Britain). The mobile phase consisted of 0.02 *M* K_2HPO_4—methanol (28:72, v/v), final pH 7.2, pumped at a flow-rate of 1.0 ml/min. The UV detector was set at 240 nm.

Using this system k' values for ketanserin and its internal standard were 1.64

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and 2.70, respectively (R_s = 6.90); total analysis time was 10—12 min. With the detector normally operated at 0.005—0.01 a.u.f.s. a nominal limit of detection of 2 ng ketanserin tartrate could be achieved. Calibration was linear over a concentration range of 10—500 ng/ml ketanserin tartrate ($y = 0.012x - 0.024$, r = 0.998) and coefficients of variation for intra- and inter-assay variation throughout this concentration range varied between 4.9—5.7% and 3.7—12.0%, respectively. An extraction efficiency of approximately 60% was normally achieved.

The above technique possesses performance characteristics almost identical to those described by Kacprowicz et al. [5]. The complementary data obtained from the two independent studies indicate that ketanserin, a compound of increasing clinical and pharmacological interest, may be accurately and reliably determined by reversed-phase HPLC at levels consistent with its clinical administration in man.

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CHROMBIO. 1643

Book Review

Radioimmunoassay of gut regulatory peptides, methods in laboratory medicine, Vol. 2, edited by S.R. Bloom and R.G. Long, Praeger, Eastbourne, New York, 1982, IX + 194 pp., price US$ 24.95, ISBN 0-03-062116-X.

One can only agree with the statement of the Editors that "the general importance of gut regulatory peptides is now widely recognized and workers in many areas need to be able to measure them". However, successful radioimmunoassay procedures require purification of antibodies and labelled peptides. The latter involves the separation of the labelled peptide and other reactants, mainly unreacted iodide and unreacted peptide hormone. This is inevitably done by chromatographic methods. Someone may say that gel permeation chromatography, which is frequently the method of choice, is uninteresting from the separation point of view if it is carried out with classical sorbents for medium- and low-pressure chromatography. Those thinking along this line should be directed to do an experiment in this way themselves to encounter all the unforeseen difficulties.

The present tiny volume offers a good deal of information and practical hints on the separation of numerous gut peptide hormones like gastrin, cholecystokinin, pancreatic glucagon, enteroglucagon, pancreatic polypeptide, gastric inhibitory polypeptide, motilin, vasoactive intestinal polypeptides, bombesin, somatostatin, neurotensin and substance P. The book is remarkably uniform in its build-up, all of the specified chapters starting with the general chemistry of the peptide, its physiology, followed by the radioimmunoassay procedure with a detailed part on its purification. There are practically none that would escape a chromatographic step. If several chromatographic procedures are applicable, they are always meticulously compared and evaluated.

In summary, the book is certainly a contribution in this field where highly purified peptides have to be obtained from very complex mixtures. Those looking for high-performance liquid chromatographic (HPLC) methods would be disappointed; the preparative procedures for obvious reasons stick strictly to the medium- and low-pressure techniques. This, however, does not devalue the volume as more specific HPLC methods with higher selectivity (that would be certainly welcomed) are not elaborated to such a level to be applicable in this diagnostic area. Small imperfections like the use of daltons for relative molecular mass are likely to be overlooked by the readers focused on the very essence of the volume.

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CHROMBIO. 1655

Book Review

Gel electrophoresis of nucleic acids: A practical approach, edited by D. Rickwood and B.O. Hames, IRL Press, Oxford, Washington, DC, 1982, XV + 242 pp., price £ 8.50, US$ 18.00, ISBN 0-904-14724-X.

This volume is a counterpart of an analogous book edited by the same authors (Gel electrophoresis of proteins: A practical approach, IRL Press, Oxford, 1981). It consists of six chapters and two appendices, all of which are full of detailed descriptions of laboratory procedures, recipes and background information extremely useful to all researchers dealing with various aspects of the biology and chemistry of nucleic acids and nucleoproteins. The first chapter (Gel electrophoresis of RNA by D. Grieson) describes both the basic tube method of polyacrylamide gel electrophoresis and some of its modifications, e.g. gradient gels, solubilisable polyacrylamide gels, composite agarose—polyacrylamide gels, denaturating gels, molecular weight estimations in denaturing and non-denaturing gels, electrophoresis in polyacrylamide slab gels and transfer of RNA from the slab gels to DAB-paper for hybridisation. Valuable are the remarks on some problems occasionally occurring during PAGE and their remedies. The second chapter (Electrophoresis of DNA by P.G. Sealey and E.M. Southern) deals with analytical techniques and with large-scale preparative gel electrophoresis of DNA. Two current types of preparative electrophoresis apparatus are described very thoroughly with many practical hints for their home-construction. The next chapter (Two-dimensional gel electrophoresis of nucleic acids by R. De Wachter and W. Fiers) contains separate descriptions of two-dimensional electrophoresis of RNAs (e.g. separation of mRNA or tRNA mixtures and analysis of endonuclease RNA digests) and DNA (separation of complex mixtures of restriction fragments in conventional and denaturing gradient gels). Chapter 4 (DNA sequencing by R.W. Davies) describes very thoroughly (on 55 pages) the methods used in both chain terminator and chemical sequencing methods. The author does not limit himself to electrophoresis, but adequately describes many essential techniques involved in DNA sequencing, e.g. manipulation of very small volumes of liquid, characteristics of primers, annealing of primer and template, the M13 cloning system, etc. Similarly, J.M. D'Alessino, the author of the fifth chapter on RNA sequencing, describes besides electrophoretic systems used in this area also topics such as radiolabelling of RNA, site specific cleavage, etc. The last chapter (Electrophoresis of nucleoprotein by G.A. Goodwin and A.G. Dahlberg) briefly describes electrophoresis of nucleosomes, ribosomes and polysomes and their constituents. Appendix I

contains tables of nucleic acid molecular weight markers, and the commercial suppliers of electrophoretic items are listed in Appendix II. Although there is some overlap between some of the chapters, e.g. some recipes for gel casting, this is clearly rather an advantage, as it enables the reader to use the chapters directly and without a lengthy search in other parts of the book. The book is quite indispensable for anyone in the field, from students to experienced workers. The authors and editors can be congratulated on this very useful publication.

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MEETINGS

5th INTERNATIONAL BIOANALYTICAL FORUM

Advances in high-performance liquid chromatography with electrochemical detection will be among the topics at the 5th International Bioanalytical Forum to be held at the University of Surrey in Guildford (near London), Great Britain, on Sept. 6–9, 1983. The topic range includes ligand approaches and covers forensic analytes (especially tissue-bound) besides anti-cancer (e.g. Pt-complex) and other drugs in blood.

For further information contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, Great Britain. Tel.: 0483-65324.

CAPILLARY CHROMATOGRAPHY – 2nd INTERNATIONAL SYMPOSIUM

The 2nd International Symposium on Capillary Chromatography will be held on Oct. 10–12, 1983, at the Westchester Marriott Hotel in Tarrytown, NY, U.S.A. The symposium will consist of invited and submitted papers on all aspects of capillary chromatography, given by leading authorities from throughout the world. Informal discussions will permit the free exchange of ideas on various current questions related to these techniques and their applications. There will also be an exhibition of chromatography instrumentation.

Further information may be obtained from: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.

3rd INTERNATIONAL SYMPOSIUM ON HPLC OF PROTEINS, PEPTIDES, AND POLYNUCLEOTIDES

The 3rd International Symposium on HPLC of Proteins, Peptides, and Polynucleotides will be held at the Loews Monte-Carlo Hotel in Monte Carlo, Monaco, on November 14–16, 1983, and will be co-sponsored by Varian Associates and Pharmacia. The symposium is expected to provide a forum for researchers utilizing high-performance liquid chromatography techniques within life science disciplines.

Further information may be obtained from: Shirley Schlessinger, HPLC Symposium, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.

WORKSHOP ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY

This workshop will be held at the Lausanne Congress Centre in Lausanne, Switzerland, on Nov. 24–25, 1983, and is organized by the International Association of Environmental Analytical Chemistry and sponsored by national organizations. The Workshop is intended to bring together specialists in this field who can give a good account of the state-of-the-art in their particular specialty and to present first-hand experience in sample handling. Strong industrial participation and ample discussion time are planned. Poster contributions to the workshop can be submitted until October 15, 1983.

A short course on "sample handling in liquid chromatography" will be offered prior to the workshop on Nov. 22–23, 1983, in the same location.

For further information contact: Prof. R.W. Frei, The Free University of Amsterdam, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

CALENDAR OF FORTHCOMING EVENTS

June 27–July 1, 1983 Gatlinburg, TN, U.S.A.	**3rd Symposium on Separation Science and Technology for Energy Applications** Contact: A.P. Malinauskas, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A. (Further details published in Vol. 245, No. 1.)
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–Sept. 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.
Aug. 29–Sept. 2, 1983 Bratislava, Czechoslovakia	**4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"** Contact: Professor J. Garaj, Department of Analytical Chemistry, Faculty of Chemical Technology, Jánska 1, 81237 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.)
Sept. 5–9, 1983 Montreux, Switzerland	**43rd International Congress on Pharmaceutical Sciences** Contact: Mr. L.G. Felix-Faure, Administrative Director, International Pharmaceutical Federation, 11 Alexanderstraat, 2514 JL The Hague, The Netherlands.
Sept. 6–9, 1983 Guildford, Great Britain	**5th International Bioanalytical Forum** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, Great Britain. Tel.: 0483-65324.
Sept. 6–9, 1983 Heidelberg, G.F.R.	**International Conference on Heavy Metals in the Environment** Contact: Heavy Metals Secretariat, CEP Consultants, Ltd., 26 Albany Street, Edinburgh EH1 3QH, Great Britain. Tel.: 031-557 2478.
Sept. 21–29, 1983 Amsterdam, The Netherlands	**het instrument** Contact: het instrument, Birkstraat 108, Postbus 152, 3760 AD Soest, The Netherlands. Tel. (02155) 18204.

Sept. 25–30, 1983 Philadelphia, PA, U.S.A.	**10th Annual Meeting of the Federation of Analytical and Spectroscopy Societies** Contact: FACSS X Program Chairman, John O. Lephardt, Philip Morris Research Center, P.O. Box 26583, Richmond, VA 23261, U.S.A.
Sept. 29–30, 1983 Schliersee, G.F.R.	**Symposium "Chiralität und Aktivität"** Contact: Gesellschaft Deutscher Chemiker, Geschäftsstelle, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: 0611/7917-366.
Oct. 3–4, 1983 Bad Nauheim, G.F.R.	**Anwender-Kolloquium über die Gaschromatographische Dampfraumanalyse** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, D-6000 Frankfurt am Main 90, G.F.R. Tel.: 0611/7917-366.
Oct. 3–6, 1983 Amsterdam, The Netherlands	**20th Anniversary – International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Department of Chemistry, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 272, No. 2.)
Oct. 10–12, 1983 Tarrytown, NY, U.S.A.	**Capillary Chromatography – 2nd International Symposium** Contact: Professor A. Zlatkis, Department of Chemistry, University of Houston, Houston, TX 77004, U.S.A.
Oct. 17–21, 1983 Neubrandenburg, G.D.R.	**Analytiktreffen 1983: Fortschritte in der Gas- und Flüssigkeits-Chromatographie** Contact: Dr. sc. W. Engewald, Karl-Marx-Universität Leipzig, Sektion Chemie, Leibigstrasse 18, DDR-7010 Leipzig, G.D.R.
Nov. 10–16, 1983 Düsseldorf, G.F.R.	**9th International Congress and Exhibition for Instrumentation and Automation (INTERKAMA 83)** Contact: INTERKAMA 83, Düsseldorfer Messegesellschaft mbH, NOWEA, Postfach 32 02 03, D-4000 Düsseldorf 30, G.F.R.
Nov. 14–16, 1983 Monte Carlo, Monaco	**3rd International Symposium on HPLC of Proteins, Peptides and Polynucleotides** Contact: Shirley E. Schlessinger, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.
Nov. 16–18, 1983 New York, NY, U.S.A.	**22nd Eastern Analytical Symposium** Contact: Norman Gardner, 73 Ethel Street, Metuchen, NJ 08840, U.S.A. Tel.: (201) 548-7377.
Nov. 24–25, 1983 Lausanne, Switzerland	**Workshop on Handling of Environmental and Biological Samples in Chromatography** Contact: Prof. R.W. Frei, The Free University of Amsterdam, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.
Dec. 7–10, 1983 Singapore, Singapore	**Chem Asia '83 Conference** Contact: Singapore Exhibition Services, Ltd., 601 Cathay Building, Singapore 0922, Singapore.

March 5–9, 1984 Atlantic City, NJ, U.S.A.	**35th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 8–13, 1984 St. Louis, MO, U.S.A.	**187th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 16–19, 1984 New York, NY, U.S.A.	**20th International Symposium on Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
April 29–May 4, 1984 Rio de Janeiro, Brazil	**12th International Congress of Clinical Chemistry, 7th Latin American Congress of Clinical Biochemistry & 12th Brazilian Congress of Clinical Analysis** Contact: 12th International Congress of Clinical Chemistry, Rua Vicente Licinio 95, Tijuca, 20270 Rio de Janeiro, RJ, Brazil.
May 9–11, 1984 Dourdan, France	**4th Weurman Flavour Research Symposium** Contact: J. Adda, Laboratoire de Recherches sur les Arômes, 17 rue Sully, 21034 Dijon Cedex, France.
May 20–26, 1984 New York, NY, U.S.A.	**8th International Symposium on Column Liquid Chromatography** Contact: Professor Cs. Horváth, Mason Laboratory, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A. (Further details published in Vol. 272, No. 2.)
July 29–Aug. 3, 1984 Washington, DC, U.S.A.	**36th National Meeting of the American Association for Clinical Chemistry** Contact: American Association for Clinical Chemistry, 1725 "K" Street, NW, Washington, DC 20006, U.S.A.
Aug. 26–31, 1984 Philadelphia, PA, U.S.A.	**188th National Meeting of the American Chemical Society** Contact: A.T. Winstead, American Chemical Society, 1155 16th Street, NW, Washington, DC 20036, U.S.A.
Aug. 26–Sept. 1, 1984 Cracow, Poland	**EUROANALYSIS V – 5th European Conference on Analytical Chemistry** Contact: Professor Zygmunt Kowalski, Secretary-General, Euroanalysis V, Academy of Mining and Metallurgy, Mickiewicza 30, 30-059 Kraków, Poland.
Sept. 2–6, 1984 Hradec Králové, Czechoslovakia	**4th International Symposium on Isotachophoresis – ITP 84** Contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo nám. 2, CS-166 10 Praha 6, Czechoslovakia. (Further details published in Vol. 272, No. 2.)
Sept. 23–28, 1984 Philadelphia, PA, U.S.A.	**11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies** Contact: R.F. Hirsch, Division of Analytical Chemistry, American Chemical Society, 304 Beach Wood, Orange, NJ 07050, U.S.A.
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.

Oct. 8–10, 1984 Tarrytown, NY, U.S.A.	**3rd International Symposium on Capillary Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
Nov. 22–24, 1984 Barcelona, Spain	**14th Annual Symposium on Analytical Chemistry of Pollutants** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.
Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques in Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; Telex: 50458 FOIMB-E.
Feb. 25–March 1, 1985 New Orleans, LA, U.S.A.	**36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Biggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 28–May 3, 1985 Miami Beach, FL, U.S.A.	**189th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
July 1–6, 1985 Edinburgh, Scotland, Great Britain	**9th International Symposium on Column Liquid Chromatography** Contact: J.H. Knox, Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland, Great Britain.

NEW BOOKS

Clinical and pharmaceutical chromatography atlas, Chrompack, Middelburg, The Netherlands, 1981, *ca.* 100 pp., price Dfl. 46.00.

The chemistry and biology of antibiotics (Pharmacochemistry Library, Vol. 5), by V. Betina, Elsevier, Amsterdam, Oxford, New York, 1983, 574 pp., price US$ 125.50 (U.S.A. and Canada), Dfl. 295.00 (rest of world), ISBN 0-444-99678-8.

Phospholipids (New Comprehensive Biochemistry, Vol. 4), edited by J.N. Hawthorne and G.B. Ansell, Elsevier Biomedical Press, Amsterdam, New York, 1982, 500 pp., price US$ 59.50 (U.S.A. and Canada), Dfl. 140.00 (rest of world), ISBN 0-444-80427-7.

Drug discrimination: Applications in CNS pharmacology (Proc. 2nd Int. Symp. on Drugs as discrimative stimuli, Beerse, June 30–July 3, 1982; Janssen Research Foundation Series, Vol. 6), edited by F.C. Colpaert and J.F. Slangen, Elsevier Biomedical Press, Amsterdam, New York, 1982, X + 448 pp., price US$ 80.75 (U.S.A. and Canada), Dfl. 190.00 (rest of world), ISBN 0-444-80475-7.

Clinical immunology update – Reviews for physicians, 1983 edition, edited by E.C. Franklin, Elsevier Biomedical Press, Amsterdam, New York, 448 pp., price US$ 42.50 (U.S.A. and Canada), Dfl. 115.00 (rest of world), ISBN 0-444-00711-3.

Serum protein abnormalities: Diagnostic and clinical aspects, edited by S.E. Ritzmann and J.C. Daniels, Alan R. Liss Inc. (Wiley), 1983, *ca.* 570 pp., price *ca.* US$ 81.80, £45.45, ISBN 0-8451-2799-3.

Immunogenetics in rheumatology: Musculoskeletal diseases and D-penicillamine (Proc. Int. Workshop, Perth, April 14–22, 1982; International Congress Series, No. 602), edited by R.L. Dawkins, F.T. Christiansen and P.J. Zilko, Excerpta Medica, Amsterdam, New York, 1982, XXII + 390 pp., price US$ 91.50 (U.S.A. and Canada), Dfl. 215.00 (rest of world), ISBN 0-444-90293-7.

CHROMATOGRAPHY

Fundamentals and Applications of Chromatographic and Electrophoretic Methods

Part A: Fundamentals and Techniques
Part B: Applications

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

Journal of Chromatography Library Vol. 22 A + B

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Part B – Applications – is a critical review of the isolation and quantification methods in current use for various classes of substances.

Both parts of this work belong in all libraries serving chromatographers at technical colleges, research institutes, universities, pharmaceutical companies and instrument manufacturers. Practising chromatographers and analysts will refer to it often when faced with new or unusual analytical problems and situations.

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PUBLICATION SCHEDULE FOR 1983

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

MONTH	D 1982	J	F	M	A	M	J	
Journal of Chromatography	252 253/1 253/2	254 255 256/1	256/2 256/3 257/1	257/2 258 259/1	259/2 259/3 260/1	260/2 261/1 261/2	261/3 262 263	The publication schedule for further issues will be published later.
Chromatographic Reviews					271/1		271/2	
Biomedical Applications		272/1	272/2	273/1	273/2	274	275/1	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications*: Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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